

The promising results of clinical trials using immune checkpoint inhibitors revived interests in cancer immunotherapy. However, it also became apparent that efficacy of immune checkpoint blockade can benefit from combining it with immunostimulatory strategies. Here, we review prior and re-emerging approaches using Toll-like Receptor 9 (TLR9) agonists, CpG oligodeoxynucleotides (ODNs), focused on the generation of antitumor immune responses in cancer patients. While numerous early clinical trials using TLR9 ligands in monotherapies provided evidence of CpG ODNs tolerability and safety, they failed to demonstrate sufficient antitumor efficacy. Recent studies unraveled multiple levels of negative regulation of immunostimulatory TLR9 signaling in immune cells by the tumor microenvironment that can stifle immune activity in cancer patients. Therefore, CpG ODNs-based strategies can greatly benefit from combination with strategies targeting immune checkpoint regulation. The most recent clinical trials of CpG ODNs together with immune checkpoint inhibitors have a chance to generate novel, more effective and safer cancer immunotherapies.

Key words: CpG-based cancer immunotherapy, Stat9, TLR-9, tumor.

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The revival of CpG oligonucleotide-based cancer immunotherapies

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The origins of TLR9-based cancer immunotherapy

The beginning of modern cancer immunotherapy is marked by experiments carried out in 1891 by Dr. William Coley, who treated patients with incurable cancers by intratumoral injections of bacterial lysates. Antitumor effects of Coley's toxins have not been understood until almost a century later, when in 1983 studies by Tokunaga *et al.* identified DNA as major immunologically active component of another bacterial immunoadjuvant [1]. It took until 1995 to identify the unmethylated, dinucleotide CpG motif present in bacterial DNA and also in synthetic oligonucleotides as responsible for immune activation [2]. In turn, these observations led Dr. Arthur Krieg to the design of single-stranded CpG oligodeoxynucleotides (CpG ODNs) as synthetic immunoadjuvants. Finally in 2001, Shizuo Akira and his group succeeded in cloning an intracellular protein, Toll-like receptor 9 (TLR9), expressed in human B cells and in plasmacytoid dendritic cells (pDCs) or in all myeloid cells in mice, as responsible for sensing of CpG motifs in the DNA [3]. It became increasingly clear that triggering CpG-TLR9 signaling pathway leads to upregulation of proinflammatory genes such as IL-6, TNF- α and type-I interferons (IFN- α and IFN β), through activation of MyD88 adaptor proteins leading to recruitment of kinases and downstream activation of IRF and NF- κ B signaling [4]. Subsequent efforts resulted in the design of various classes of CpG ODNs differing in structural characteristics and immunomodulatory activities. The CpG class A (also known as type D), form multimers through interaction of G-rich 3' termini and are known to stimulate pDCs maturation and secretion of IFN- α . The monomeric CpG class B (or type K), strongly activate B cells, induce maturation of pDCs and production of TNF- α . Finally, the dimeric CpG class C, seem to combine effects of both previous CpG ODN types albeit with intermediate intensity. All classes of CpG ODNs are equipped with partly (CpG-A) or completely (CpG-B/C) phosphorothioated (PS) sugar backbone to prevent their degradation by serum nucleases, which enhances their *in vitro* and *in vivo* activity. Promising results of preclinical studies, which demonstrated potent immunostimulatory and antitumor effects of CpG ODNs, aroused interest in clinical application of these immunoadjuvants to treatment of human cancers.

The rise and the fall of CpG-based cancer immunotherapies

Initial studies on CpG ODNs demonstrated their efficacy in several preclinical tumor models, especially in hematologic malignancies, such as B cell leukemia and lymphoma. The CpG ODN triggered activation of the downstream TLR9 signaling and secretion of proinflammatory cytokines was shown to induce CD4⁺T_H1 cells activity, thereby resulting in cytotoxic CD8⁺T cell responses *in vivo* [5]. Several clinical trials explored the potential of using CpG ODNs as an immunoadjuvants for cancer vaccines. Tumor vaccination using CpG7909 (class B) administered together with synthetic peptide

antigens induced tumor antigen-specific cells CD8⁺T cells in NSCLC, melanoma, breast cancer and sarcoma patients [6,7,8]. Beyond cancer vaccines, CpG ODNs were tested as single-agents and in combination with standard therapies, such as chemo-, radiotherapy, and also as immunoadjuvants for cancer vaccines. Encouraging results and the evidence of humoral and cellular immune responses resulting from TLR9 stimulation, generated strong rationale for clinical testing of CpG ODNs not only for cancer treatment but also for therapy of infectious and allergic diseases. In mid-2000s, first clinical studies focused on highly immunogenic melanomas and other skin cancers. The single agent CpG ODNs trials using local administration in melanoma patients were met with limited success. The most promising effects were the augmented release of proinflammatory cytokines only occasionally followed by the elevated percentage of NK cells and CD8⁺T cells limited to the periphery and not detectable in tumor tissues [9,10]. Another effort combining CpG7909 with standard dacarbazine treatment in metastatic melanoma patients showed no evidence of clinical benefits [11]. Locally administered TLR9 agonists were also evaluated in patients with recurrent glioblastoma (GBM), non-small-cell lung cancer, and metastatic colorectal cancer generating minor antitumor response with moderate increase of patients' survival [12,13,14,15]. Compared to systemic administration, the results of local CpG treatments were more promising. The clinical trial in Non-Hodgkin's lymphoma (NHL) patients treated with CpG7909 in combination with rituximab or local tumor radiotherapy showed partial responses including recruitment of tumor-infiltrating CD8⁺T cell [16–21].

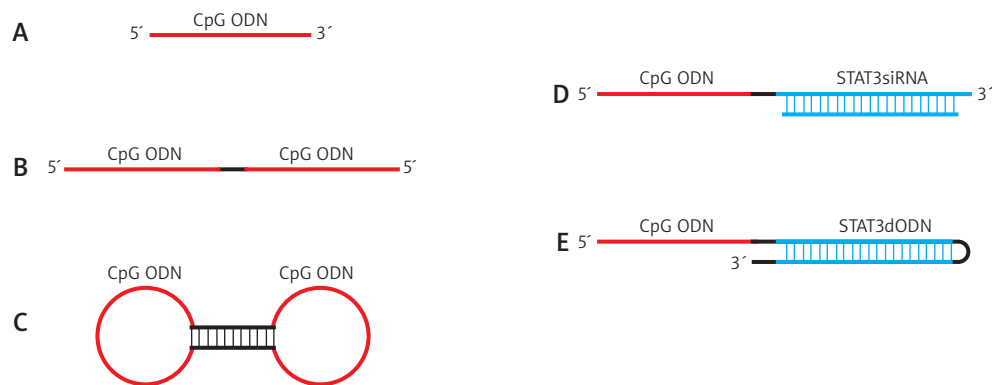
Discrepancy between promising preclinical results and rather unimpressive clinical outcomes at least partly resulted from different patterns of TLR9 expression in humans (selective in pDCs and B cells) and more broad in rodents (in all myeloid cells). Consequently, in mice CpG ODNs monotherapy is often sufficient for induction of potent antitumor effects. Unfortunately, these effects are far less likely to occur in patients with established cancers and potentially immunosuppressive tumor microenvironment. The positive conclusion of clinical trials using CpG ODN was undoubtedly the evidence that TLR9 agonists can be well tolerated by cancer patients. Primary adverse effects were mostly related to immunostimulation and indicated by dose-dependent local injection reactions (e.g. erythema, pain, swelling) or systemic flu-like reactions (e.g. headache, rigors, nausea and vomiting) [22]. Nevertheless, prolonged treatment with CpG ODNs and chronic secretion of IFNs could possibly lead to autoimmune disorders [23]. While PS modified ODNs are known for potential liver and kidney toxicities, these effects are more likely to occur in case of systemic administration of antisense ODN requiring higher dosing than immunostimulatory CpG ODN. Thus, continued efforts have been made to further improve immunomodulatory properties, safety and delivery of CpG ODNs. Among others, protein/peptide-CpG ODNs conjugates and nanomaterial-CpG ODNs complexes have shown successful preclinical results and undergone clinical evaluations [24].

It takes two: TLR9 stimulation and immune checkpoint blockade

Studies of molecular mechanisms underscoring tumor immune evasion resulted in the identification of so called immune checkpoint regulators (ICR). The immune checkpoints under normal physiologic conditions ensure the negative control over immune responses to prevent dangerous, unrestricted inflammation. The two best characterized ICRs are: cytotoxic T lymphocyte antigen 4 (CTLA-4) and programmed cell death protein 1 (PD-1). The CTLA-4 expressed by T-cells and plays critical role in limiting their early immune responses in lymphoid tissue. PD-1 is more broadly expressed on activated T-cells, B cells and myeloid cells and is thought to regulate late immune responses in peripheral tissues [25]. Two different PD-1 ligands, PD-L1 and PD-L2 respectively, are expressed on both tumor cells and tumor infiltrating immune cells. Interestingly PD-L2, but not PD-L1, triggers signaling in DCs cells leading to IL-12 production and thus differentiation of T cells into T_H1 cells [26]. Therefore PD-L1 was chosen to be a perfect candidate for immune checkpoint inhibitor without disrupting native immune activity. Therapies targeting CTLA-4 and PD-1/PD-L1 showed notable efficacy in clinical trials in patients with various cancer types, including certain solid tumors. In spite of significant responses percent of patients still failing to respond remains high and some types of human cancers, for example advanced prostate cancer, showed resistance to ICR [27], suggesting the need for combinatorial immunotherapeutic approaches. Beyond standard therapies, recent preclinical studies identified CpG ODNs as ideal candidates for supporting ICR targeted cancer therapy. Preclinical studies demonstrated synergy between blocking CTLA-4 expression in order to revive T cell activity and CpG ODN-mediated activation of antigen presenting cells in melanoma mouse model [28]. Synergistic effect was also observed when CpG ODNs were combined with CTLA-4 or PD-1 antibodies in murine bladder cancer, resulted in improved long term survival [29]. Another study showed that limited responsiveness to anti-PD-1 treatment of colon carcinoma, can be significantly increased by simultaneous administration of TLR agonists, to generate durable T-cell response [30]. These studies provided strong rationale for combined clinical regimens based on the stimulation using CpG ODNs and immune checkpoint inhibitors (Table 1). The ongoing clinical trial in metastatic melanoma patients focuses on the combination of a modified TLR9 agonist in proprietary 3'-3' dimer design (IMO-2125; Idera Pharmaceutical) with CTLA4 blockade (ipilimumab) to enhance immunostimulatory properties in (Fig. 1B). The competing approach for the treatment of metastatic melanoma utilizes CpG class C (SD-101; Dynavax) together with PD1 inhibition (pembrolizumab). Another original strategy utilizes an innovative design of TLR9 agonist in the circular form (MGN1703; Mologen), which at the same time improves both immunostimulatory activity and stability of the ODN. The MGN1703 contains two loops with three CpG motifs each, linked together through stable double-stranded linker (Fig. 1C). The molecule showed superior activation of immune cells compared to single stranded CpG ODNs,

Table 1. The new combinatorial CpG ODN-based cancer immunotherapies in clinical trials

Product	Target	Institution	Development phase
IMO-2125 CpG-3'-3' fusion	TLR9/CTLA-4	Idera Pharmaceutical	Phase 1/2 (metastatic melanoma): in combination with ipilimumab
SD-101 CpG-C	TLR9/PD-1	Dynavax	Phase 1/2 (metastatic melanoma): in combination with pembrolizumab
MGN1703 double loop CpG	TLR9/CTLA-4	Mologene AG	Phase 1 (advanced solid tumors): in combination with ipilimumab
CMP-001 CpG-A/VLP	TLR9/PD-1	Checkmate Pharmaceuticals	Phase 1/2 (advanced melanoma): in combination with pembrolizumab
CSI-2 CpG-B conjugate	TLR9/STAT3	City of Hope	Phase 1 planned for 2019 (Non-Hodgkin's B cell lymphoma)

**Fig. 1.** Various designs of CpG ODN-based immunotherapeutics. **A)** Single-stranded CpG ODN. **B)** IMO-2125 consists of two 3'-3' linked single-stranded CpG ODNs. **C.** MGN1703 molecule comprises two loops, each containing three CpG motifs, connected with double stranded stem. **D–E)** CSIs – dual function CpG-conjugates with STAT3 inhibitors, CpG-STAT3siRNA (**D**) and CpG-STAT3dODN (**E**).

while toxicity studies revealed no changes in liver, spleen or lymph node weight³¹. MGN1703 is currently being evaluated for therapy of advanced solid tumors in combination with anti-CTLA4 inhibitor. Finally, a recent clinical study focused on the unmodified and highly potent TLR9 agonists (CMP-001) encapsulated into noninfectious virus-like particles (VLPs) to ensure oligonucleotide stability. Preclinical studies showed that CMP-001 formulation had improved immunogenic effect on TLR9-positive target immune cells due to the lack of backbone phosphorylation [24]. CMP-001 is in clinical testing in combination with anti-PD-1 inhibitor treatment in patients with advanced melanoma.

Dual function CpG-siRNA/dODN conjugates

Signal transducer and activator of transcription 3 (STAT3) is an oncogenic transcription factor commonly activated both in cancer cells and tumor-associated immune cells. STAT3 plays pivotal role in a crosstalk between cancer cells and the tumor microenvironment, including immune cells, endothelial cells and fibroblasts. Persistent activation of STAT3 corresponds to increased proliferation, survival and invasion of cancer cells regulated by c-MYC, Cyclin D1/2, BCL-X_L and many others [32]. Furthermore, STAT3 suppresses antitumor immunity through multiple mechanisms from inhibition of expression of immunostimulatory mediators, through degradation of MHC class II molecules to secretion of immunosuppressive mediators such as Arginase-1, IL-10 or VEGF [33, 34]. Importantly, STAT3 is also

known to control PD-L1 expression directly contributing to immune checkpoint control [35, 36]. As shown before, STAT3 activity is also triggered by cytokines release in order to stress and inflammation, downstream from Toll-like receptor (TLR) and NF- κ B signaling [37]. Given that STAT3 is a nodal point in multiple oncogenic pathways and a central immune checkpoint regulator, it is a unique and highly desirable target for cancer immunotherapy. Small molecules inhibitors targeting STAT3 itself, or its upstream regulators, such as Janus kinase (JAK) or Vascular Endothelial Growth Factor Receptor (VEGFR) did not meet expectations in selective and potent STAT3 blocking [38]. The direct STAT3 targeting proved challenging for pharmacologic approaches due to lack of the enzymatic activity leading to the development of alternative oligonucleotide-based strategies [39, 40]. One of the most advanced approaches is STAT3 antisense oligonucleotide (ASO), AZD9150 (Astra-Zeneca). Preclinical studies on AZD9150 demonstrated efficient target knockdown in cancer cells and growth inhibition of various tumor models and led to phase I clinical trial. While the clinical trial provided evidence of antitumor efficacy, the biodistribution studies revealed that STAT3 inhibition occurred mainly in the tumor-associated myeloid cells, such as macrophages, but not in cancer cells [41]. This is likely due to limited internalization rate of ASO by tumor cells compared to immune cells. Consequently, the follow up clinical trials shifted attention to the combination of AZD9150 with immune checkpoint blockade,

namely PD-L1 inhibitor (MEDI4673; durvalumab). These strategies should synergize in disrupting the immunosuppressive tumor microenvironment, although partial functional overlap of both strategies is possible based on the known role of STAT3 in PD-L1 upregulation [36].

Among multiple functions of STAT3 as a negative feedback regulator, it is specifically involved in restricting the outcome of TLR9 signaling through secretion of IL-6/IL-10. Therefore, STAT3 inhibition provides an opportunity for augmenting the potency of TLR9 agonists in the tumor microenvironment. The proof-of-concept studies in mice demonstrated that in the absence of STAT3 signaling in hematopoietic cells, even a single intratumoral injection of CpG ODN can cause complete and long term regression of large (> 1 cm) B16 melanoma tumors. These therapeutic effects resulted from an unleashed innate and adaptive antitumor immunity without any indication of autoimmune disorders within the treatment window [42]. These findings provided a strong rationale for combining CpG ODNs with STAT3 oligonucleotide inhibitor (CSIs) into a single molecule. The simultaneous release of checkpoint blockade and immune stimulation leads to the gain-of-function effect amplifying therapeutic efficacy of CSIs. Such design that STAT3 gene silencing coincides with immunostimulation of TLR9-positive tumor-associated myeloid cells, thereby generating potent gain-of-function effect. The first generation of CpG-STAT3 inhibitor (CSI-1) utilized RNA interference (RNAi) for STAT3 silencing (Figure 1D). The CpG-STAT3siRNA conjugates are quickly internalized by TLR9-positive target cells, resulting in the downregulation of STAT3 expression and augmented TLR9 signaling. Both CpG-STAT3siRNA effects act in concert to stimulate production of interleukin 12 (IL-12) and interferon- γ (IFN- γ), which are critical mediators of antigen-presentation and T_H1 immune responses [43].

Local and systemic treatments using CSI-1 can effectively inhibit growth of various syngeneic solid tumor models, such as melanoma, glioma, bladder and colon cancers in mice mainly through stimulatory effect on innate and adaptive immunity [44]. Some of these therapeutic effects were also validated using human immune cells. For example, when treated using CpG-STAT3siRNA, myeloid-derived suppressor cells (MDSCs) derived from prostate cancer patients showed reduced inhibitory effect on T cell proliferation and cytotoxicity [45]. CpG-STAT3siRNA showed also remarkable efficacy against hematopoietic malignancies, including acute myeloid leukemia and B cell lymphoma [46]. The repeated systemic administration of CSI-1 triggered immunogenic effects in AML cells and resulted in significantly reduced leukemia-initiating potential.

In spite of the efficacy in mouse tumor models, relatively short half-life in human serum limits further clinical application of CpG-siRNA conjugates to local administration. To overcome this issue, the second generation CpG-STAT3 inhibitor (CSI-2) utilized nuclease-resistant decoy oligodeoxynucleotide (dODN) design (Fig. 1E). CpG-STAT3dODN comprises high affinity consensus DNA sequence recognizing and binding STAT3 to inhibit its activity. In target cells, CSI-2 binds the activated STAT3 and prevents its migration to the nucleus and downstream gene regulation

[47]. Preclinical evaluation showed that CSI-2 is effective when injected intravenously against mouse models of disseminated acute myeloid leukemia (AML) [48] and B cell lymphoma (Zhao and Kortylewski, unpublished data). Reduced tumor burden correlated with recruitment of CD8⁺ T cells into leukemia and lymphoma reservoirs in bone marrow, spleen or lymph nodes. Synergistic effect of TLR9 activation and STAT3 blocking was essential for the generation of memory T cells and long-term antitumor immunity. CSI-2 is currently undergoing toxicology studies in preparation for clinical testing in phase 1 clinical trial in B cell lymphoma patients (Table 1).

Future of CpG oligonucleotide-based cancer immunotherapies

There is a growing consensus that maximizing benefits of immunostimulatory agents for cancer therapy requires elimination of the negative influence of the tumor microenvironment. Current early clinical trials explore the possibility of combining TLR agonists and also other emerging immunoadjuvants, like for example STING, together with the FDA-approved immune checkpoint inhibitors. The growing list of ICRs creates additional combinatorial opportunities, which can address intricacies of various types of human cancers and their immunosuppressive effects. At the same time, the growing arsenal of oligonucleotide strategies, including siRNA, antisense or decoy oligonucleotides, makes it possible to target central regulators of tumor immune tolerance, such as STAT3, which until now remained undruggable. Combination of these gene- or protein-selective inhibitors with CpG-mediated delivery to immune cells provides a unique opportunity to overcome limitations in both oligonucleotide delivery as well as therapeutic efficacy. Cancer immunotherapies addressing the complexity of cellular and molecular networks operating in the tumor microenvironment can provide new avenues for more precise and effective treatments for human malignancies.

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