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Recent progress concerning CpG DNA and its use as a vaccine adjuvant

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Abstract

CpG Oligonucleotides (ODN) are immunomodulatory synthetic oligonucleotides designed to specifically agonize Toll-like receptor 9. Here, we review recent progress in understanding the mechanism of action of CpG ODN and provide an overview of human clinical trial results using CpG ODN to improve the vaccines for cancer, allergy and infectious disease.

Keywords

allergy; cancer; CpG oligonucleotide; infection; Toll-like receptor; vaccine adjuvant

The host immune response induced by exposure to an infectious agent requires immediate recognition of the pathogen. This recognition event initially triggers the innate immune system. Janeway was the first to postulate the existence of an innate immune recognition system (pattern recognition theory) and suggest the principles of innate control of adaptive immunity [1]. His predictions were confirmed and expanded upon in subsequent years. Hoffmann and collaborators described the function of the Toll gene in innate protective responses in flies while Beutler and collaborators discovered its mammalian homologs, the Toll-like family of receptors (TLRs) [2,3]. This series of Nobel prize winning discoveries form a conceptual framework for the current understanding of the innate immune system, the links between innate and adaptive immunity, and strategies to improve vaccine development.

Upon infection, an innate immune response is triggered by evolutionarily conserved molecular structures expressed by the infectious organism. These structures, termed pathogen-associated molecular patterns, are recognized by host TLRs [4]. Pathogen-associated molecular patterns are expressed by a wide variety of infectious microorganisms. For example, lipopolysaccharide and lipoprotein are commonly found on bacterial cell walls and are recognized by TLR4 and TLR2. Flagellin, a major component of bacterial flagella, is recognized by TLR5. dsRNA is recognized by TLR3 while ssRNA is detected by TLRs

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7/8. Unmethylated CpG motifs present in bacterial and viral DNA (referred to as CpG DNA) are recognized by TLR9 [4].

The innate immune response elicited by TLR activation is characterized by the production of proinflammatory cytokines, chemokines, type I IFNs and antimicrobial peptides [5]. Subsequently, the host mounts an adaptive immune response characterized by the expansion of antigen-specific T and B cells resulting in the production of high-affinity antibodies (Abs) and the generation of cytotoxic T cells that provide sterilizing immunity and ensure long-lasting memory to protect against subsequent infection [6]. Since agents that support the efficient induction of innate immune responses thus contribute to adaptive immunity, their potential utility as vaccine adjuvants is of considerable interest.

However, there was some concern over the safety of TLR ligands, particularly the possibility that they might increase susceptibility to toxic shock or autoimmune disease. Ligands for TLR2 and TLR4 were of particular concern in this context, as they did induce septic shock in animal models [7]. Similarly, experimental asthma was exacerbated by TLR2 and TLR4 ligands, as they elicit an immune response dominated by a Th2 (pro-allergic) profile [8,9]. These findings increased interest in TLR agonists with a strong safety profile.

TLR9 recognizes and is activated by CpG motifs (consisting of a central unmethylated CG dinucleotide plus flanking regions). TLR9 engagement triggers a protective immune response that improves the host's elimination of infectious pathogens [10,11]. Oligonucleotides (ODN) containing CpG motifs trigger cells that express TLR9, thereby promoting the maturation of APCs. When CpG ODN are co-administered with antigen (Ag), the resulting response is biased toward Th1 immunity [12]. Plasmacytoid dendritic cells (pDC) recognize CpG ODN and play a key role initiating the ensuing immune response. Upon stimulation they can secrete large amounts of type 1 IFNs and support the induction of Ag-specific T cells [13]. pDC do not express TLRs 2, 3, 4, 5 or 6 [14]. While several TLR signaling pathways (those triggered via TLRs 3, 4, 7 and 9) induce the production of type I IFNs, only TLR7 and TLR9 elicit both IFN-α and IFN-β (TLR3 and 4 induce only IFN-b) [15]. Through secretion of IFN-α, pDC enhance the cytotoxicity of NK, $CD8^+$ T cells and Th1 cells, since these CpG-adjuvanted effects were not observed in IFN-α βR KO mice [16,17]. Of importance, a series of studies showed that CpG ODN did not induce toxic shock or increase susceptibility to autoimmune disease. Moreover, TLR9 ligands actually confer protection against allergic asthma, as they primarily elicit a Th1 biased immune response [18,19]. Thus, a practical advantage to the use of CpG ODN is their safety profile.

Recognition of CpG motifs

It was initially believed that TLR9 recognized bacterial but not mammalian DNA and that this ability primarily reflected differences in the structure of DNA from those sources since mammalian DNA has far few unmethylated CpG motifs than bacterial DNA due to a combination of CpG suppression and CpG methylation (mammalian stop codons contain CG dinucleotides and thus are both selected against and methylated in mammalian genomes). However, some unmethylated CpG motifs are present in the genome of mammals and should be detectable by pDC, suggesting that stimulation of TLR9 is determined by the relative

frequency of such motifs. Recent findings indicate that additional factors are also relevant. TLR9 is located in the endoplasmic reticulum and endolysosomal compartments of cells [20]. These locations contribute to receptor identification of pathogenic DNA, as they are normally devoid of self DNA but may contain nucleic acids released by pathogens (e.g., following the phagocytosis and degradation of infectious viruses) [21]. Moreover, self DNA is accessible to rapid degradation by extracellular DNAses whereas foreign DNA is typically encapsulated by the bacterial cell wall or in viral particles and thus protected. Thus, DNA derived from pathogens can reach and be sensed by TLR9 in endosomes whereas self DNA cannot. Of interest, when cells were experimentally induced to express TLR9 on their surface membrane, self DNA in serum was recognized and the animals developed lethal inflammation [22]. Similarly, when self DNA forms complexes with anti-DNA Abs, the antimicrobial peptide LL37, or various cationic lipids, such complexes are able to activate TLR9 and induce the production of type I IFNs [23–25].

The binding of CpG DNA to TLR9 induces proteolytic cleavage of the receptor [26,27]. After exiting the ER, the TLR9 ectodomain is cleaved by asparagine endopeptidase and/or cathepsins [28,29]. It is this truncated (rather than full-length) form of TLR9 that then recruits myeloid differentiation factor 88 (MyD88). Indeed, if proteolysis of the receptor is prevented, it becomes non-functional. The requirement for ectodomain cleavage provides another mechanism by which receptor activation is restricted to endolysosomal compartments, further preventing TLR9 from responding to self DNA.

The signaling pathway triggered by the interaction of CpG DNA with TLR9 proceeds through the recruitment of MyD88, IL-1R-associated kinase (IRAK), and tumor necrosis factor receptor-associated factor 6 (TRAF6), and subsequently involves the activation of several mitogen-activated kinases (MAPK) and transcription factors (such as NF-κB and AP-1) culminating in the transcription of proinflammatory chemokines and cytokines [5].

Classes of CpG ODN

Four classes of CpG ODN have been identified that differ in structure and immunological activity. ‘K’-type ODNs (also referred to as ‘B’ type) are composed of one or multiple CpG motifs typically on a phosphorothioate backbone. This backbone enhances resistance to nuclease digestion when compared with native phosphodiester nucleotides, providing the ODNs with a substantially longer half-life *in vivo* (30–60 min compared with 5–10 min for phosphodiester) [30]. Phosphodiester K ODN can also be used, but these need to be protected from nucleases by incorporation into liposomes or DOTAP. K-type ODNs trigger pDC to differentiate and produce TNF-α and stimulate B cells to proliferate and secrete IgM [31,32].

‘D’-type ODNs (also referred to as ‘A’ type) have a phosphodiester core flanked by phosphorothioate terminal nucleotides. They carry a single CpG motif flanked by palindromic sequences and have poly G tails at the 3′ and 5′ ends (these facilitate the formation of concatamers). D-type ODNs trigger pDC to mature and secrete IFN-α but have no effect on B cells [33,34]. The distinct activities of K- versus D-type ODNs are largely due to differences in the retention times of CpG/TLR9 complexes in the endosomes of pDC

[35,36]. Whereas K-type ODNs are rapidly transported through early endosomes into late endosomes, D-type ODNs are retained for longer periods in the early endosome. It is in the early endosomes that D-type ODNs interact with MyD88/IRF-7 complexes, triggering a signaling cascade that supports IFN-a production [36]. While the utility of K-type ODN has been explored in a number of clinical trials, human testing of D-type ODN has been difficult due to the proclivity of their poly-G tails to form complex multimers in solution. D-type CpG ODN were recently packaged into stable virus-like particles (VLP) and used as adjuvants in preclinical and clinical studies [37].

C-type ODNs resemble K-type in being composed entirely of phosphorothioate nucleotides but resemble D-type in containing palindromic CpG motifs and thus can form stem loop structures or dimers. This class of ODN stimulates B cells to secrete IL-6 and pDC to produce IFN-α. C-type ODNs have activity in both early and late endosomes, and thus express properties in common with both K- and D-type ODNs [38,32]. Phosphodiester linkages can be introduced into the CG dinucleotides (referred to as semi-soft C) a modification reported to further enhance the activity of C-type ODN [201].

P-Class CpG ODN contain double palindromes that can form hairpins at their GC-rich 3′ ends as well as concatamerize due to the presence of the 5′ palindromes. These highly ordered structures are credited with inducing the strongest type I IFN production of any class of CpG ODN [39].

TLR9 expression in B cells & pDC

In humans, TLR9 is primarily expressed by B cells and pDC while in mice multiple additional myeloid cells (including monocytes, macrophages and mDC) express TLR9 and thus can respond to CpG stimulation. Although this difference can present a problem in transitioning from preclinical animal studies to clinical use, results derived from mouse experiments have been reasonably successful in predicting the therapeutic potential of CpG ODNs in clinical trials.

In humans, TLR9 is primarily present within B cells and pDC [40]. Whereas TLR9 activation alone is sufficient to induce human memory B cells to proliferate, undergo class switching to IgG2a and secrete Abs in a T-cell-independent response, naïve B cells express only very low levels of TLR9 and do not respond directly to CpG ODN [41,42]. Ag stimulation through the B-cell receptor induces naïve B cells to upregulate TLR9 expression and acquire CpG responsiveness [42,43]. Physiologically, the requirement that naïve B cells be activated by Ag before becoming responsive to CpG stimulation would restrict stimulation to Ag-specific B cells and thus prevent unwanted polyclonal B cell activation. To substantiate the synergy between BCR ligation and CpG ODN stimulation, Eckl-Dorna and Batista showed that crosslinking of CpG and Ag enhanced the generation of class switched Ab *in vivo* [43]. This observation is also consistent with the ability of CpG ODN to act as vaccine adjuvants, inducing strong humoral immune response when co-presented with Ag.

TLR expression by human pDC is restricted to TLRs 7 and 9 whereas mDC express a broad profile of TLRs (including TLRs −2, −3, −4, −5, −6 and −8). These divergent patterns of

TLR expression support the concept that individual DC subsets generate distinct/tailored responses to different pathogens (mDC primarily recognize infectious bacteria whereas pDC primarily recognize viruses). Thus, CpG ODN can be used as a vaccine adjuvant to trigger the specific type of response elicited by pDC [14].

pDC play a central role in the mediation of CpG-induced immune responses. pDC are characterized by their ability to rapidly produce large amounts of type I IFNs in response to viral infection, and it is by this means that host protection from viruses is initiated [13]. CpG ODN similarly elicit the production of IFN-α by pDC (especially the D/A and C classes) and in addition trigger a program of cell activation and differentiation characterized by upregulation of co-stimulatory molecules including CD80, CD86 and CD40, expression of the functional marker CCR7 and the maturation marker CD83 [44]. These effects improve the ability of pDC to support T-cell stimulation. These findings suggest that CpG ODN should be effective as adjuvants for antiviral vaccines (particularly the induction of strong cytotoxic T lymphocyte [CTL]). It should be noted that in addition to their direct effect on B cells and pDC, CpG ODN indirectly support the activation of additional cell types, including T cells and myeloid DC through the intensive crosstalk that occurs between mDC and pDC [45,46].

CpG ODN as vaccine adjuvants: preclinical findings

Numerous preclinical studies examining the immunogenicity of CpG-adjuvanted vaccines have been conducted. Results show that CpG ODN enhance both humoral and cellular (Th1 cells and CTL) immunity against pathogens, allergens and tumor antigens in mice. More recent work has focused on vaccine strategies designed to extend the utility of CpG ODN as vaccine adjuvants. These include altering the method of delivery, conjugation to vaccine Ag and in combination with other TLR ligands (Table 1).

Impact of delivery method

To optimize vaccine responses, APCs should be exposed to CpG ODN plus Ag simultaneously. This was initially established in murine studies involving the model Ag ovalbumin [47], and extended to a number of vaccine candidates. Studies of a vaccine directed against the hepatitis B surface Ag showed that administering CpG ODN at the same time to the same site significantly enhanced the resultant Ab response. By contrast, when CpG ODN was administered to a different site than the Hep B vaccine, no significant improvement in immunity was observed over vaccine alone [30].

A number of delivery strategies to insure that CpG ODN and Ag are co-delivered to the same APCs have been devised. These include synthesizing CpG–Ag conjugates, co-encapsulation in liposomes, incorporation into biodegradable microparticles (including alginate and cationic microparticles) and multicomponent nanorods (Table 1). Animal studies show that co-delivery of CpG ODN plus Ag generally increases the speed and duration of the resultant immune response and tends to improve the immunogenicity of weak Ags. In some studies, conjugating CpG ODN to Ag boosted immunity by up to 100-fold over that induced by simply mixing CpG ODN with the immunogen [19,48,49].

Similarly, conjugating CpG ODN to cancer vaccines composed of apoptotic tumor cells enhanced antitumor immunity [50]. Immunization with CpG-conjugated tumor cells triggered the expansion of tumor-specific CTL that reduced the growth of established tumors and prevented their metastatic spread in mice. The mechanisms by which CpG ODN–Ag conjugates enhance immunogenicity includes ensuring that both Ag and TLR agonist are taken up by the same APC and improving such uptake via DNA-binding receptors on these APCs (the latter effect is independent of the nature of the ODN but requires physical conjugation of DNA to target Ag) [51,52]. Thus, in multiple scenarios conjugating CpG ODN to vaccine Ag is of considerable benefit.

CpG ODN in combination with other TLR agonists

Many groups are trying to identify TLR agonist combinations that synergistically enhance cytokine and cellular responses. Understanding the signaling pathways utilized by various ligands assists in this effort. For example, the TLR3 signaling pathway is MyD88 independent and distinct from that engaged by TLR9, raising the possibility that agonists directed against these two ligands may interact synergistically [53,54]. In microarray studies of gene expression, the administration of both ligands accelerated and synergistically enhanced the activation of genes associated with immune function [55]. In murine experiments where CpG ODN plus Poly(I:C) were co-delivered with a DNA-encoded HIV vaccine, the combination increased protection against viral challenge, enhanced the functional avidity and magnitude of the Ag-specific CD8 T-cell response and increased IL-12/IL-15 production by DC [56–59]. More recently, GlaxoSmithKline (GSK) developed an adjuvant formulation that combines CpG ODN with the TLR4 ligand, Monophosphoryl lipid A (MPL) in liposomes [60]. That adjuvant, AS15, is being evaluated in clinical trials for treatment of non-small-cell lung cancer (NSCLC) and melanoma [61,62].

CpG ODN in combination with alum or oil-based adjuvants

The adjuvant activity of CpG ODN was initially examined by administering it in combination with Incomplete Freud’s adjuvant (IFA) [63]. In those studies, the inclusion of CpG ODN improved both humoral and cellular immunity in mice, especially Th1-biased and cytotoxic immune responses, when compared with IFA alone [63–65]. CpG ODN also boosted the immunogenicity of alum-based vaccines against infectious diseases including hepatitis B [66,67], anthrax [68] and influenza [69], and was effective at improving the CD8 T-cell responses elicited by cancer vaccines [70]. The effect of adding CpG ODN has also been examined. Results indicate that TLR9 agonists enhance the immune response to vaccine antigens presented in Quil A; Emulsigen® (a mineral oil-in-water emulsion) [71,72], and Montanide® (a water-in-mineral oil emulsion) [73–75].

CpG ODN for use with mucosal vaccines

Cholera toxin (CT) has emerged as the leading experimental adjuvant for the induction of strong mucosal immune responses [76]. The B subunit of CT (CTB) binds to cellular GM1 ganglioside receptors and facilitates the entry of the toxigenic A subunit. CTB alone has strong immune-enhancing effects on mucosal membranes that support its use in conjunction

with vaccines targeting the lungs and GI tract [77,78]. Recent studies indicate that the inclusion of CpG ODN further enhances the utility of CT as a mucosal adjuvant [79].

In separate animal studies, co-administration of CpG ODN with CT plus Ag conferred protection following challenge with pathogens including chlamydia, *Helicobacter pylori* and HPV. Delivery of a chlamydia vaccine combined with CpG ODN and CT by the intranasal (in.) route induced the secretion of Ag-specific IgG and IgA into the bronchioalveolar fluid (BAL) whereas subcutaneous (sc.) immunization had no such effect. In addition, cells isolated from the draining lymph nodes of animals immunized intranasally showed a 20-fold increase in IFN-γ mRNA expression versus non-immunized controls. Ten days post-challenge, naïve animals had >7000 Inclusion Forming Unit (IFU) in their lungs, whereas animals vaccinated in. and sc. had <50 and <1500 IFU, respectively [80].

The effect of immunizing with Ag plus CpG and/or CT was also examined. Following *H. pylori* challenge, the addition of CpG ODN alone provided no significant protection whereas the addition of CT alone resulted in partial/incomplete protection. However, when CpG ODN plus CT were combined with the vaccine and delivered in., no bacterial colonization was detected, demonstrating synergy between CpG ODN and CT [81,82]. In murine studies of a vaccine targeting influenza virus, once again the combination of CpG ODN plus CT prevented/delayed mortality and reduced weight loss caused by infection when compared with vaccine alone or vaccine with only CpG or CT [83]. Studies of Ab responses induced by vaccines delivered in. against hepatitis, malaria, HPV, HSV and anthrax all found that the addition of CpG ODN plus CT led to higher titers of Ag-specific Abs compared with single adjuvant systems [84–87]. In this context, several recent studies also showed that mucosal immunization with CpG ODN alone (absent CT) could also induce strong mucosal immune responses [88,89].

Clinical trials in which CpG ODN was used as avaccine adjuvant

More than 100 clinical trials involving the use of CpG ODN as adjuvants are currently underway or have been completed [202]. These fall into one of several general categories, including vaccines targeting infectious agents, vaccines targeting allergens and vaccines targeting cancer. Table 2 presents a subset of clinical trials (focusing on those whose results have been published and deemed most informative).

Vaccines targeting infectious pathogens

Human clinical trials have evaluated the utility of CpG ODN in combination with vaccines designed to prevent malaria, hepatitis B (HBV), pneumococcus, influenza and anthrax (Table 2).

The first CpG-containing vaccine to complete Phase III trials was HEPLISAV, targeting the hepatitis B virus. The currently approved HBV vaccine is alum adjuvanted and requires three immunizations over a period of 6 months to achieve protective Ab titers. Despite receiving a complete series of this vaccine, 5–10% of immune-competent individuals still fail to achieve long-lasting seroprotection. Moreover, a large fraction of immunocompromised patients fail to achieve protective Ab titers using available vaccines.

The inclusion of CpG ODN in HEPLISAV was found to improve the induction of humoral and cell-mediated immune responses against HBV. In Phase III clinical trials, HEPLISAV elicited higher seroprotective Ab titers with fewer immunizations than did currently licensed alum-adjuvanted vaccines [90–92]. The US FDA is reviewing the Biologic License Application (BLA) containing Phase III clinical data for HEPLISAV, and the decision on licensure is awaiting the provision of additional safety data.

A CpG-containing anthrax vaccine is undergoing Phase II clinical trials. The licensed anthrax vaccine (BioThrax®) requires five immunizations at 0, 1, 6, 12 and 18 months to induce a persistent protective response. For biodefense preparedness in the USA, there is considerable interest in a vaccine capable of inducing faster, higher titered and longer-lasting immunity against anthrax. In animal studies, combining CpG ODN with the licensed human anthrax vaccine significantly increased the speed, magnitude and duration of the resultant immune response, generating Ab titers that provided significantly greater protection from challenge when compared with animals vaccinated with the licensed anthrax vaccine adsorbed (AVA) alone ($p < 0.001$) [93–95]. Unexpectedly, a majority of mice immunized with the CpG-adjuvanted vaccine maintained resistance to anthrax for >1 year, despite Ab titers declining below protective levels ($p < 0.01$ vs AVA alone). Results indicate that this prolonged survival was mediated by the rapid *de novo* production of protective Abs by memory B cells. The pool of memory B cells from mice vaccinated with the CpG-adjuvanted vaccine was threefold larger and contained sixfold more high-affinity B cells than mice immunized with AVA alone ($p < 0.01$ for each parameter) [96].

Phase I clinical trials showed that adding CpG to Bio-Thrax accelerated and enhanced the immune response elicited in healthy volunteers. Those who received the adjuvanted vaccine developed six- to eightfold higher Ab titers 3 weeks more rapidly than recipients of BioThrax alone. No serious adverse events (AEs) related to study agents were reported, and the combination was considered to be reasonably well tolerated [97,98].

Malaria vaccines supplemented with CpG ODN are undergoing Phase I and Phase IIa trials in the USA, Australia and Mali. These vaccines are designed to generate immune responses against the merozoite surface protein 1 (MSP1) and apical membrane antigen 1 (AMA1) of the parasite. Results show that volunteers mounted significantly stronger Ab responses to these malaria Ags when they were co-delivered with CpG ODN. Inclusion of CpG ODN with AMA1 increased the mean anti-AMA1 Ab titer by sixfold when compared with AMA1 alone. This enhanced Ab response was persistent: at 236 days after vaccination, those immunized with AMA1 plus CpG ODN maintained serum Ab titers 4.6-fold higher than those vaccinated with just AMA1 [99–103]. In a separate study, the addition of CpG enhanced anti-MSP1 antibody responses by 49-fold after the second immunization and 8-fold after the third immunization when compared with MSP1/Alhydrogel alone ($p < 0.0001$) [101].

Vaccines targeting allergens

The characteristic features of allergic inflammation include eosinophilia and elevated serum IgE levels. These features are orchestrated when Th2 cells engage an allergen. Suppression

or deviation of Th2-dominated immune responses is an important target of anti-allergic immunotherapy. Th1 cells inhibit the pro-allergenic effects of Th2 cells by secreting Th1 cytokines (such as IFN-γ) that inhibit Th2-mediated eosinophilia. Indeed, Th1 and Th2 cells with the same antigenic specificity mutually inhibit one another's effector function. In animal models, co-administering CpG ODN with allergen generates a Th1-skewed response that opposes the induction of allergic inflammation [18,19,104]. Indeed, CpG ODN could deviate even an established Th2 response toward one that was anti-allergic.

These findings were initially evaluated in clinical trials designed to treat humans with ragweed allergy (Table 2). Studies found that CpG ODN coupled to ragweed allergen (TOLAMBA) reduced the responsiveness of immune cells in the nasal mucosa to allergen stimulation, reduced disease severity for two seasons and led to a prolonged shift from Th2 immunity toward Th1 immunity [105,106]. Despite these encouraging results, a multicenter multiyear Phase II trial yielded inconclusive results in failing to achieve the primary efficacy end point. This lack of reproducibility was attributed to the low level of active disease among volunteers in the second study and a problem in the timing of vaccine administration (which did not properly match the onset of ragweed season).

More recently, an A/D class CpG ODN was stabilized by incorporation into VLP and used as an adjuvant for sc. immunotherapy in combination with the house dust mite allergen, QbG10. All patients achieved nearly complete alleviation of their allergic symptoms after 10 weeks of immunotherapy. This promising clinical outcome calls for larger placebo-controlled Phase II studies [107,108].

Vaccines targeting cancer

Perhaps the greatest number of clinical trials involving CpG ODN have been conducted in the oncology field. One major approach has been to combine CpG ODN with tumor-associated antigens (TAAs) or tumor cells. These are summarized in Table 2. Animal studies and clinical trials utilizing tumor-derived proteins/peptides demonstrated the feasibility of generating tumor-specific CD8 T cells capable of eradicating established tumors and tumor metastases. For example, the addition of CpG ODN to the MART-1 peptide vaccine in patients with melanoma resulted in a 10-fold increase in the frequency of Ag-specific CD8 T cells [70]. Vaccination with the tumor Ag NY-ESO-1 plus CpG ODN yielded a high titered Ag-specific Ab response and CD8 T-cell responses in patients with melanoma, NSCLC, prostate and breast cancer (when those tumors expressed NY-ESO-1) [109–111]. Anti-MAGE-3 Ab titers were enhanced by the addition of CpG ODN to a MAGE-3 vaccine in a Phase I/II trial, with one patient raising a durable objective response [112]. The most advanced cancer vaccine trials involve two Phase III, randomized, controlled, clinical studies of MAGE-A3 with CpG ODN for patients with completely resected NSCLC whose tumors express that Ag. CpG ODN are also being studied as vaccine adjuvants in patients with metastatic renal cell carcinoma. Preliminary results show that vaccination with autologous tumor cells, CpG and GM-CSF followed by maintenance therapy with IFN-α and CpG led to a partial remission or stable disease in three of seven patients [113]. Finally, studies in which A/D class CpG ODN were stabilized in VLP and coupled to Melan-A/MART-1 peptide were evaluated as adjuvants for a tumor vaccine targeting MelQbg10. In a Phase IIa

clinical study involving patients with advanced stage melanoma, vaccination promoted the *ex vivo* generation of detectable Melan-A/MART-1-specific T-cell responses in all (11/11) participants [114].

The ability of free CpG ODN to induce tumor-specific CTLs is also being examined. The concept involves delivering the ODN to patients who have undergone cytotoxic tumor therapy (including radiation, chemotherapy or treatment with tumoricidal monoclonal Abs). Under such conditions, CpG ODN promote the uptake of tumor antigens released by dying cells and improve the ensuing immune response [115,116]. Pilot studies in patients with malignant glioma and lymphoma support the hypothesis that TLR9 agonists can synergistically enhance the efficacy of more conventional antitumor strategies [117,118]. To date, such combinations showed clinical benefit and an excellent safety profile (Table 2).

In a randomized Phase II study, the addition of the K/B-type TLR9 agonist PF-3512676 (CpG 7909) to first-line treatment with platinum-based chemotherapy in stage IIIb–IV NSCLC improved the overall objective tumor response and prolonged survival (12.3 vs 6.8 months; $p = 0.18$) [119]. CpG ODN were also studied in combination with monoclonal Abs such as anti-CD20 (rituximab) on the basis of preclinical results showing that CpG ODN upregulates CD20 expression on malignant human B cells and increases the expression of several IFN-inducible genes [120,121]. A Phase II study confirmed the safety of this approach. While the contribution of CpG ODN to clinical and immune responses can only be determined in a randomized controlled trial, results from that Phase II study found significant $CD8^+$ T cell infiltrates in tumors after treatment, consistent with an enhanced immune response.

Another approach to the administration of CpG ODN involves intratumoral injection. This strategy seeks to activate DC present in the cancer microenvironment, thereby facilitating Ag presentation *in situ*. Several clinical trials were conducted in which CpG ODN were delivered intratumorally to treat malignant skin tumors or lymphoma, and these yielded promising results [122]. Hofmann *et al*. reported that this strategy induced complete or partial tumor remission in half of all patients with basal cell carcinoma or melanoma, whereas Molenkamp *et al*. and Brody *et al*. showed that intratumoral CpG administration (alone or combined with radiation therapy) induced systemic tumor regression by improving the generation of tumor-specific CD8 T cells in lymphoma and melanoma patients [123,124]. Kim et al. showed that intratumoral injection of CpG ODN combined with radiation therapy induced an increase in $CD123^+$ pDC and decrease in $FoxP3^+$ T cells (Tregs) at the site of delivery accompanied by tumor regression at distant sites in patients with lymphoma [118].

Tumors utilize a variety of mechanisms to evade/suppress the host’s immune response. Indeed, established tumors are frequently surrounded by an immunosuppressive microenvironment consisting of cells and factors that limit the tumoricidal activity of effectors that might otherwise eliminate the cancer [125]. Myeloid-derived suppressor cells (MDSC) are key contributors to this inhibitory milieu [126]. Successful immunotherapy might therefore need to both induce antitumor immunity and nullify the suppressive environment maintained by such cells. Recent studies show that CpG ODN have the unique

ability to induce MDSC (which express TLR9) to differentiate into tumoricidal macrophages and lose their ability to suppress CD8 T-cell function [127,128].

Averse events in clinical trials

Thousands of patients have received CpG ODN (5000 patients were enrolled in the HEPLISAV trial). In general, vaccines containing CpG ODN were well tolerated. Common AEs involved local injection site reactions (erythema, edema, inflammation and pain) or systemic flu-like symptoms (headache, rigors, pyrexia, nausea and fever) [129]. Those symptoms typically developed within 24 h of administration and were transient, lasting for less than 2 days. With few exceptions they were of mild-to-moderate severity (Common Terminology Criteria grade 1/2). There were no major AEs related to this CpG-adjuvanted vaccine when it was administered to healthy adults [129,130].

Doses of CpG ODN in the range of 0.5–3 mg were generally used in vaccine adjuvant trials. By comparison, doses of free CpG ODN ranging up to 10–20 mg (~0.5 mg/kg) were administered to cancer patients in combination or intratumoral therapy. Despite these high doses, CpG ODN injections were generally tolerated well by cancer patients, with the dominant complaints being transient and mild flu-like symptoms and induration at the injection site. While other AEs were reported in the cancer trials, the majority of these were deemed unrelated to the administration of CpG ODN [131].

However, one concern that was not fully addressed was the potential of CpG-adjuvanted vaccines to trigger autoimmune disease or inflammation [132,133]. The immune stimulation induced by CpG ODN inhibited the apoptotic death of stimulated lymphocytes, induced polyclonal B-cell activation and increased the production of auto-Abs and proinflammatory cytokines in preclinical studies [134–136]. These effects could potentially increase the risk of autoimmune disease [137–139]. When mice predisposed to developed lupus were repeatedly injected with immunostimulatory doses of CpG ODN, the number of IgG anti-DNA secreting B cells rose by two- to threefold and serum IgG anti-ssDNA antibody titers rose by 60% [140,141]. The magnitude of these effects was insufficient to induce or worsen systemic autoimmunity. Karbach *et al.* reported the development of anti-CpG Ab in 21 of 37 patients receiving CpG ODN [142]. However, these Ab did not cross-react with natural DNA. Clinically, no effect on anti-dsDNA or anti-nuclear Abs (ANA) was observed following the administration of CpG ODN in the HEPLISAV trial. Similarly, there was no difference in autoimmune events between recipients of HEPLISAV versus recipients of the alum-adjuvanted Engerix-B vaccine, leading a FDA advisory committee to conclude that HEPLISAV raised no safety concerns [143]. Despite this safety record, the FDA did not approve HEPLISAV, instead requesting additional safety data. The FDA asked the manufacturer to add to their trial volunteers who were ethnically more diverse and of younger age that those originally enrolled, and to monitor safety in these individuals for a minimum of 12 months. Dynavax recently announced the design of another large-scale Phase III clinical study of HEPLISAV to meet these criteria.

Expert commentary

The ability of synthetic CpG ODN to activate the innate immune system was first reported in 1995 [144]. Since that time, several additional classes of CpG ODN were identified and their ability to promote vaccine-specific immunity established by numerous preclinical studies. That body of work supported the conduct of >100 clinical trials involving CpG ODN as vaccine adjuvants. While no vaccine has yet been approved that incorporates CpG as an adjuvant, several are in Phase II/III trials and nearing evaluation by the FDA for licensure. The safety profile of CpG ODN combined with their ability to support the induction of both humoral and cell-mediated immunity strongly suggests that this class of agent will be of considerable use in the clinic. While market forces may drive their incorporation into immunotherapeutic vaccines targeting cancer, the authors believe that their use in prophylactic vaccines targeting infectious pathogens are the most likely to succeed. This conclusion reflects both the safety record and ease of use of CpG ODN in prophylactic vaccines (such as HELPISAV) and the difficulties associated with generating effective cancer vaccines (which include difficulty in effectively targeting tumor Ags, immune suppression of even Ag-specific responses in the tumor microenvironment and regulatory hurdles associated with the adverse events observed in all trials of patients with advanced disease).

Five-year view

Barring the unexpected development of severe/frequent AEs, we expect that one or more vaccines incorporating CpG ODN will achieve FDA approval within the next 5 years. We further predict that combination adjuvants that contain CpG ODN plus other immunomodulatory agents will make considerable progress during that period. Most of this work will utilize K-class CpG ODN, although D-class ODNs may prove effective if a means of preventing their uncontrolled oligomerization is achieved. There have also been efforts to synthesize agents that mimic the ability of CpG ODN to trigger TLR9. Whether such agents offer utility beyond that already provided by CpG DNA should become clear over the next few years.

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Financial & competing interests disclosure

DM Klinman and members of his laboratory are inventors/co-inventors on a number of patents pertaining to CpG ODN. All rights to these patents have been assigned to the Federal Government. This work was supported by the Intramural Research Program of the National Institutes of Health, National Cancer Institute. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

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• of interest

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Key issues

- Toll-like receptor 9 (TLR9) responds to the unmethylated CpG motifs present at high frequency in bacterial DNA. TLR9 engagement triggers a protective immune response involving plasmacytoid dendritic cells and B lymphocytes that improves host elimination of infectious pathogens.
- TLR9 is expressed in early and late endosomes. The precise location of receptor interaction with CpG oligonucleotides (ODN) determines the nature of the ensuing response.
- To optimize immunogenicity, APCs should be exposed to CpG ODN plus antigen simultaneously. Co-delivery of CpG ODN plus antigen increases the speed, magnitude and duration of the resultant immune response.
- The activity of CpG ODN can be further improved by combining them with other adjuvants and TLR agonists. For example, combining CpG ODN with cholera toxin improve the response induced by mucosal vaccines.
- Clinical trials demonstrate that CpG-adjuvanted vaccines induce stronger and faster immune responses against both HBV and anthrax.
- The ability of CpG ODN to induce a Th1-biased immmune response supports their use in vaccines targeting allergens. However, clinical results using a CpG-adjuvanted vaccine against ragweed were inconsistent.
- CpG ODN have been evaluated in a number of cancer trials. Their ability to boost immunity sufficiently to eradicate established cancers remains unclear.
- CpG ODN have a good safety profile when used as vaccine adjuvants.

Table 1.

Methods used to co-deliver CpG ODN with antigen.

Method	Materials	Antigen	Effect on immune response	Ref.
CpG ODN and antigen conjugates	Biotin-avidin Sulfo-SMCC Sulfo-MB	Amba1 Cryj2 Der f gp120 Cancer cell	10–100 × ↑ Ab titer vs Ag alone ↑ cross-presentation and CTL induction ↑ Ag uptake by APCs	[19,48–50]
Liposomes	DOPE:CHEMS DOTAP/cholesterol	Listeriolysin O Cancer cell rLmSTI1 TM4SF5	↑ Ag uptake by DC 20× ↑ IFN-$_{\gamma}$ production by T cells ↑ Th1 cytokine production ↑ IgG2a production, ↑ protection	[145–149]
Multicomponent nanorods	Gold Nickel	OVA	10× ↑ Ag-specific CD8 responses	[150]
Alginate microparticles *Burkholderia pseudomallei*	Alginate	HBsAg *Leishmania*	↑ mucosal immunity ↑ protection against *Leishmania* and *Burkholderia* ↑ mucosal humoral immunity ↑ protection, ↑ IFN-$_{\gamma}$ production	[151–153]
Cationic microparticles	CTAB, DOTAP	Her2/neu Der p 1	↑ antigen-specific Ab titer and the production of IgG2a ↑ allergen-specific Th1 and Th2 responses	[95,154,155]
Biodegradable microparticles	PLA, PGA PLGA	OVA Tumor lysate Bacteria lysate	Protect CpG ODN from nuclease degradation ↑ 20× Th1 Ag-specific response ↑ Ag-specific Ab responses	[156–159]

Ab: Antibody; Ag: Antigen; CTL: Cytotoxic T lymphocyte; DC: Dendritic cell; ODN: Oligonucleotides; OVA: Ovalbumin; PGA: Polyglycolic acid; PLA: Polylactic acid; PLGA: Polylactic-co-glycolic acid

Table 2.

Use of CpG ODN for vaccines targeting infectious disease, allergy and cancer.

Agent	Disease	Trial phase	Outcome	Ref.
Infectious disease				
HEPLISAV	Hepatitis B	Phase III	Faster seroprotection Anti-HBs Ab titers 45-fold higher vs HBs alone 100% seroconversion after 6 wk vs 63% in controls Seroprotection faster and higher vs Engerix-B	[90–92]
Biothrax® + CpG	Anthrax	Phase II	8-fold ↑ Ab response. Peak Ab production induced 3 wk Faster vs BioThrax alone	[96]
Malaria Ag + CpG MSPI/alhydrogel AMA1 + CpG	Malaria	Phase II	Anti-MSP1 Ab titer ↑49-fold after second and 8-fold after third vaccination vs MSP1/alhydrogel alone Anti-AMA1 Ab titer ↑ 6-fold vs AMA1 alone	[99,100,102] [101]
Allergy				
TOLAMBA CpG-conjugated ragweed allergen	Allergy	Phase II	Immune response shifted from Th2 toward Th1 Reduced allergic Sx's (allergic rhinitis) for in 2 consecutive seasons	[106,107]
CYT003-QbG10 (A-CpG ODN/house dust mite allergen packaged into Qbeta coat protein)	Allergy	Phase II	Amelioration of Sx's by 10 wk, lasting >38 wk ↑ in allergen-specific IgG, transient ↑ increase IgE	[108,109,160]
Cancer				
CpG + T cells MelanA/MART1	Melanoma	Phase I	No clinical response but 10× ↑ tumor Ag-specific CD8 (>3%)	[70]
QbG10 + MelanA/MART1	Melanoma	Phase II	Induced tumor Ag-specific T cells responses (detected *ex vivo*) in all patients. Immune reactions detected by PET/CT in 13/15 subjects	[114]
CpG + responses NY-ESO-1	Prostate	Phase I	Tumor Ag-specific Ab (100%) and cellular (69%) elicited Disease reduced in 2/13 and stable in 8/13 patients	[161]
CpG + MAGE-3	Melanoma	Phase II	2/5 patients with PR at Rx with 1 mg CpG ODN No response in recipients of the 0.5 mg	[112]
CpG/MPL/QS21 HER2	Breast cancer	Phase I	Tumor-specific Ab induced in all patients, HER2-specific T cells in 1 patient	[162]
Cancer combination therapy				
CpG + Improved chemotherapy (PTX/CBDCA)	NSCLC	Phase II Phase III	Higher objective response rate, trend toward survival No effect of adding CpG to chemotherapy observed vs chemotherapy alone	[119,163]
CpG + chemotherapy (GEM/CBDCA)	NSCLC	Phase III	No improvement observed vs chemotherapy alone	[164]
CpG + of anti-CD20 Ab (rituximab)	NHL	Phase II	CD20 expression by malignant B cells and expression several IFN-inducible genes increased	[114,115]

Agent	Disease	Trial phase	Outcome	Ref.
CpG + response, Radiation	Lymphoma	Phase II	Four of 15 patients with complete or partial clinical Induction of tumor reactive memory CD8 T cells	[122]
Cancer CpG monotherapy				
CpG	Basal cell cancer	Phase I	Local regression: 1 complete, 4 partial Metastatic regression: 1 complete in 10 patients	[123]
CpG	Melanoma	Phase I	↑ NK cell numbers and tumor Ag-specific CD8 T cells after one dose of CpG	[123,124]

This list represents only a subset of clinical trials (focusing on those whose results have been published and deemed most informative).

Ab: Antibody; Ag: Antigen; CBDCA: Carboplatin; CT: Computed tomography; GEM: Gemcitabine; NHL: Non-Hodgkin lymphoma; NK: Natural killer; NSCLC: Non-small-cell lung cancer; ODN: Oligonucleotides; PR: Progesterone receptor; PTX: Paclitaxel