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.
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 rational 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 effect 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 ODNs 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,1 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 TLR9 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 Pharmaceuticals) with CTLA4 blockade (ipilimumab) to enhance immunostimulatory properties in (Fig. 1B). The competing approach for the treatment of metastatic melanoma utilizes Cp G class C (SD-101; Dynavax) together with PDI 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,
while toxicity studies revealed no changes in liver, spleen or lymph node weight [31]. 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 phosphorothioation [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 in both 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, 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-κB 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 molecule 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.

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

| Product    | Target | Institution          | Development phase                                      |
|------------|--------|----------------------|-------------------------------------------------------|
| IMO-2125   | TLR9/CTLA-4 | Idera Pharmaceutical | Phase 1/2 (metastatic melanoma): in combination with ipilimumab |
| SD-101     | TLR9/PO-1  | Dynavax              | Phase 1/2 (metastatic melanoma): in combination with pembrolizumab |
| MGN1703    | TLR9/CTLA-4 | Mologene AG          | Phase 1 (advanced solid tumors): in combination with ipilimumab |
| CMP-001    | TLR9/PO-1  | Checkmate Pharmaceuticals | Phase 1/2 (advanced melanoma): in combination with pembrolizumab |
| CSI-2      | 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).
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 know 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 (CSI-1) 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 the CpG-STAT3 oligonucleotide expression and cytotoxicity [45]. CpG-STAT3siRNA showed also remarkable efficacy against hematopoietic malignancies, 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.

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 though 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 oligodeoxyribonucleotide (dODN) design (Figure 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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