

## REVIEW ARTICLE



## Translational Therapeutics

# Toll-like receptor 9 agonists and combination therapies: strategies to modulate the tumour immune microenvironment for systemic anti-tumour immunity

Zhangchi Dongye<sup>1,2</sup>, Jian Li<sup>2</sup>✉ and Yuzhang Wu<sup>2</sup>✉

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Over the past decade, tremendous progress has taken place in tumour immunotherapy, relying on the fast development of combination therapy strategies that target multiple immunosuppressive signaling pathways in the immune system of cancer patients to achieve a high response rate in clinical practice. Toll-like receptor 9 (TLR9) agonists have been extensively investigated as therapeutics in monotherapy or combination therapies for the treatment of cancer, infectious diseases and allergies. TLR9 agonists monotherapy shows limited efficacy in cancer patients; whereas, in combination with other therapies including antigen vaccines, radiotherapies, chemotherapies and immunotherapies exhibit great potential. Synthetic unmethylated CpG oligodeoxynucleotide (ODN), a commonly used agonist for TLR9, stimulate various antigen-presenting cells in the tumour microenvironment, which can initiate innate and adaptive immune responses. Novel combination therapy approaches, which co-deliver immunostimulatory CpG-ODN with other therapeutics, have been tested in animal models and early human clinical trials to induce anti-tumour immune responses. In this review, we describe the basic understanding of TLR9 signaling pathway; the delivery methods in most studies; discuss the key challenges of each of the above mentioned TLR9 agonist-based combination immunotherapies and provide an overview of the ongoing clinical trial results from CpG-ODN based combination therapies in cancer patients.

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## INTRODUCTION

Immunotherapy represented by immune checkpoint inhibitors (ICI) has made great clinical breakthroughs [1]. So far, ICI targeting programmed cell death 1 (PD-1), programmed death-ligand 1 (PD-L1) and cytotoxic T lymphocyte-associated protein 4 (CTLA-4) have been approved for clinical treatment by the US Food and Drug Administration (FDA) [2, 3]. Cell-based immunotherapies, such as chimeric antigen receptor (CAR) T cells [4], have also been shown to be effective in treating refractory or recurrent haematopoietic malignancies. These immunotherapies have been effective in improving survival and quality of life for cancer patients. However, due to the complex heterogeneity of tumours, the response rate of checkpoint inhibitors to solid tumours is only 20–30% [5], and most tumours remain insensitive to ICI therapy.

Tumours are usually classified as having a “hot” (inflamed) or “cold” (non-inflamed) phenotype [6]. “Hot” tumours are classified by high infiltration of cytotoxic lymphocytes (CTLs) and leukocytes in the tumour microenvironment (TME) [7]. Such tumours usually respond well to immunotherapy. In contrast, “cold” tumours are poorly immunogenic and characterised by lower lymphocyte infiltration into the tumour and a lack of pre-existing tumour-specific T cell response [8]. In this case, the tumour

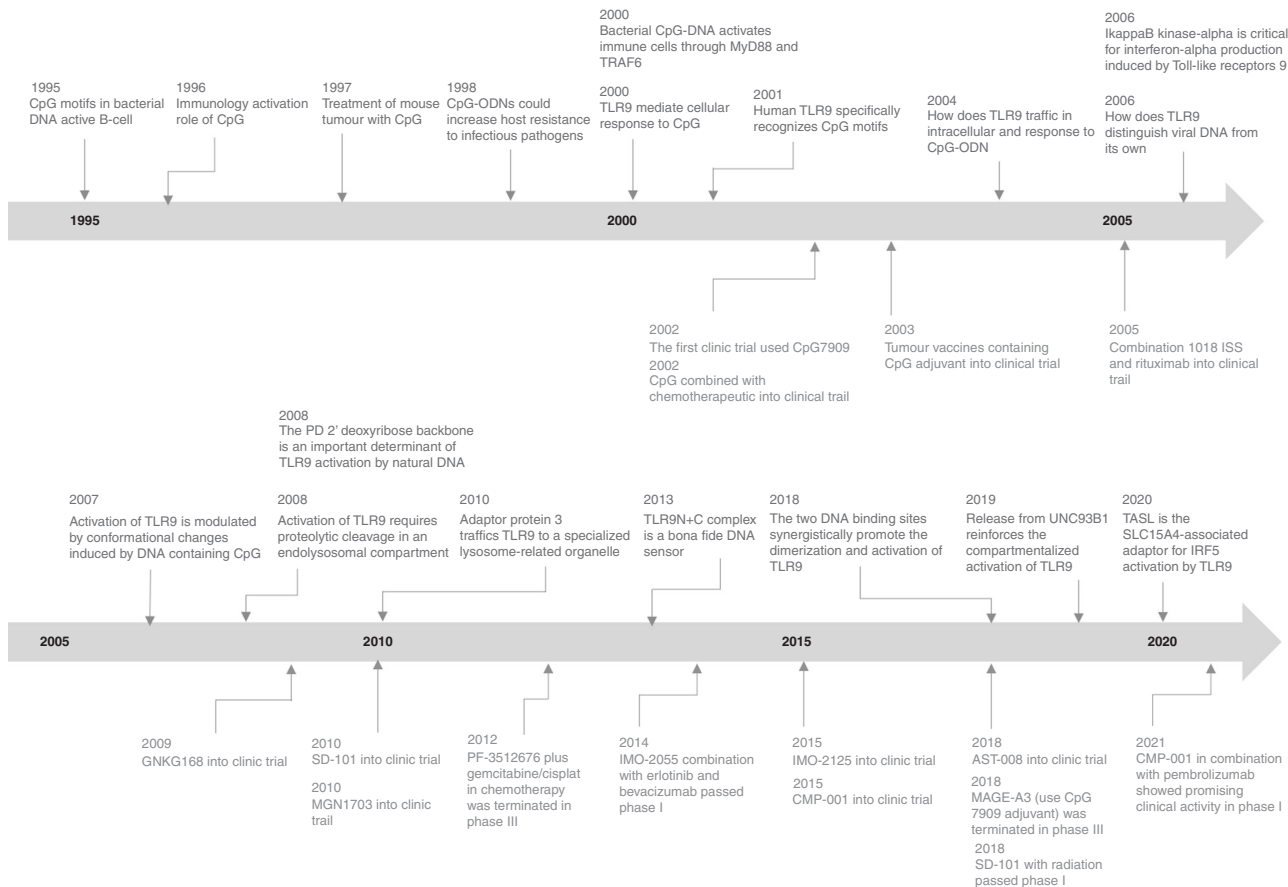
microenvironment has a low tumour mutation burden, lack of tumour neoantigens, lack of chemokines necessary for T cell homing and presence of immunosuppressive tumour signals (such as PD-L1 expression), unique vascular barriers, these phenomena may be part of the reasons for immune tolerance [9, 10]. To increase the efficacy of immunotherapy, novel combination therapy to convert non-inflamed “cold” tumours into an inflamed microenvironment with increased infiltration of CTLs is needed [11, 12]. Therefore, extensive efforts have been made to target multiple immune-suppressive mechanisms, or target different steps for tumour immune response, or give different drug combinations at different time points, all of which have shown encouraging synergistic effects to some extent.

As reviewed above, the ability of tumours to escape and suppress the immune system has led to the development of combination therapies that can modulate multiple suppression signaling pathways in order to increase the response rate of tumour patients. TLR9 agonists have shown great potential in combination therapies to synergise with other therapeutics for augmented anti-tumour immune response. Studies have found that TLR9 agonists activate B cells and plasmacytoid dendritic cells (pDC), increasing the release of T<sub>H</sub>1-promoting chemokines and

<sup>1</sup>Department of Immunology, Medical College of Qingdao University, 266071 Qingdao, Shandong, PR China. <sup>2</sup>Institute of Immunology, PLA, Third Military Medical University (Army Medical University), Chongqing, China. ✉email: seraph-2004@163.com; wuyuzhang@iiicq.vip

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**Fig. 1** A brief history of the development of TLR9 agonists CpG in immunology and its application in combined clinical trials.

cytokines such as IFN-inducible genes, which then improve the tumour suppression microenvironment and promote the T-cell-mediated immune response [13, 14]. However, the use of TLR9 agonists alone in clinical trials did not achieve the expected results [15]. The use of TLR9 agonists in combination with antigen vaccines, radiotherapies, chemotherapies and immunotherapies has been investigated in several animal models and clinical trials. In this review article, we describe the TLR9 signaling, the application of TLR9 agonists CpG-ODN in tumour biology, the delivery methods and focus on the combination strategies with different therapeutics in tumour immunotherapy and the clinical trial results from CpG-ODN-based combination therapies (Fig. 1).

## TLR9 OVERVIEW: TLR TYPES, TLR9 STRUCTURE, CLINICAL AGONISTS AND SIGNALING PATHWAYS

### TLR types

The discovery of Toll-like receptors is one of the milestones in immunology. Together with the discovery of dendritic cells, they won the 2011 Nobel Prize in Physiology or Medicine [16]. Toll-like receptors (TLR) are a type of pattern recognition receptors (PRR), which are highly conserved and recognise various types of microbial pathogen-related pattern molecules (PAMP) [17]. There are 10 members of Toll-like receptor family (TLR1-10) identified in humans and 12 members in mice (TLR1-9 and TLR11 and 13) [18]. TLR1-6 are distributed on the cell surface; the intracellular toll-like receptors are distributed in the endosome, including TLR3, TLR7, TLR8, TLR9 and TLR10 [19].

### TLR9 structure

Structurally, toll-like receptors belong to type I transmembrane glycoproteins, which consist of a transmembrane helix,

extracellular N-terminal ligand recognition domain, and an intracellular C-terminal cytoplasmic signal domain [20]. The extracellular region is the Leucine-rich repeat sequence (LRR), which directly binds to specific sites of PAMP [20]. TLR usually contains 16–28 leucine-rich repeats, each part consists of 20–30 amino acids, including the conserved LxxLxLxxN motifs (L is leucine and can also be isoleucine, valine or phenylalanine, x is any amino acid and N is asparagine) and variable regions [21]. Cysteine lysosomes cut the hinge region between LRR14 and LRR15 in the extracellular region of TLR9 to form the hydrolysed TLR9 [22]. whether hydrolysed or not, TLR9 can bind to CpG, but only the hydrolysed TLR9 can activate the MyD88 signaling pathway to transmit the activation signal [23]. The cleaved fragments are still related to each other and play an important role in inflammation [24]. Structural analysis revealed that full-length proteins are unable to form the contact necessary for receptor dimerisation and signal transduction [13]. In general, TLR9 crystal structure is non-ligand which could bind to CpG-DNA or inhibitory DNA (iDNA) [23].

### TLR9 agonists in clinical

TLR9 mainly recognises unusual unmethylated CpG (cytosine-phosphate-guanine dideoxynucleotide) motifs (human 5'-GTCGTT-3 and mouse 5'-GACGTT-3) while vertebrate genomes are severely methylated and lack unmethylated CpG motifs [25]. Synthetic CpG-ODN is divided into A, B and C types according to the number and position of CpG sequence and ODN structure. Class A such as CMP-001 which can activate NK cells, induces pDCs to produce lots of IFN- $\alpha$  and TNF- $\alpha$ , but it has a weak stimulating effect on B cells [26]. Type B CpG-ODN significantly increases the production of cytokines such as interleukin (IL)-6 and TNF- $\alpha$  and then induces a strong Th1 response, but it only induces a small

amount of IFN- $\alpha$  secretion while activates B cells very well [27]. Type C CpG-ODN shares the common characteristics of CpG-A and CpG-B ODN [28]. In addition to its anti-tumour properties, class C also promotes wound healing [29]. Studies have shown that low doses of CpG-B can significantly inhibit tumour growth, which requires high doses of CPG-C to achieve [30]. Compared with the natural structure of TLR9 agonists, the artificially designed agonists have a different structure, which can resist nuclease degradation, thereby increasing the half-life of the drug in the body, and resulting in a stronger activation ability [31]. In addition, in order to meet the research needs of TLR9 agonists, it is essential to design human and mouse TLR9 agonists separately [32].

### TLR9 signaling pathway

After TLR9 is synthesised in the endoplasmic reticulum, the transmembrane protein UNC93B1 is essential for TLR9 to leave the endoplasmic reticulum (ER) and be transported to the endosomes via classical secretory pathways [33, 34]. Before reaching the endosome, TLR9 is transported to the plasma membrane, where it is internalised by adaptor protein complex 2 (AP-2)-mediated endocytosis. At this point, UNC93B1 still has a regulatory function, then UNC93B1 is separated from TLR9 after reaching the endosome [33]. The extracellular domain of TLR9 is cleaved in the endolysosome, and the full-length protein cannot be detected in the compartment which recognises the ligand [35]. In the early endosomes TLR9 signaling leads to the production of IFN- $\alpha$  by pDCs, while TLR9 signaling induces pDCs maturation, IL-6 and TNF- $\alpha$  secretion in the late endosomes [36]. In general, the agonist CpG-DNA binds to TLR9 in a 2:2 ratio to form a symmetric TLR9-CpG-DNA complex [37]. Ligand binding to the leucine-rich domains of TLR9 causes physical interactions and the formation of TLR9 dimers. They recognise CpG-DNA mainly by the amino-terminal fragment (LRRNT-LRR10) from one of the dimers and the carboxy-terminal fragment (LRR20-LRR22) from the other [23]. Both methylated and unmethylated CpG bind to TLR9 receptors and the immunostimulatory activity of oligodeoxynucleotides depends on their ability to co-locate with TLR9 in late endosomes [38]. In addition to CpG-ODN, TLR9 can also be activated by endogenous ligands, including heat shock proteins [39], anti-microbial peptide LL37 [40] and high mobility group protein B1 (HMGB1) [41] and so on.

After ligand binding TLR9 agonist, the dimerisation of the extracellular domain promotes intracellular signal transduction which in turn recruits corresponding junction proteins [42]. TLR9 transmits downstream signals through the MyD88 dependent pathway [43]. TIR domain of TLR9 binding to MyD88 activates interleukin-1 receptor-associated kinase 4 (IRAK-4) and then through the death domain of MyD88 transmits a downstream signal of IRAK-1 [44]. Activation of IRAK-4 is followed by recruitment of tumour necrosis factor receptor-associated factor 6 (TRAF6), which further activates transforming growth factor- $\beta$ -associated kinase 1 (TAK1) [45]. TAK1 phosphorylates the I $\kappa$ B kinase (IKK) complex, activates NF- $\kappa$ B and mitogen-activated protein kinase (MAPK) and ultimately promotes gene transcription of inflammatory cytokines, including increased IL-6, IL-12 and TNF, via transcription factors NF- $\kappa$ B and AP-1, and co-stimulatory molecules such as CD80 and CD86 [46] (Scheme 1).

### TLR9 IN CANCER IMMUNOTHERAPY

Toll-like receptors are sensors of PAMPs that serve as components of the intrinsic immune system and protect the host from pathogen infection [13]. At the same time, Toll-like receptor is one of the links between innate and adaptive immunity. When the CpG-ODN injects into the body, antigen-presenting cells (APC) such as DCs and macrophages will be activated to generate antigen-independent innate immunity, and then followed by the initiation of the adaptive immune response. Koster et al. found

that in early melanoma tissues, CpG-B injection could induce concerted recruitment of CLEC9A<sup>+</sup> CD141<sup>+</sup> cDC1 and CD14<sup>+</sup> APC to the injection site and its draining lymph nodes, this may explain how are T cells infiltrated after CpG injection [47]. Although tumour microenvironment (TME) normally inhibits DC activation in this model, CpG-ODN treatment enables tumour DCs to efficiently cross-present tumour antigens to activate CD8<sup>+</sup> T cells and promote anti-tumour immune response [48]. B cells, another class of APCs activated by CpG, undergo antibody class transformation and differentiate into plasma cells that produce high-affinity antigen-specific antibodies, as well as produce IL-6 and IL-12 via NF- $\kappa$ B pathway activation and various chemokines [49, 50]. In addition, circulating anti-tumour antibodies and tumour-associated tertiary lymphoid structures can significantly affect the clinical efficacy and prognosis of cancer patients, which means that B cells also play an important role in anti-tumour immunity [51]. Currently, the mechanisms of B-cell anti-tumour immune response are not clear and need to be further investigated. For immunosuppressive cells in TME, it has also been shown that delivery of CpG-ODN into tumours can reduce the immunosuppressive activity of MDSC (expressing TLR9) [52]. Moreover, delivery of nanoparticles encapsulating CpG, baicalin and melanoma antigen peptide fragments to tumour macrophages by nanomaterials targeting macrophages, repolarised M2 TAMs to the M1 type [53].

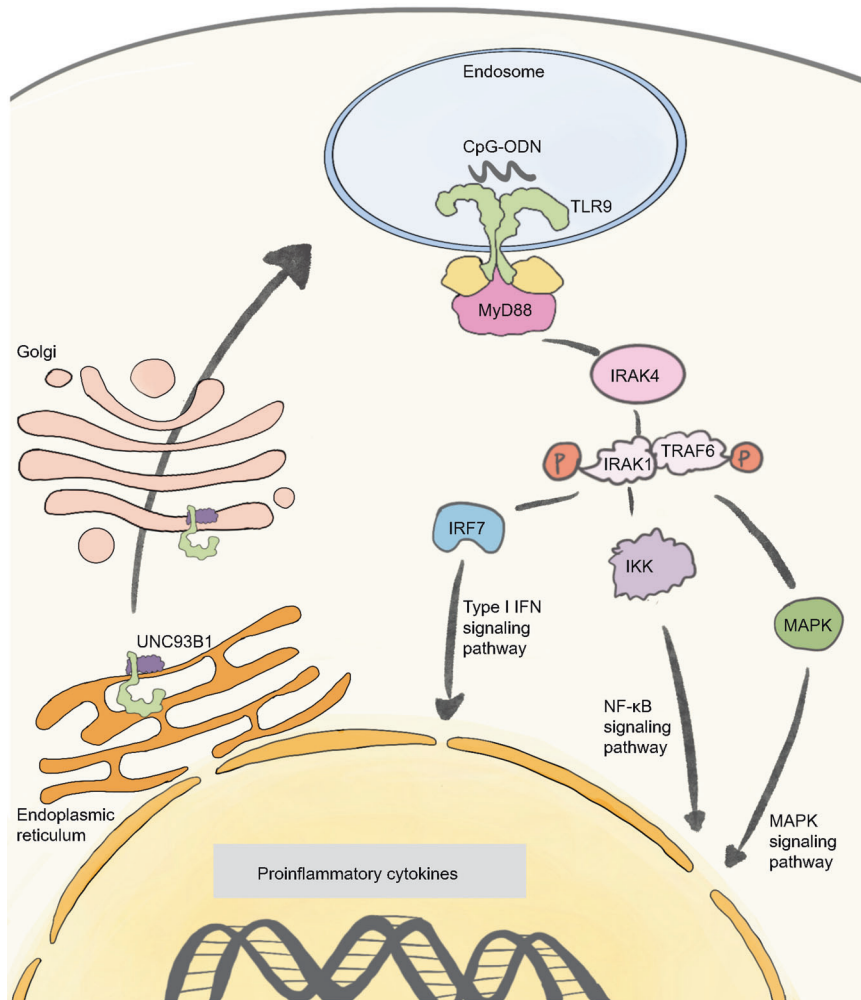
The immunotherapy strategies that simultaneously target innate and adaptive immunity are effective in large tumours, suggesting that the involvement of both innate and adaptive immune responses is necessary for effective cancer immunotherapy [54]. Based on preclinical studies that TLR9 agonists induce an intrinsic immune response, indirectly promote T cell activation and control tumour growth, they have been investigated as a therapeutic anti-tumour agent in clinical trials [55], but clinical trials have not yielded the expected results as animal trials have [56]. At present, Monotherapy in most cancer patients is not enough to completely eradicate cancer, combined immunotherapy is the trend in cancer therapy [11, 12]. It is necessary to explore the combination of CpG-ODN with existing cancer therapies.

### TLR9 AND COMBINATION IMMUNOTHERAPY

Tumour microenvironments lack pre-existing immune-infiltrating lymphocytes. However, bone marrow cells and lymphocyte populations retain expression of TLR9 in tumour microenvironments, which may sense endogenous damage-associated molecular patterns (DAMPs) and activate innate immunity in response. A deep understanding of the TLRs-mediated immune signaling pathways has led to the development of new strategies combining TLR9 agonists and other therapeutics for tumour immunotherapy. Several combination therapies have been shown to stimulate the innate immune response that contributes to the initiation of the adaptive immune response against tumours (Scheme 2). Table 1 shows the clinical trials of TLR9 agonist combinations over the last 5 years.

### TLR9 and immune checkpoint inhibitor (ICI) combination therapies

In recent years, ICI therapies have made remarkable progress in the field of oncology. ICI therapies improve tumour immunosuppression, increase the body's response to tumours, and generate immune memory which keeps durable anti-tumour immunity [11, 57]. Since 2011, the FDA has approved Ipilimumab [anti-CTLA-4 monoclonal antibody (mAb)] for the treatment of melanoma patients. It is the first ICI therapy approved by the FDA, and then more ICI therapies have been approved in recent years [58]. Now, the most widely studied immune checkpoints are CTLA-4, PD-1 and PD-L1 [59]. New ICIs have also emerged, such as new targets inhibitory (e.g. lymphocyte activation gene/LAG-3, T cell

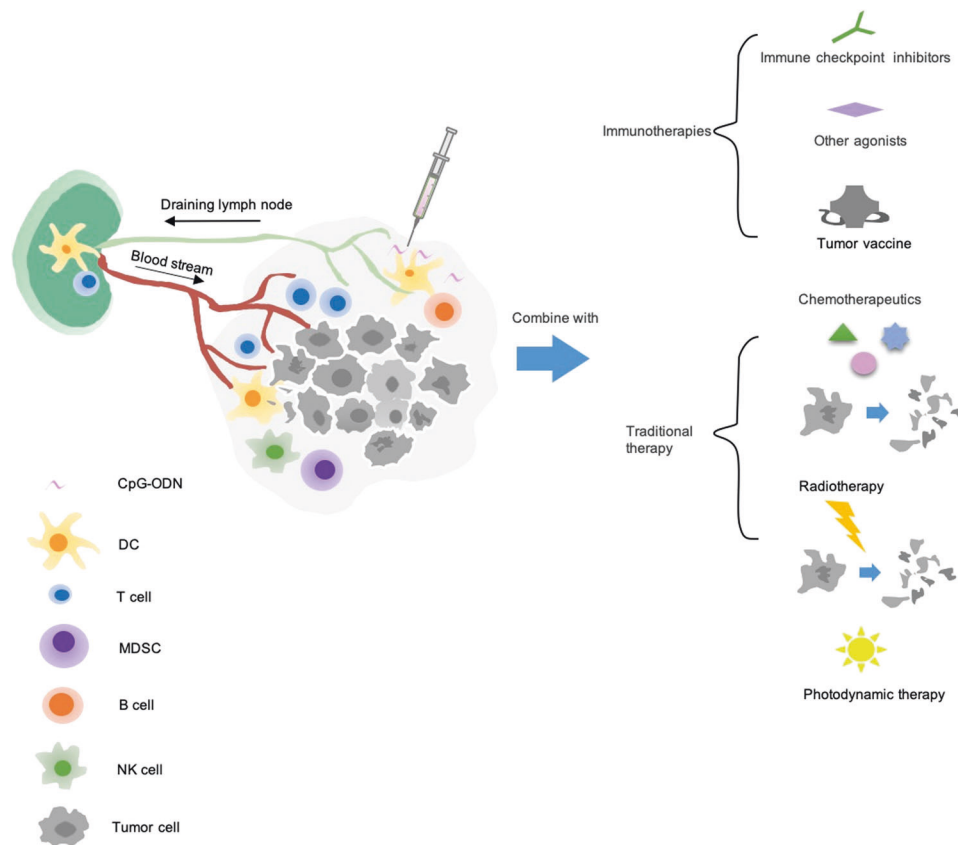


**Scheme 1 The TLR9 signaling pathways.** The binding of CpG-ODN to TLR9 leads to the activation of type I IFN signaling pathway, NF-κB signaling pathway and MAPK signaling pathway, which promotes the expression and release of pro-inflammatory cytokines in target cells.

immunoglobulin/TIM-3, V-domain Ig suppressor of T cell activation/VISTA) and stimulatory (e.g. inducible co-stimulator/ICOS, OX40, 4-1BB) [59]. We hypothesise that ICI immunotherapy may have advantages over conventional radiotherapy and chemotherapy [11]. However, the overall response rate of patients to ICI therapies is low, with response rates ranging from 10 to 35% [7, 60]. Furthermore, there have been other issues concerning ICI therapies including adverse effects associated with autoimmune-like systemic symptoms (fatigue or fever) or organ-specific damage which may lead to rashes, colitis, pneumonia and adrenal or thyroid dysfunction [61, 62]. Researchers found that ICIs only work in specific groups of people with certain types of cancers, such as melanoma, non-small-cell lung cancer (NSCLC), small cell lung cancer (SCLC), renal cell carcinoma (RCC), colorectal cancer (CRC), classical Hodgkin lymphoma (cHL), head and neck squamous cell carcinoma (HNSCC), hepatocellular carcinoma (HCC), primary mediastinal large B-cell lymphoma (PMLBCL), Merkel cell carcinoma (MCC), etc [63], while patients with other types of cancer showed a poor therapeutic effect. Therefore, scientists look for combination therapies combining ICI with other agents in the hope of achieving better results.

For tumours such as NSCLC that respond to PD-1 antibody therapy, the combination of TLR9 agonists with ICI further enhances the anti-tumour effect. Scientists showed that intratumorally injection of CMP-001 (CpG-A ODN) combine with anti-PD-1 in C57BL/6 mice to treat head and neck squamous cell

carcinoma (HNSCC) tumour, inhibited the growth of tumours at the injection site and distally, prolonged the mice's survival, further enhancing the therapeutic effect compared to single-drug treatment [64]. The combination of Vidutolimod (formerly CMP-001) and pembrolizumab (PD-1 Ab) entered Phase Ib clinical studies to cure patients with advanced melanoma, in which 25% of patients experienced tumour regression (including non-intra-tumoral injected tumours) [65]. The synergistic effect of TLR9 agonists cooperating with anti-PD-1 also has been validated in multiple mouse tumour models, including pancreatic ductal adenocarcinoma (PDAC) [66], lung cancer [67], breast cancer [68], colorectal cancer [69] and lymphoma [70]. In immunogenic B16/OVA melanoma, the combination of CpG1826 with anti-CTLA-4 resulted in bilateral tumour reduction, which was associated with increased tumour-antigen-specific T cell infiltration and decreased Tregs and inflammatory cytokines [71]. Moreover, in the non-immunogenic B16/F10 melanoma mouse model, intratumoral CpG-ODN1826 injection combined with anti-PD-1 or anti-CTLA-4 treatment is also effective on the treated side of the tumour but the uninjected tumour rarely regressed [72]. However, with the use of a better effective TLR9 agonist (MGN1703) and an enhanced CTLA-4 antibody (9D9-IgG2a) cures 50% of bilateral B16-F10 mouse melanoma [72]. Thus, it seems that in addition to choosing different steps in activating or enhancing the anti-tumour immune response, selecting the appropriate drug representative is also important.



**Scheme 2 The application of CpG-ODN in combination therapy.** CpG-ODN intra-tumoral injection activates DC, resulting in enhanced antigen presentation and subsequent tumour-antigen presentation to CD8<sup>+</sup> T cells in tumour-draining lymph nodes. In addition, activated DCs secrete cytokines, recruit tumour-specific CD8<sup>+</sup> T cells to the tumour site and directly kill tumour cells. By combining different therapeutic approaches including immunotherapy, chemotherapy, radiation therapy, photodynamic therapy and so on, the tumour microenvironment can be modulated to improve the anti-tumour responses.

Co-stimulatory signaling pathways play a key role in T cell activation, differentiation, effector function and survival [73]. With the in-depth study of the mechanism of CpG-ODN, it was found that the intra-tumoral administration of CpG-ODN increases the expression of OX40 co-stimulatory receptor in Treg cells, and the intra-tumoral injection of SD -101 with anti-OX40 antibody successfully protected mice with spontaneous breast cancer with good therapeutic effect [74]. Now, this combination is being tested in clinical trials (NCT03831295, NCT03410901), and research advances have shown great potential for the combination treatment.

In other studies, Zhou et al. found that TLR9 activation in HCC cells affected PARP1 and STAT3 pathways, resulting in PD-L1 expression and ultimately inducing immune escape of cancer cells [75]. The latest results suggest that if TLR9 signaling activation in macrophages of breast cancer, the co-administration of anti-PD-1 antibodies with TLR9 agonists may induce macrophages to reprogram and polarise into an immunosuppressive phenotype [76]. This interesting observation is contrary to the prevailing results of the current two-drug combination trials, most of which have shown that TLR9 agonists are effective in combination with PD-1. The mechanism of drug combination is still not clear, and more studies are needed.

#### TLR9 and tumour vaccine

Vaccine platforms include DNA [77], RNA [78], peptides [79] or direct use of DC cells [80], promoting DC activation, and ultimately generating an anti-tumour immune response and immune memory. Factors influencing vaccine efficacy include antigen quality, DC activation, whether induces strong and sustained

CD4<sup>+</sup> T helper cells and cytotoxic T lymphocyte (CTL) responses, TME infiltration and persistence and maintenance of the immune response [81]. Currently, the only landmark cancer vaccine product approved by FDA is Sipuleucel-T (Provenge®) for prostate cancer [82]. Since then, no more cancer vaccine has been approved, and it appears that the tumour vaccine field has reached a plateau [81]. Unlike other types of vaccines which predominantly induce humoral immunity, the most common aluminum adjuvants are not suitable for tumour vaccines. As a TLR agonist, CpG-ODN is one of the adjuvants used in tumour vaccines. A large number of clinical trials of tumour vaccines with CpG-ODN are underway for various types of tumours such as lymphoma [55] (NCT00490529), melanoma (NCT00145145, NCT00112242, NCT00471471, NCT00112242), non-small cell lung cancer (NCT00199836) and prostate cancer (NCT00292045).

In addition to the one-by-one combination which is based on the theory that breaking down the tumour's immunosuppressive microenvironment enhances the body's immune response; researchers have also tried multiple drug combinations that attempt to influence multiple steps of the immune response. The development of material science makes this idea more feasible than before and greatly promotes advances in tumour vaccines. Mai et al. designed a combination of 2'3' -CGAMP, CpG-ODN and antigenic peptide nanoporous microparticles ( $\mu$  GCVax) to achieve functional healing in HER2-positive breast cancer mice, and effectively inhibit lung metastatic melanoma, primary breast cancer and subcutaneous colorectal cancer in mouse models [83]. It has been shown that high-density lipoprotein-mimicking nanodiscs coupled with antigen and CpG adjuvant can eliminate established MC-38 and B16-F10 tumours when combined with ICI

**Table 1.** Combination of TLR9 agonists and other therapies in clinical trials.

TLR9 agonist	Interventions	Conditions	Phase	Status	NCT number
CMP-001	Drug: Pembrolizumab	Carcinoma, Squamous cell of head and neck	Phase 2	Recruiting	NCT04633278
	Drug: Nivolumab	Melanoma, Advanced melanoma, Metastatic melanoma, Unresectable melanoma	Phase 2	Recruiting	NCT04698187
	Drug: Cemiplimab-rwlc	Advanced cancer, Metastatic cancer	Phase 2	Recruiting	NCT04916002
	Drug: Nivolumab	Melanoma, Advanced melanoma, Metastatic melanoma, Unresectable melanoma	Phase 2, 3	Recruiting	NCT04695977
	Radiation: Stereotactic body radiotherapy	Triple negative breast cancer	Phase 2	Recruiting	NCT04807192
	Biological: Nivolumab	Melanoma, Lymph node cancer	Phase 2	Completed	NCT03618641
	Biological: Agonistic Anti-OX40 Monoclonal Antibody INCAGN01949	Locally advanced malignant solid Neoplasm, Metastatic pancreatic Adenocarcinoma, Stage IV pancreatic cancer AJCC v8, Unresectable malignant solid Neoplasm	Phase 1, 2	Recruiting	NCT04387071
	Biological: Nivolumab, Other: [18 F]F-AraG PET/CT	Melanoma	Phase 2	Recruiting	NCT04401995
	Biological: Pembrolizumab, Procedure: Surgical Procedure	Clinical stage III cutaneous Melanoma AJCC v8, Melanoma of unknown primary, Pathologic stage IIIB cutaneous Melanoma AJCC v8, (and 3 more...)	Phase 2	Recruiting	NCT04708418
	Drug: Pembrolizumab	Lymphoma	Phase 1, 2	Recruiting	NCT03983668
	Radiation: Liver radiation therapy Drug: Nivolumab Injection [Opdivo] Drug: Ipilimumab Injection [Yervoy]	Colorectal neoplasms malignant, Liver metastases	Phase 1	Unknown	NCT03507699
CpG-7909	Biologica: ELI-002 immunotherapy peptide-based antigens	Minimal residual disease, KRAS G12D, KRAS G12R, (and 9 more...)	Phase 1	Recruiting	NCT04853017
SD-101	Biological: Nivolumab, Ipilimumab	Metastatic uveal melanoma in the liver	Phase 1	Recruiting	NCT04935229
	Biological: Anti-OX40 Antibody BMS 986178	Advanced malignant solid neoplasm, Extracranial solid neoplasm, Metastatic malignant solid neoplasm	Phase 1	Active, not recruiting	NCT03831295
	Biological: Anti-OX40 Antibody BMS 986178 Other: Laboratory Biomarker Analysis Radiation: Radiation Therapy	B-cell non-Hodgkin lymphoma, Grade 1 follicular lymphoma, Grade 2 follicular lymphoma, (and 5 more...)	Phase 1	Active, not recruiting	NCT03410901
	Biological: Nivolumab, Radiation: Radiation Therapy	Metastatic pancreatic Adenocarcinoma, Refractory pancreatic Adenocarcinoma, Stage IV pancreatic cancer AJCC v8	Phase 1	Active, not recruiting	NCT04050085
	Drug: Epacadostat Radiation: Radiotherapy	Advanced solid tumours, Lymphoma	Phase 1, 2	Completed	NCT03322384
	Biological: Nivolumab Biological: Ipilimumab Biological: Pembrolizumab	Hepatocellular carcinoma, Intrahepatic cholangiocarcinoma	Phase 1, 2	Recruiting	NCT05220722

Source: ClinicalTrials.gov.

therapy [84]. Our group developed an injectable host-guest hydrogel system that co-deliver gold nanoparticle (AuNPs) conjugated tumour-antigen peptide and CpG-ODN1826 as nano-vaccine to induce robust anti-tumour T cell response in B16 melanoma mice model [85]. Recently a study reported combining AIRISE-02 (nanoparticles encapsulating CpG-ODN1826 and STAT3 siRNA) with PD-1 and CTLA-4, and the combination achieved significant results in multiple tumour models, both in situ injected tumours and distal tumours regressed, 63% of melanoma mice were completely cured, and this anti-tumour effect also had immunological memory [86]. AIRISE-02 subsequently underwent investigational new drug (IND) evaluation and will soon enter clinical trials. With the in-depth study of drug mechanisms, appropriate drug combinations were designed according to the characteristics of various drugs. AIRISE-02 not only active TLR9 pathway like CpG-ODN, but also overcome the disadvantage of upregulating STAT3 pathway. Meanwhile, PD-1 was used to strengthen the function of effector T cells, which accumulated experience for the rational combination of drugs in the future. There are also a number of CpG-ODN containing cancer vaccines in animal experiments, including breast cancer [83], colorectal cancer [87], melanoma [88] and subcutaneous xenograft cervical cancer [89].

Nowadays, therapeutic cancer vaccines are still in their infancy, although tumour vaccines with advanced materials modification have shown some preventive effects in many animal models, overall the therapeutic effects have been limited. As we all know, tumour heterogeneity varies with different people, and tumour development is also influenced by plenty of factors, it is very difficult to completely eliminate tumours by injecting tumour vaccines alone. Secondly, the screening process of tumour neoantigen is also a limiting factor in the development of tumour vaccines. It takes a lot of time to obtain a truly effective broad-spectrum antigen epitope or to develop personalised tumour vaccines. In this situation, both clinical stratification criteria and epitope screening techniques need to be improved in order to fully support the cancer vaccine development process [90]. The tumour models we usually use and the generic epitopes screened by experimental animals may not be suitable for humans, which is also a problem to be considered in future research. Finally, TME is also a limiting factor for the efficacy of tumour vaccines, as TME not only inhibits antigen uptake and presentation but also inhibits DCs activation and T cells infiltration, all of them making tumour vaccines not as therapeutically effective as desired.

### TLR9 and other agonists

New strategies to trigger multiple PRRs, including different TLRs and STING with specific agonists, have been shown to simultaneously activate multiple signaling pathways to generate robust immune responses for tumour vaccines [91, 92]. Typically, the anti-tumour effects of agonists are more effective in small tumours, Zhao et al. successfully eradicated large primary tumours with the 3M-052 (TLR7/8 agonist) and CpG-ODN [93]. STING agonist recognises circulating dinucleotides (CDNs), which are the second messenger regulating bacterial vital activity. Temizoz et al. evaluated the effect of TLR9 and STING agonists combination in Pan02 peritoneal dissemination model of pancreatic cancer, where CD8<sup>+</sup> T cells and CD4<sup>+</sup> T cells cooperated to control tumour growth [94]. TLR3 recognises the double-stranded RNA (dsRNA) of the viral genome or replication intermediates, and Poly (I:C) mimics this structure. The combination of Poly (I:C) and CpG-ODN has been evaluated in various animal models, such as mouse model of melanoma [95], TC-1-grafted mouse model [96] and ErbB2<sup>+</sup> breast cancer [97]. Aerosol delivery of CpG-ODN plus Poly (I:C) has been shown to effectively treat B16 melanoma lung metastases in C57BL/6 mice [98]. It has been shown that the phosphonothioate modification of CpG-ODN prevents poly (I:C) from entering tumour cells when they are administered

simultaneously. However, using CpG-ODN followed by poly (I:C) administration could avoid this entry blockade [99]. This finding suggests that when combining TLR9 agonists with other agonists for tumour therapy, the order of administration is also important and the relevant mechanisms need to be explored.

### TLR9 and radiation therapy

For decades, radiation therapy (RT) has been an important part of routine treatment for about 40–50% of cancer patients [100]. It has been thought that radiation irreversibly damaged DNA and other large molecules, causing cancer cells to lose their ability to divide and eventually cell death. Recent studies have shown that in addition to directly killing tumour cells, local radiation can also trigger immunogenic cell death (ICD), the release of tumour antigen and produce an abscopal effect, indirectly killing tumour cells through the immune system [101, 102]. After ICD occurs in tumour cells, they release a series of damage-associated molecular patterns (DAMP), such as calreticulin (CRT), ATP and high mobility group protein B1 (HMGB1), which promote APCs maturation and activate CTLs to kill tumour cells [103]. When TLR9 agonist is added to the tumour microenvironment, it may further enhance activation of APCs and promotes cross-presentation of antigens from tumour cells through MHC class I molecules, leading to CD8<sup>+</sup> T cell responses that kill tumour tissue.

Several preclinical studies evaluating the combination of TLR9 agonists and RT have demonstrated their synergistic effects in immunoreactive mouse tumour models. RT therapy cooperates with TLR agonists and has been demonstrated in metastatic lung adenocarcinoma and colon cancer to significantly inhibit tumour growth at both primary and distal tumour sites [104]. Domankevich et al. demonstrated that diffusing alpha-emitters radiation therapy (DaRT) in combination with CpG-ODN delayed tumour growth and cured 41% of colon cancer CT26 mouse models compared to DaRT alone. When DaRT was used in combination with CpG-ODN, Treg inhibitor cyclophosphamide and MDSC inhibitor sildenafil, it cured 51% of the mice. And all of them had immune memory [105]. Based on the fact that CpG-ODN activates the STAT3 pathway, Dayson Moreira and colleagues designed CpG-STAT3ASO in combination with radiotherapy against homologous HPV + mEERL and HPV-MOC2 HNSCC tumours in mice, which induced tumour regression and/or prolonged survival [106]. Zhang et al. designed BC-NF κBODN (NF-κB-specific DECOy DNA linked to CpG-ODN) which could target TLR9-expressing B-cell lymphoma cells, and its combination with local 3-Gy dose radiation successfully blocks the progression of xenografted human lymphoma [107]. In neuroblastoma tumours in which CTLA-4 checkpoint blockade was ineffective, combining radiation therapy with TLR9 agonists achieved an effective anti-tumour response, suggesting that choosing the right combination of treatments is critical for efficacy [108].

### TLR9 and chemotherapy

Chemotherapy drugs are also one of the traditional drugs for tumour treatment, and play an important role in oncology in plenty of aspects: In addition to causing the ICD of tumour cells, it also changes the tumour microenvironment, increase the tumour-infiltrating T cells and NK cells, induces the transformation of M2 macrophages into M1-type macrophages, and reduces the number of immunosuppressive cells such as Treg and MDSC to damage their function [109, 110]. Moreover, chemotherapy increases the permeability of the tumour cell membrane to granzyme B (GrzB) and makes them more sensitive to the cytotoxic effects of CTLs [111].

A recent study has shown that the combined injection of CpG-ODN, α-OX40, and anthracycline completely eliminated local and distant 4T1 breast cancer without significant recurrence [112]. Doxorubicin and CpG-ODN self-crosslinking nanoparticles (CpG-ODN NP) were delivered to mice by hydrogel, which showed

synergistic anti-tumour effects [113]. Research also combined ibrutinib with CpG-ODN and achieved good anti-tumour effects in a mouse model of lymphoma [114]. The combined injection of cyclophosphamide (CXT) hydrogel and CpG-ODN into CT26 mice effectively inhibited tumour formation, and 90% of cured mice were reinoculated with tumour stock for more than 60 days. The combination not only reduced the toxicity of CTX but also produced immune memory [115]. This study demonstrated that chemotherapy drugs cooperate with TLR9 agonists represent a powerful strategy for tumour therapy. In mice genital orthotopic HPV16 TC-1 model, carboplatin/paclitaxel (C + P) chemotherapy combined with HPV16-E7 synthetic peptide (E7LP) vaccine, followed by CpG for intravaginal immune stimulation, significantly improved mice survival as compared to any of the dual treatment [116]. This combination is now being tested in the phase I/II trial (NCT02128126).

However, in practical application, we should also focus on the cytotoxic and myelosuppressive effects of chemotherapy, and how balancing effectiveness and safety remains a key issue [117]. At the same time, the dose ratio and time point when combining chemotherapy with immunotherapy should be considered in order to effectively exert synergistic effects.

### TLR9 and photodynamic therapy

Photodynamic therapy (PDT) is a noninvasive therapeutic that has shown great potential in treating primary tumours with negligible systemic toxicity. PDT has been approved for the treatment of some types of cancers including lung cancer, oesophageal cancer, cervical cancer, etc [118]. However, due to the penetration limitations, the application of PDT has been challenged for the treatment of metastatic tumours or deep-seated tumour. PDT can activate immune response through triggering tumour cell death and the release of tumour antigens [119]. Immunoadjuvants such as CpG can specifically bind to TLR9 in APCs and stimulate a systemic immune response. The combination of CpG with PDT may overcome their own limitations and augment the ability to activate immune system. In fact, it has been reported that a multifunctional nanoplatform combine PDT, photothermal therapy (PTT), docetaxel (DTX) and CpG can markedly inhibit tumour growth in 4T1 tumour-bearing mice model [120]. Xu et al. reported the design of a nanomaterial system combining PDT and personalised cancer immunotherapy. Neoantigen peptides, CpG and photosensitiser chlorin e6 were coloaded into the nanomaterial system. This combination of PDT and personalised cancer vaccine synergistically inhibit both local and distant tumour growth in multiple murine tumour models [121]. Cai et al. reported the design of metal-organic framework (MOF)-based nanoparticles combining PDT, antihypoxic signaling and CpG adjuvant. This nanoparticle inhibits the HIF-1 $\alpha$  induced survival and metastasis [122]. Taken together, new strategies combining PDT and immunoadjuvants represent a suitable therapeutic option for advanced cancer.

### DELIVERY STRATEGIES

Using two different models of immunogenic melanoma, Lou et al. demonstrated the importance of intra-tumoral injection in drug combination therapy. Comparing intra-tumoral injection of a CpG oligonucleotide with intravenous administration, the results showed that intra-tumoral administration was able to generate more infiltration of antigen-specific T cells and more inflammatory chemokines (RANTES, IP-10, MCP-1, MCP5, MIP1 $\alpha$  and MIP1 $\beta$ ) in TME, at the same time intra-tumoral injection was able to trigger anti-tumour response at injection site and distal position [123]. From a pharmacological perspective, the use of intra-tumoral administration increases the bioavailability of TLR9 agonists in TME, controls the scope of drug action, then limits systemic toxicity that may lead to immune-related adverse events (AEs).

However, human tumours are rarely able to be directly injected intra-tumorally, which is one of the factors limits the effect of CpG-ODN in the clinical application.

It has also been shown that inhaled TLR9 agonist administration combined with PD-1 antibody induces CD8<sup>+</sup> T cells to become highly functional CTLs that persistently reject lung tumours and extrapulmonary neoplasms [67]. In addition, this study demonstrates that TLR9 agonists may also have a promising future as adjuvants in combination with inhaled vaccines.

Advances in biomaterials and nanomaterials chemistry have also greatly advanced the course of immunotherapy [124]. A variety of advanced materials are developed for the delivery of immune agents, such as hydrogels [88, 113, 115], microneedle patches [125], polylactic-co-glycolic acid (PLGA) [53] and liposomes [126], albumin nanoparticles [127], inorganic particles such as silica particles [128] and gold nanoparticle [129], iron oxide [130] which can also be used as diagnostic carriers. CpG-ODN is often loaded into materials through electrostatic adsorption, covalent bonding, hydrophilic and hydrophobic interactions, DNA self-assembly and so on [131]. Compared with direct injection, material encapsulated drugs can reduce side effects to a certain extent, allow multiple drugs to act synergistically on the same cells, in the meantime achieve targeted drug delivery and sustained release, overcome the adverse drug kinetics and high miss rate of the drug itself. Thus, it is possible to reduce the drug doses and precisely delivery to target cells through muscle or intravenous injection can still play an excellent anti-tumour effect in clinical practice.

### CONCLUSIONS AND PERSPECTIVES

Clinical trials told that TLR9 receptor agonist CpG-ODN, as a single tumour treatment drug, showed a good anti-tumour effect in animal experiments, but did not completely cure patients in clinical trials. Therefore, appropriate CpG dose should be selected in combination with other drugs to improve the anti-tumour effect while minimising side effects. At present, TLR9 agonists and tumour immune checkpoint inhibitors are expected to have great potential in tumour immunotherapy. With the development of the nanotechnology, the use of different packaging materials in drug design also improves targeting and therapeutic effectiveness. Furthermore, in order to achieve the ideal therapeutic effect, we should also strengthen the basic research of each drug, only through in-depth understanding of its potential mechanism of action, can we achieve better therapeutic effects.

Due to the complexity of tumorigenesis and development, multiple factors may limit the efficacy of single immunotherapy in solid tumours. The mechanisms of primary and acquired drug resistance to tumours are possibly due to lack of adequate antigen presentation (insufficient neoantigen or impaired antigen processing or presentation); insufficient immune cell infiltration into TME; T cell exclusion; T cell unresponsiveness; impaired interferon- $\gamma$  signaling; presence of immunosuppressive cells; expression of multiple suppressive immune checkpoints and T cell loss of function/T cell failure [132]. To conquer above limitations, it may be necessary to target multiple steps of tumour immunity, at the same time apply multiple drugs together to achieve complete elimination of tumour. Combination drugs may be able to strengthen each step of various cells in the process of immune response in a balanced way, reduce the harm caused by excessive activation of immune cells, then achieve the therapeutic effect. So far, researchers have tried hundreds of drug combinations, and have accumulated a lot of raw data that might lead to a generalised formula for combinations. Further research works are needed to determine which of these combination strategies are capable of improving outcomes in patients.



**DATA AVAILABILITY**

Not applicable.

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ZCDY, JL and YZW: conceptualisation, writing and editing.

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#### COMPETING INTERESTS

The authors declare no competing interests.

#### ADDITIONAL INFORMATION

**Correspondence** and requests for materials should be addressed to Jian Li or Yuzhang Wu.

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