Clinical evaluation of CpG oligonucleotides as adjuvants for vaccines targeting infectious diseases and cancer

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Abstract
Synthetic oligonucleotides (ODN) that express unmethylated “CpG motifs” trigger cells that express Toll-like receptor 9. In humans this includes plasmacytoid dendritic cells and B cells. CpG ODN induce an innate immune response characterized by the production of Th1 and pro-inflammatory cytokines. Their utility as vaccine adjuvants was evaluated in a number of clinical trials. Results indicate that CpG ODN improve antigen presentation and the generation of vaccine-specific cellular and humoral responses. This work provides an up-to-date overview of the utility of CpG ODN as adjuvants for vaccines targeting infectious agents and cancer.

Keywords
CpG oligonucleotide; Adjuvant; Infection; Cancer; Toll-like receptor

1. Introduction
Vaccines are highly effective public health interventions, having saved millions of lives by preventing infectious diseases. The efficacy of a vaccine is determined by the magnitude, duration and quality of the immune response it induces. Depending upon the disease, both the innate and adaptive arms of the immune system may contribute to protection. The innate immune response involves the activation of multiple cell types, including dendritic cells, macrophages, monocytes, neutrophils, basophils, eosinophils, lymphocytes and/or NK cells. The innate immune system provides a rapid response to pathogens, initiates pathogen clearance, and helps in the healing of damaged tissue. Cells of the adaptive immune system include primarily B and T lymphocytes. Adaptive immune responses develop more slowly but are highly specific and therefore critical for providing sterilizing immunity and long-term memory.

Successful vaccination requires a complex series of immune interactions. Professional APCs (dendritic cells and macrophages) as well as certain other immune cells (e.g. B lymphocytes)

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Conflict of interest
Dr. Klinman and members of his lab have patents related to the use of CpG ODN. All rights to such patents have been assigned to the Federal Government.
take up the vaccine Ag. The Ag is then digested and fragments presented to T lymphocytes via cell–cell interactions that require additional surface receptors. Ag-activated CD4 T cells provide help to Ag-specific B cells, supporting their proliferation, switch recombination and somatic hypermutation, resulting in the production of high-affinity Ab (an outcome measured in many clinical trials) [1–3]. CD8 T cells are also stimulated to proliferate and mature into cytotoxic effectors. These are typically measured by their production of Th1 cytokines, such as IFN-γ. Over the course of the vaccine-elicited response, Ag-specific memory B and T lymphocytes arise that persist long-term and provide protection from subsequent challenge [4–7].

Toll-like receptors (TLR) belong to the family of pathogen recognition receptors that are triggered by pathogen-associated molecular patterns expressed by bacteria, viruses, fungi and protozoa. The basic structure of these receptors include an extracellular leucine-rich repeat region, a transmembrane domain and a cytosolic Toll/Interleukin-1 receptor domain. TLR stimulation contributes to the induction and maintenance of innate and adaptive immune pathways as well as memory function [3]. Eleven human and 13 mouse TLRs have been identified. These TLRs have been categorized based on their ligand specificity, signal transduction pathways, cellular expression profiles and subcellular localization. While human TLRs 1, 2, 4, 5 and 6 are localized on the cell membrane, TLRs 3, 7, 8, 9 and 10 are embedded in intracellular vesicles [8,9], although TLR9 was found on the cellular membrane of human CD19+ B cells [10].

TLRs are expressed primarily on immune cells although other cells are known to contribute to host protection, such as barrier epithelial cells, can also express these receptors. Once triggered by pathogen, these cells produce pro-inflammatory cytokines and chemokines that support the recruitment of additional inflammatory mediators, type I interferon and antimicrobial peptides [11]. TLRs are also expressed on malignant cells and may play a role in oncogenesis and tumor progression by influencing the tumor microenvironment [9,12–14].

2. Toll-like receptor 9

TLR9 recognizes the unmethylated CpG motifs present at high frequency in bacterial but rare in mammalian DNA. The TLR9 receptor is localized to the endoplasmatic reticulum, late endosomal and lysosomal compartments of the intracellular milieu. Thus, internalization of pathogen-derived DNA is required for TLR9 triggering, an outcome that results from either intracellular infection or uptake of bacterial/viral particles by immune cells [15]. Once stimulated, TLR9 initiates a response biased towards pro-inflammatory/Th1 biased immunity [16].

TLR9 molecules differ between species, with the structure of human versus mouse TLR9 varying by 24% [17]. There is also variation between species in terms of which cell types express TLR9. For example, the TLR9 receptor is present in rodent but not in primate macrophages and myeloid dendritic cells (mDC). In humans, TLR9 is expressed primarily by plasmacytoid CD (pDC) and B cells [18–21]. Reflecting their utility as vaccine adjuvants, B lymphocytes exposed to TLR9 agonists become more susceptible to activation.
by Ag [22–24] while TLR9 stimulated pDC produce type I interferons and more efficiently present Ag to T cells [25–27]. Non-human primates (NHP) also respond to the same CpG motifs as humans. Thus, results from NHP are better predictors of human immunological responses to CpG ODN than rodents.

3. TLR9-mediated signaling cascade

The binding of CpG DNA to TLR9 induces proteolytic cleavage of the receptor [28,29]. After exiting the ER, the TLR9 ectodomain is cleaved by asparagine endopeptidase and/or cathepsins [30,31]. This truncated (rather than full length) form of TLR9 recruits myeloid differentiation factor 88 (MyD88) such that if proteolysis of the receptor is prevented, it becomes non-functional. The requirement for ectodomain cleavage provides an ancillary mechanism to restrict receptor activation to endolysosomal compartments and further prevents 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 [32]. While TLR9 expressing cells recognize unmethylated CpG motifs, other molecules may support the signal transduction. For example DEC-205, a multi-lectin receptor that can bind CpG motifs and has been shown to facilitate the uptake of CpG ODN by DC and B cells [33].

4. CpG ODN

Bacterial DNA is the native ligand for TLR9 and synthetic oligonucleotides (ODN) that mimic the structure of bacterial DNA duplicate this activity. In humans, four distinct classes of CpG ODN have been identified based on differences in structure and the nature of the immune response they induce. Although each class contains at least one motif composed of a central unmethylated CG dinucleotide plus flanking regions, they differ in structure and immunological activity.

K-type ODNs (also referred to as B-type) contain from 1 to 5 CpG motifs typically on a phosphorothioate backbone. This backbone enhances resistance to nuclease digestion and substantially prolongs in vivo half-life (30–60 min compared with 5–10 min for phosphodiester) [34]. K-type ODNs trigger pDC to differentiate and produce TNF-α and stimulate B cells to proliferate and secrete IgM [35,36]. Extensive clinical trials involving K-type ODN have been conducted (as reviewed below).

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 that enables the formation of a stem-loop structure. D-type ODN also has poly G motifs at the 3’ and 5’ ends that facilitate concatamer formation. D-type ODN trigger pDC to mature and secrete IFN-α but have no effect on B cells [35,37]. 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 [38,39]. 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-α production [39]. D-type ODN tend to form complex multimers in solution due to interactions between their poly-G tails, and thus has posed a barrier to their use in clinical trials. However D-type ODN were recently packaged into stable virus like particles (VLP) and used as adjuvants in preclinical and clinical studies [40–42].

C-type ODNs resemble K-type in being composed entirely of phosphorothioate nucleotides but resemble D-type in containing palindromic CpG motifs that 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 [43,44]. 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. 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 [45,46].

Stimulation via TLR9 results in the rapid activation of the innate immune system that in turn supports the induction of an adaptive immune response. This series of effects provides a mechanism by which CpG ODN might be harnessed as a vaccine adjuvant. Extensive animal testing showed that CpG ODN could support the induction of Ag-specific immunity against co-administered peptides and vaccines [47,48]. Below, we review the literature by focusing on clinical studies that examined the utility of CpG ODN as an adjuvant for vaccines targeting infectious diseases (Table 1) and cancer (Table 2). Most clinical trials evaluated the activity of CpG ODN adjuvanted vaccines by monitoring specific responses such as Ab titers, cytokine levels, cell proliferation and changes in the frequency of CTL, NK cells, CD8 and CD4 T cells. For vaccines targeting infectious agents, the efficacy of adding CpG ODN was typically examined by comparison to a control group that received vaccine only. In cancer vaccine trials, this critical control group was often missing which complicates interpretation of the reported results.

5. Clinical trials that include CpG ODN in vaccines targeting infectious diseases

5.1. Anthrax

Anthrax is caused by the toxin-producing gram positive bacterium Bacillus Anthracis. The spores of this pathogen are highly resistant to environmental degradation (desiccation, heat, UV light) and many disinfectant solutions [49]. Protection against anthrax is provided by Abs targeting the ‘protective antigen’ (PA) component of the toxin.

The currently licensed human vaccine, Anthrax Vaccine Adsorbed (AVA) is administered as 6 subcutaneous (SC) injections over 18 months followed by annual boosters. A range of adverse events (AEs), including injection site pain, joint pain, flu-like symptoms and even pneumonia was observed as the number of vaccinations increases [50,51]. In an effort to
reduce AEs and accelerate the induction and duration of protective immunity, the effect of adding K-type CpG ODN to AVA was examined. In multiple animal models combining CpG ODN with AVA improved immunogenicity and accelerated the generation of PA-specific Abs [48,52]. Such findings supported the initiation of several clinical trials which consistently showed that adding CpG ODN to AVA increased serum IgG anti-PA titers by 6–8 fold ($p < 0.001$) while reducing the time needed to induce a protective titer by nearly 3 weeks ($p < 0.001$) [53]. A similar increase and acceleration in toxin neutralizing Ab levels was also observed. More recently, a study evaluating two doses of AVA delivered IM found that adding CpG 7909 again significantly improved Ab titers and accelerated the induction of immunity [54].

### 5.2. Hepatitis B

Hepatitis B (HBV) is a hepadnavirus that predominantly infects hepatocytes. The resulting infection can lead to acute or chronic hepatitis, the latter being associated with long-term complications including cirrhosis and hepatocellular carcinoma. Vaccination has reduced the incidence and complications of HBV infection worldwide in the past 3 decades. Yet HBV remains a major public health issue among individuals who respond poorly to the licensed vaccines [55].

Efforts to produce a more immunogenic HBV vaccine have focused on use of HbsAg which is expressed on the surface of HBV and is released into the serum following infection. It acts as the major antigenic component of current HBV vaccines. Pre-clinical studies showed that CpG 2216 and CpG 2395 increased the recognition of HBV epitopes [56] and triggered a 2–4 fold increase in the production of IFN-$\gamma$ and IL-4 by PBMC from patients with chronic HBV infection [57]. Rodents and primates treated with HBsAg adjuvanted with CpG 2006 had higher response rates (a dose-dependent effect) and produced significantly higher levels of IgG2A and IgG2B anti-HBsAg Ab (2–10 fold; $p \leq 0.005$) when compared to vaccine alone [58–60]. Similar improvements were observed when CpG 7909 and ISS 1018 were evaluated in clinical trials: normal volunteers generated significantly 3–10 fold higher anti-HBsAg Ab titers when immunized with adjuvanted vaccine vs. vaccine alone ($p \leq 0.005$) [61]. In a phase I dose escalation study of healthy adults, 1–3 mg of ISS 1018 (K/B-type ODN) generated higher anti-HBsAg in a greater fraction of participants after the first and second immunization than vaccine alone ($p \leq 0.001$) [62]. A recent phase III trial compared the effect of recombinant HBsAg plus 3 m of ISS 1018 to the Engerix-B® vaccine in healthy individuals. CpG-HBsAg co-vaccination yielded a higher percentage of seroprotected participants at 4 (23% vs. 3%), 8 (88% vs. 26%), 24 (98% vs. 32%) and 28 weeks (97% vs. 81%) and generated peak Ab titers after only 2 vaccinations vs. 3 vaccinations for the Engerix-B® group [64]. The use of HBsAg plus ISS 1018 vs. Engerix-B® was also evaluated in adults 40–70 years of age. As above, seroprotective titers were achieved in more participants vaccinated with ISS-HBsAg at 4 weeks (96% vs. 24%) and 28 weeks (100% vs. 73%; $p < 0.0001$). Moreover, titers of anti-HBsAg Ab exceeding 100 mIU/mL were induced in 97% vs. 58% at week 28 and were maintained in 90% vs. 43% through week 50.
compared to Engerix-B® ($p < 0.0001$) [65]. These trials clearly establish the improved immunogenicity of a CpG ODN adjuvanted vaccine.

5.3. Malaria

A number of clinical trials evaluated the use of CpG ODN as adjuvants for vaccines against malaria [66,67]. In the blood stage of malaria infection, parasite-specific Plasmodium falciparum (PF) Abs play a critical role in controlling parasite numbers [67,68]. Natural infection elicits memory B cells and long lived plasma cell response that provided malaria-specific Abs and reduced disease burden [69,70]. An object of vaccination is to reproduce the natural protection. Preclinical studies showed that immunization with a vaccine containing the apical membrane antigen 1 (AMA1) of malaria, a surface protein expressed during the asexual blood-stage of PF, induced Abs and that protected against homologous parasite challenge in rodents [71]. Yet studies in Aotus monkeys showed that in vivo protection required considerably higher anti-AMA 1 Ab titers than were achieved by vaccination with the CpG ODN adjuvanted vaccine [72].

In human clinical trials involving malaria-exposed adults, supplementing an AMA1-C1 vaccine formulated in Alhydrogel® with CpG 7909 improved the resultant anti-AMA-1 Ab response by 2–3 fold ($p = 0.013$) [73,74]. An even greater improvement in AMA1-specific Ab titers (up to 14-fold) was observed when CpG 7909 was added to this vaccine and administered to malaria-naïve individuals [75–77]. A positive correlation was observed between the frequency of vaccine-specific memory B cells and Ab levels after re-immunization (AMA1-C1: $r = 0.80$; MSP42: $r = 0.86$). Serum from subjects vaccinated with the CpG 7909 adjuvanted vaccine provided 3–4 fold higher inhibition of PF growth in vitro than serum from individuals vaccinated with AMA 1 alone ($p < 0.0001$) [76,78]. Unfortunately, the Ab titers achieved in subjects vaccinated with the CpG 7909 adjuvanted vaccine were insufficient to protect against malaria infection or even reduce the multiplication rate of PF rate in vivo ($p = 0.7$) [78]. This data elicit special difficulties in generating vaccine strategy to provide immunity to malaria as an intracellular parasite.

5.4. Influenza

The addition of CpG ODN to licensed influenza vaccines was found to improve immunogenicity in multiple animal models [79–81]. For example, adding CpG ODN to the human influenza vaccine Fluviral® (including antigens A/Sydney, A/Beijing and B/Harbin) induced higher virus-specific Ab titers resulting in better protection in ferrets as manifest by a 20% reduction in viral load when compared to Fluviral® alone [81].

A randomized, blinded, phase Iib trial was conducted in which subjects were vaccinated with either 1/10th or a full-dose of Fluarix® alone or co-administered with 1 mg of the CpG 7909 ($n = 15$ per group). The inclusion of CpG 7909 did not improve hemagglutinin inhibition (HI) Ab titers or total IgG levels in the serum ($p > 0.05$). However, recipients of the 1/10th dose of Fluarix® vaccine combined with CpG 7909 had 4–7 fold higher IFN-γ producing PBMCs after stimulation with the antigens A/Beijing/262/95 ($p = 0.048$) and B/Harbin/7/94 ($p = 0.0057$) on day 28 compared to low dose vaccine recipients alone, which was nearly
equivalent to a full dose of Fluarix® in the absence of adjuvant. These results are consistent with a dose-sparing effect of CpG ODN in humans [82].

6. CpG ODN as an adjuvant in HIV-infected individuals

Chronic HIV infection is characterized by latent infection and impairment of CD4 T cells and the cytolytic activity of CD8 T cells. This contributes to a diminution in the immunogenicity of standard vaccines, placing HIV patients at higher risk for associated infections and increased mortality [83,84]. CpG ODN were found to boost immunity in mice with depressed immune systems. However their utility in HIV-infected patients was uncertain, since they could potentially increase virus replication levels by stimulating the proliferation of latently infected immune cells [85–87] as was observed in HIV patients with opportunistic bacterial infections [88,89].

Clinical trials described above (see Hepatitis B section) established the safety and efficacy of using CpG 7909 or ISS 1018 as adjuvants for a recombinant HBsAg hepatitis vaccine in healthy volunteers. Those findings supported the initiation of clinical trials of HBV vaccines in patients with HIV infection [90–92]. Anti-HBsAg Ab titers in subjects receiving the CpG 7909 adjuvanted vaccines were 8 fold higher after the second vaccination (week 4) and remained high (4–7 fold) at week 48 of follow up ($p < 0.05$). 89% of the CpG 7909 adjuvanted group responded to vaccination and achieved protective titers by week 6, which was 4 weeks earlier than recipients of Enderix-B® alone. Seroprotective titers (>10 mIU/L) were reached by all individuals in the CpG group by week 10 versus 89% in the control group. Also, protective titers persisted in all members of the CpG group at week 48 after vaccination versus 63% of those immunized with vaccine alone ($p = 0.008$). The frequency of seroprotective Ab levels persisted in the CpG 7909 treated group through 54 months of follow up when compared to the non-CpG group ($p \leq 0.02$). Even 60 months after vaccination 80% of the CpG 7909 vaccinated individuals maintained seroprotective titers compared to 40% in the non-CpG group ($p = 0.004$). It was also reported that helper T cells from individuals receiving the adjuvanted vaccine mounted 2–3 fold higher responses to Ag-specific stimulation ex vivo that did vaccine alone controls for up to 48 months ($p = 0.04$). However, there were no changes in total CD4 or CD8 T cell counts in the peripheral blood nor were significant differences in plasma (HIV p24) viremia observed in these HIV patients, all of whom were maintained under HAART throughout the study [90–92].

A phase Ib/IIa trial was conducted in HAART-naive and HAART-treated HIV-infected patients vaccinated with the pneumococcal Ag PCV7 (Prevnar®) and PPV-23 (PneumoNovum®) vaccines [93–95]. After 3 vaccinations, there was a significantly higher percentage of responders (seroprotective titers >10 mIU/mL) in the group that received the CpG 7909 adjuvanted vaccines versus the control group (48.8% vs. 25%, $p = 0.02$) [93]. In the same cohort of patients, increased Ab dependent cellular immunity was detected after the second vaccination accompanied by significantly higher PBMC cytokine responses involving IL-1 ($p = 0.0046$), IL-6 ($p = 0.0051$), IFN-α ($p = 0.0047$), MIP-β ($p = 0.0086$), and IL-2R ($p = 0.0062$). PBMC from the CpG 7909 adjuvanted group responded to LPS stimulation by producing IL-1β ($p = 0.038$) and IFN-α ($p = 0.019$) at significantly higher levels when compared to non-CpG controls [94]. HIV pro-viral DNA levels fell by 12.6% in
the CpG 7909 treated group versus a rise of 6.7% in the placebo group after the 3rd vaccination (at 10 months) \( p = 0.056 \). There were no changes in HIV-specific Ab levels or the frequency of total or CD4 T cells after immunization [95].

7. Clinical trials that include CpG ODN in vaccines targeting cancer

7.1. Use of CpG ODN as stand-alone anti-tumor agents

CpG ODN were evaluated as stand alone anti-tumor agents in several clinical trials. While distinct from their use as vaccine adjuvants, results from those trials are described below as they provide insights into the immunostimulatory activity of CpG ODN. Based on the observation that CpG ODN can stimulate an innate immune response capable of decreasing tumor burden in animal models, CpG 7909 alone at doses up to 0.08 mg/kg SQ and 0.32 mg/kg IV was tested in humans. Evidence of in vivo activity was provided by elevations in the concentration of serum IP-10, IFN-\( \alpha \), MIP-1\( \alpha \) and IL-12p40 levels in the serum with increasing dosage of CpG ODN [96]. Phase I clinical trials of healthy volunteers showed that CpG ODN were generally well tolerated without significant toxicity. In most of these trials CpG ODN was administered SQ or IV. The route of administration impacted the nature/strength of the resultant immune response and the occurrence of adverse events. For example, increases in serum cytokine levels and injection site reactions were more common when CpG ODN was administered SQ versus IV [96].

The effect of intra-tumoral administration of CpG ODN was also investigated. A number of preclinical animal studies showed that intra-tumoral injections of CpG ODN elicited a stronger protective response than did systemic delivery of the same agent [97–99]. The mechanism underlying this finding was linked to the ability of TLR-activated cells in the tumor microenvironment to (i) better recognize and present tumor Ag and (ii) convert that tumor milieu from immunosuppressive to immunostimulatory. Intra-tumoral delivery induced stronger long-term immunity and enhanced tumor rejection when compared to systemic administration of CpG ODN in mice. Encouraged by these preclinical results, phase I and II clinical trials in patients with glioblastoma were initiated. Unfortunately, overall results were disappointing as no significant clinical improvement was observed. The continuous intra-tumoral delivery of CpG ODN (4 mg/h) resulted in a modest but not statistically significant improvement in median survival time (6–7 vs. 5 months). After 6 months, 19% of these subjects experienced progression free survival (PFS) versus 15–20% among patients receiving standard radiotherapy and chemotherapy [100,101]. Survival of CpG treated subjects after 1 year was 24%, which is superior to that of historic controls (15%). Due the absence of a concurrent control group, care must be taken not to over-interpret this finding [100,101]. Yet the generally poor prognosis of patients with glioblastoma raises hopes when even a modest prolongation in survival is achieved. Thus, the value of local CpG ODN administration awaits the conduct of a randomized control study.

7.2. Use of CpG ODN as tumor vaccine adjuvants

CpG ODN as stand-alone therapy was generally safe but induced immune responses of insufficient magnitude to clear established tumors. Peptide based vaccines by themselves
generally failed to elicit strong immune responses. Investigation studies of peptide vaccines included against Melan-A/Mart-1, gp100, EGF, Mage-A10, NY-ESO-1, IDM-2101, MAGE-A3 and Wilms’ Tumor nuclear protein 1 (WT-1) [102–111]. Thus, a number of clinical trials examined the utility of adding adjuvants such as CpG ODN to peptide-based vaccines targeting various tumor associated antigens (TAAs). Of note, many of these studies simultaneously evaluated other immunostimulatory agents such as Montanide\textsuperscript{\textregistered} ISA-51, granulocyte-macrophage colony stimulating factor (GM-CSF) and Incomplete Freund’s adjuvant (IFA) in combination with CpG ODN. Although several different CpG ODN were included in these clinical trials, most used the K-type ODN CpG 7909 (also referred as PF 3512676). The early phase I trials focused on determining whether CpG ODN were safe and could improve the immunogenicity of co-administered vaccines. Few conclusions concerning clinical efficacy could be reached due to low patient numbers and lack of relevant controls and randomization. As the results of earlier studies were previously reviewed [112–114], this work will focus on more recent clinical trials in which CpG ODN was used as an adjuvant for peptide vaccines (excluding chemo-and/or radiotherapy, Table 2).

Patients with malignant melanoma were included in several clinical studies of CpG ODN adjuvanted vaccines (Table 2). A prospective Phase I trial involved 24 patients with stage II–IV metastatic melanoma who were vaccinated monthly with Melan-A/MART-1 peptide, Montanide\textsuperscript{\textregistered} ISA-51 and CpG 7909 SQ. 10 fold more Melan-A/MART-1 specific T cells were generated by patients immunized with the vaccine containing CpG 7909 vs. the same vaccine lacking CpG ODN. When T cells from CpG 7909 co-vaccinated subjects were re-stimulated \textit{in vitro} with a vaccine derived peptide, their cytokine production of IFN-\(\alpha\), TNF-\(\alpha\), IL-2 and CD107a were increased 2–10 fold (\(p < 0.01\)) when compared to the non CpG-group [115]. In another clinical trial, 22 patients with stage III–IV melanoma were vaccinated SQ up to 13 times with a combination of MART-1, gp100 and tyrosinase peptides emulsified in Montanide\textsuperscript{\textregistered} ISA-51 with CpG 7909 (600 \(\mu\)g) and GM-CSF. Over 2–7 months of follow up, 10% of these patients had partial remission (PR) and 38% stable disease (SD). Half of the patients with SD developed IFN-\(\gamma\) secreting T cells that recognized the MART-1 Ag post vaccination. The median PFS time in this study was 1.9 months and the median overall survival (OS) was 13.4 months. These outcomes were not superior to the therapeutic alternatives commonly used in patients with stage IV and recurrent melanoma [116,117].

Several studies were published investigating the role of D/Atype CpG ODN incorporated in VLP [41,42]. MelQbG10 consists of the Qb coat protein of bacteriophage coupled to the G10 CpG ODN and the tumor peptides Melan-A/MART-1. A phase I/II clinical trial first tested MelQbG10 in melanoma patients with stage II–IV disease found that this vaccine was safe and induced Melan-A/MART-1 specific T cell responses in 14/22 (66%) patients. There were no differences in response associated with daily versus weekly vaccination strategies or the SQ versus the ID routes of delivery. The inclusion of CpG ODN significantly increased the percentage of central memory T cells compared to patients vaccinated with peptide Melan-A peptide plus IFA alone (\(p < 0.05\)). Unfortunately the clinical response was poor: even 7 of 22 (32%) patients were free of disease, one patient had PR and one patient
had SD, 2 patients died and the remaining half of the patients had PD [42]. The recent trial tested MelQbG10 in combination with Montanide® ISA-51 and topical 5% Imiquimod cream in 21 patients with stage III/IV malignant melanoma [41]. The primary immune outcome of this study was the appearance of Melan-A-specific T cells. Patients were immunized with MelQbG10 + Montanide® ISA-51 by the SQ, ID or intranodal (IN) routes, with two groups also being treated additionally with topical 5% Imiquimod cream. In total, 76% of the patients responded to MelQbG10 vaccination by generating at least a 2 fold increase in Melan-A specific T cells when compared to pre-vaccination conditions. Significantly higher T cell frequencies were seen in patients vaccinated with MelQbG10 plus Montanide® ISA-51 versus MelQbG10 alone \((p = 0.003)\). The group vaccinated SQ generated \(\approx 2\) fold higher numbers of Ag-specific T cells than those vaccinated IN \((p = 0.001)\). Despite these promising immunological improvements, there were no significant differences between groups regarding clinical outcome, with their cancer progressing in \(\approx \) half of all patients in each treatment arm. SD was observed in a quarter of the patients in all treatment groups [41].

A phase I study examined the safety, immunogenicity and clinical effect of a vaccine targeting WT-1 peptide in 28 patients [118]. The first group of patients was vaccinated with WT-1 peptide plus Montanide ISA-51 ID. 80% of these individuals failed to complete the 9 planned vaccinations due to disease progression. A second group received the same vaccine supplemented with GM-CSF and was administered SQ. Disease progressed in 75% of patients in this group. The final set of patients received the vaccine supplemented with CpG 7909. 60% of these patients had SD, a frequency much higher than observed in the other two groups [118]. No data on the statistical significance of this result is provided, but the beneficial trend in outcome warrants further investigation.

7.3. Safety

More than a thousand subjects participated in the clinical trials described in this review. The dose of ODN commonly used in these trials was 1.5–15 µg/kg and the schedule of ODN administration ranged from weekly to monthly. In preclinical studies, much higher doses of CpG ODN (2.5 mg/kg) were administered daily to mice. These led to a number of serious AEs including liver toxicity, enlargement of the spleen and lymph nodes, extramedullary hematopoiesis and systemic inflammation [119,120]. Yet the lower dose and frequency of CpG ODN used in human vaccine trials, combined with differences in the cell types expressing TLR9 in primates versus rodents, yielded a relatively benign toxicity profile.

Flu-like symptoms including fatigue, rigors, myalgia and pyrexia, and injection site reaction were the most commonly described treatment related AEs. These events were primarily grade 1–2 and their frequency tended to increase with CpG ODN dose. The likelihood of an injection site reaction arose significantly as subjects received additional doses of CpG ODN adjuvanted vaccines, but the severity of these reactions remained mild to moderate \((p < 0.05)\) [90]. For instance 1 of 24 patients (4%) developed generalized pruritus and required oral antihistamin after the first immunization with AMA1-C1/Alhydrogel® + CpG 7909 [74].
CpG ODN also induced biphasic changes in leukocyte frequency in the peripheral blood when administered SQ. In one study, neutrophil numbers doubled at 12 h and then fell to half of baseline levels at 72 h after each vaccination, in some cases reaching a nadir of 800 cells/µl. These changes in the peripheral blood may reflect the movement of immune cells through the blood to the site of CpG ODN vaccination and associated draining lymph nodes [121–124]. Lymphadenopathy was reported in two studies [41,42] and enlarged lymph nodes were detected in 9 of 21 patients (42%) after vaccination with MelQbG10 in combination with Montanide® ISA-51 regardless the disease status [41].

Concern was raised early in their development that CpG ODN might activate autoreactive B cells and thus increase the risk of autoimmune disease [125]. This possibility was examined in multiple studies. Most detected no significant change in serum autoAb levels against dsDNA, rheumatoid factor, thyroglobulin or nuclear Abs, nor were symptoms of autoimmunity reported [74,116,126,127]. However, 1 of 56 healthy volunteers (2%) vaccinated with Engerix-B® plus 1 mg of CpG 7909 developed moderate anti-dsDNA Abs at week 6 which returned to baseline by week 24 [61]. Elevated ANA and anti-dsDNA Ab levels were found in 8/75 (10%) subjects vaccinated with AMA1-C1/Alhydrogel® + CpG ODN but these generally returned to baseline within 6 weeks [76]. Thus it appears that CpG ODN are safe and well tolerated when used as an adjuvants in normal healthy individuals.

More frequent and serious AEs were observed in studies involving HIV-infected individuals and patients with cancer. In many situations it was unclear whether the reported effects were causally linked to vaccination or due to the severe nature of the underlying disease (such as the heart attack observed in one subject onetrial). Although 3 of 29 (10%) HIV-infected patients vaccinated with Engerix-B® plus CpG 7909 developed severe AEs, the nature and timing of these AEs is inconsistent with the vaccine having been their cause. These occurred from 0.5 to 4 months post vaccination [90]. Four of 29 patients in this study developed transient elevations in serum anti-ds DNA autoAb levels although no clinical symptoms of autoimmunity were observed [90]. One case of Sjogren’s like syndrome was reported in a single trial of 50 patients (2%) with refractory B cell non-Hodgkin’s lymphoma. Symptoms in that case included inflammation of the salivary glands and kerato-conjunctivitis sicca after treatment with rituximab and 4 weekly vaccinations with CpG 7909, findings possibly related to development of a paraneoplastic autoimmune disease [122].

Autoimmune responses were developed more frequently in cancer patients [128,129]. In a small trial of patients with prostate cancer, Ab reactive with CpG ODN were detected in 13% of participants without clinical sequelae [128]. Similarly, anti-dsDNA Abs were detected in 4/8 (50%) melanoma patients after four vaccinations with Melan-A, Montanide® ISA-51 and CpG 7909, although no clinical signs of autoimmunity disease were observed [129]. When CpG ODN were administered intra-cerebrally to patients with glioblastoma, treatment-related general and partial seizures developed in 10% of patients [101]. In the same study, grade 3–4 hematologic events occurred in 2% of recipients of either 10 or 40 mg of CpG ODN [101]. In overview, concerns remain that CpG ODN may have serious side effects when administered to patients with severe infectious disease or cancer, requiring close monitoring of such individuals.
7.4. Limitations

Several considerations limit the interpretation of results from the clinical trials described above and in Table 2. Most tumor vaccine trials suffer from one or more deficiencies that compromise conclusions concerning clinical efficacy. These included (i) low patient number (leaving most trials underpowered), (ii) short duration (proving insufficient follow up to determine whether therapy altered tumor growth) and (iii) inclusion of patients with different types and severity of cancer (preventing intra-group comparison of outcome).

Efforts to evaluate the effect of vaccination on cellular and humoral immune responses were also compromised. Individual studies monitored different immunologic outcomes (ranging from changes in the frequency to activation state of primary and/or memory T cells and NK cells) reflecting a lack of consensus regarding which (if any) are relevant markers of anti-tumor efficacy. Moreover, while animal studies show that the tumor-infiltrating immune cells can provide a useful metric of vaccine efficacy, clinical trials typically rely on peripheral blood samples to monitor the nature and magnitude of the T and B cell responses elicited (as it is difficult to obtain repeated tumor biopsies from cancer patients). This raises the concern that cells in the blood may not reflect the behavior of the functionally relevant cells infiltrating the tumor [130].

8. Conclusion and perspectives

This review focuses on the outcome of clinical trials in which CpG ODN were combined with vaccines targeting infectious diseases and cancer. Overall findings indicate that CpG ODN improve humoral and cellular immune responses when vaccines are administered to healthy individuals. CpG ODN accelerated the induction of protective Abs and supported the generation of higher and more persistent Ab titers with protein vaccines. This was best demonstrated by the extensive trials involving vaccines against anthrax and HBV [53,54,62]. An additional advantage of using CpG ODN as adjuvant was improved immunity even in subjects with compromised immune systems [90,91,61].

Less definitive conclusions can be drawn concerning cancer vaccines. In most studies, CpG ODN increased the immunogenicity of co-administered peptide vaccines (Table 2). Patients who received the adjuvanted vaccines generated stronger Ag-specific serum Ab, CD8 and CD4 T cell responses when compared to peptide vaccines alone. Anti-tumor immunity also arose more rapidly in patients vaccinated with CpG adjuvanted vaccines. These effects were exemplified in the controlled phase I trial of patients with metastatic melanoma. For example, recipients of the CpG ODN adjuvanted vaccine developed Ag-specific CD8 T cells earlier (at the second vs. fourth vaccination) and with significant higher frequency (1.15% ± 0.93% vs. 0.13% ± 0.11%, p < 0.01) than the non-CpG group [129]. These CD8 T cells were of the effector memory phenotype (CD45RA−, CCR7− and expressed elevated levels of granzyme B, perforin, IFN-γ, and TNF-α) [129]. However the magnitude of immune effects varied between trials and there was little reproducibility in the effect of CpG ODN on other immune parameters such as the frequency of NK cells or DC [118,131–133]. Of greater concern, the immune responses observed rarely correlated with clinical outcome. Indeed, evidence is lacking that any of the vaccine/adjuvant formulations examined reproducibly altered the progression of large established tumors.
Given the absence of clinical efficacy, we conclude that the magnitude of the immune response needed to eliminate large established tumors exceeds that required to prevent the proliferation of an infectious pathogen, and that available vaccine/CpG ODN combinations do not achieve such a high levels of immunity. The recent success of vaccines targeting prostate tumors provides guidance on how to best structure future clinical trials examining CpG ODN based adjuvants [134]. They suggest that vaccination should be initiated early in the disease process (rather than waiting for all conventional therapies to fail) and targeting small slow growing tumors rather than patients with large tumor burden. Enrolling patients with the same type of cancer and clinical presentation would also facilitate analysis of the results. Finally, we recommend that the raw data from all clinical trials be made freely available, a step that would facilitate the meta-analysis of such data and aid in the rational design of future clinical trials.

**Abbreviations**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>Ab</td>
<td>antibody</td>
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<td>AE</td>
<td>adverse event</td>
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<td>Ag</td>
<td>antigen</td>
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<td>APC</td>
<td>Ag presenting cell</td>
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<td>CTL</td>
<td>cytotoxic T cell</td>
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<td>CR</td>
<td>complete response</td>
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<td>DC</td>
<td>dendritic cell</td>
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<td>GIA</td>
<td>growth inhibition assay</td>
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<td>GMA</td>
<td>geometric mean Ab titer</td>
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<td>HI</td>
<td>hemagglutinin inhibition</td>
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<td>ID</td>
<td>intradermal</td>
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<td>IM</td>
<td>intramuscular</td>
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<td>IN</td>
<td>intranodal</td>
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<td>IV</td>
<td>intravenous</td>
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<tr>
<td>ISS</td>
<td>immunostimmulatory DNA sequence</td>
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<tr>
<td>NA</td>
<td>not available</td>
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<tr>
<td>NK</td>
<td>natural killer cell</td>
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<td>NSCLC</td>
<td>non-small cell lung cancer</td>
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<td>OS</td>
<td>overall survival</td>
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<td>PBMC</td>
<td>peripheral blood mononuclear cells</td>
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<tr>
<td>PD</td>
<td>progressive disease</td>
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<tr>
<td>PF</td>
<td>Plasmodium falciparum</td>
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</table>

*Vaccine. Author manuscript; available in PMC 2015 November 12.*
**PFS** progression free survival

**PMR** parasite multiplication rate

**PR** partial response

**SD** stable disease

**SQ** subcutaneous

**TNA** toxin neutralizing assay

### References


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Table 1

<table>
<thead>
<tr>
<th>Trial no.</th>
<th>Infection</th>
<th>Treatment</th>
<th>Route</th>
<th>Phase</th>
<th>Date</th>
<th>Results</th>
<th>Reference</th>
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<tbody>
<tr>
<td>NCT00344539</td>
<td>Malaria</td>
<td>AMA1-C1/Alhydrogel&lt;sup&gt;®&lt;/sup&gt; (20, 80 μg) ± CpG 7909 (564 μg) n = 75 in 3 groups days: 0, 28, 56</td>
<td>IM</td>
<td>I</td>
<td>04/05–03/07</td>
<td>AMA-1 + CpG vaccination supported an 11–14 fold increase in anti-AMA1 Ab titers (p &lt; 0.02), PF GIA was 3–4 fold higher (p &lt; 0.0001) than AMA-1 alone, but similar growth inhibitory activity against Pf in standardized AMA1-IgG titer.</td>
<td>[76,135]</td>
</tr>
<tr>
<td>NCT00414336</td>
<td>Malaria</td>
<td>AMA1-C1/Alhydrogel&lt;sup&gt;®&lt;/sup&gt; (80 μg) ± CpG 7909 (564 μg) n = 24 in 2 groups days: 0, 28</td>
<td>IM</td>
<td>I</td>
<td>10/07–11/07</td>
<td>AMA-1 + CpG vaccination of malaria exposed adults increased AMA1-specific Abs by 2–3 fold vs. AMA-1 alone; no significant differences in Ag specific memory B cells or GIA between groups.</td>
<td>[74,136]</td>
</tr>
<tr>
<td>NCT00320658</td>
<td>Malaria</td>
<td>AMA1-C1/Alhydrogel&lt;sup&gt;®&lt;/sup&gt; (20, 80 μg) + MSP1&lt;sub&gt;42&lt;/sub&gt;-C1/Alhydrogel&lt;sup&gt;®&lt;/sup&gt; (80 μg) ± CpG 7909 (564 μg) n = 24 in 2 groups days: 0, 28, 56</td>
<td>IM</td>
<td>I/IIa</td>
<td>07/06–07/07</td>
<td>CpG 7909 enhanced significantly the appearance or AMA1-C1-specific memory B cells (2–10 fold at days 35, 56, 99; p &lt; 0.01) and MSP&lt;sub&gt;42&lt;/sub&gt;-specific memory B cells (7–10 fold at day 3 and 84 after 3rd vaccination) in PBMC.</td>
<td>[75,126]</td>
</tr>
<tr>
<td>NCT00984763</td>
<td>Malaria</td>
<td>AMA1-C1/Alhydrogel&lt;sup&gt;®&lt;/sup&gt; (80 μg) ± CpG 7909 (564 μg) n = 10 in 2 groups days: 0, 28, 56</td>
<td>IM</td>
<td>I/IIa</td>
<td>07/09–09/10</td>
<td>Inclusion of CpG significantly increased GIA titers (63% versus 13%; p &lt; 0.01) and correlated with lower PMR.</td>
<td>[78]</td>
</tr>
<tr>
<td>NCT00889616</td>
<td>Malaria</td>
<td>BSAM2/Alhydrogel&lt;sup&gt;®&lt;/sup&gt; (40, 160 μg) + CPG 7909 (564 μg) n = 30 in 2 groups days: 0, 56, 180</td>
<td>IM</td>
<td>I</td>
<td>06/09–03/10</td>
<td>Both doses of BSAM2/Alhydrogel&lt;sup&gt;®&lt;/sup&gt; + CPG 7909 were immunogenic: increase of anti-AMA1 (86 and 113 μg/mL) and anti-MSP1&lt;sub&gt;42&lt;/sub&gt; titers (57 and 76 μg/mL) Ab at day 194 with boosting effect after the second vaccination; GIA increased 7–41%.</td>
<td>[137]</td>
</tr>
<tr>
<td>NA</td>
<td>Influenza</td>
<td>Fluarix&lt;sup&gt;®&lt;/sup&gt; (0.5, 0.05 mL) ± CpG 7909 (1 mg) n = 60 in 4 groups Single dose</td>
<td>IM</td>
<td>Ib</td>
<td>08/99–10/99</td>
<td>CpG 7909 did not increase anti-HI Ab titers vs. vaccine alone (p &gt; 0.05). Upon Ag restimulation, PBMC at 4 week showed significantly higher IFN-γ production for A/Beijing/262/95 (p = 0.048) and B/Harbin/7/94 (p = 0.0057) in 1/10th dose Fluarix&lt;sup&gt;®&lt;/sup&gt; + CpG 7909 compared to 1/10th dose Fluarix&lt;sup&gt;®&lt;/sup&gt; alone.</td>
<td>[82]</td>
</tr>
<tr>
<td>NCT00562939</td>
<td>Pneumococcus in HIV-infected adults</td>
<td>PCV7 (Prevnar&lt;sup&gt;®&lt;/sup&gt;), PPV-23 (PneumoNovum&lt;sup&gt;®&lt;/sup&gt;) (1 mL) ± CPG 7909 (1 mg) n = 97 in 2 groups 0, 3, 9 months</td>
<td>IM</td>
<td>Ib/Ia</td>
<td>01/08–01/09</td>
<td>CpG group showed a mean reduction in proviral DNA of 12.6% vs. a 6.7% increase in the non-GpG group (p = 0.02). No significant changes in HIV specific Ab or T cell immunity. PBMC from the CpG group produced significantly more IL-1β (p = 0.0046), IL-6 (p = 0.0051), IFN-γ (p = 0.0047), MIP-β (p = 0.0086), IL-2R (p = 0.0002) levels. PBMC of CpG vaccinated patients stimulated with LPS showed elevated production of L-1β (p = 0.038) and IFN-γ (p = 0.019) vs. vaccine alone.</td>
<td>[93–95]</td>
</tr>
<tr>
<td>NCT00722839</td>
<td>Cytomegalovirus (CMV)</td>
<td>PADRE-CMV (1.5, 2.5, 10 mg), Tetanus-CMV fusion peptide, ±CpG 7909 (1 mg) n = 63 in 4 groups days: 0, 21, 42, 63</td>
<td>SQ</td>
<td>Ib</td>
<td>05/10–10/10</td>
<td>CMV alone induced no response. CD8 T cell responses induced in 30% of CpG plus PADRE-CMV and 70% of Tetanus-CMV plus CpG groups (p = 0.002), pp65 specific CD8 T cells increased 3–10 fold in tetramer binding assay compared to vaccine alone (p = 0.004) and 4–5 fold vs. prevaccination levels (p = 0.008).</td>
<td>[138]</td>
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<tr>
<td>Trial no.</td>
<td>Infection</td>
<td>Treatment</td>
<td>Route</td>
<td>Phase</td>
<td>Date</td>
<td>Results</td>
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<tr>
<td>NA</td>
<td>HBV</td>
<td>HBsAg (20 μg) + 1018 ISS (300, 650, 1000, 3000 μg)</td>
<td>IM</td>
<td>I</td>
<td>NA</td>
<td>Seroprotective titers (&gt;10 mIU/L) were reached by 0, 25, 75 and 87% of patients after 1st vaccination vs. 62, 100, 100, 100% after 2nd vaccination with increasing CpG ODN doses. GMA anti-HBsAg were 1.22, 5.78, 24.75, 206.5 after 1st and 65.37, 877.6, 1545, 3045 mIU/mL after 2nd vaccination. Injection site reactions were more frequent in CpG group (p ≤ 0.05) but all doses were well tolerated.</td>
<td>[62]</td>
</tr>
<tr>
<td>NA</td>
<td>HBV</td>
<td>rHBsAg (20 μg) + 1018 ISS (3 mg), Engerix-B®</td>
<td>IM</td>
<td>II</td>
<td>NA</td>
<td>CpG increased the percentage of participants achieving seroprotection after 1st, 2nd and 3rd vaccination compared to Engerix-B® alone (79% vs. 12%; 100% vs. 18%; 100% vs. 64%; p &lt; 0.001); GMC of anti-HBsAg Ab were 1.22, 5.78, 24.75, 206.5 after 1st and 65.37, 877.6, 1545, 3045 mIU/mL after 2nd vaccination.</td>
<td>[63]</td>
</tr>
<tr>
<td>NA</td>
<td>HBV</td>
<td>rHBsAg (20 μg) + 1018 ISS (3 mg), Engerix-B® (1 mL)</td>
<td>IM</td>
<td>III</td>
<td>12/06–07/07</td>
<td>Higher anti-HBV titers and improved seroprotection was achieved in the CpG group at 4, 8, 24 and 28 weeks post vaccination (23% vs. 3%; 88% vs. 26%; 98% vs. 32%; 97% vs. 81%). GMC were &gt;20-fold higher after the 2nd vaccination and at similar levels as Engerix-B® after the 3rd vaccination.</td>
<td>[64]</td>
</tr>
<tr>
<td>NA</td>
<td>HBV in adults 40–70 years</td>
<td>rHBsAg (20 μg) + 1018 ISS (3 mg), Engerix-B® (1 mL)</td>
<td>IM</td>
<td>III</td>
<td>NA</td>
<td>Seroprotective anti-HBsAg Ab titers (≥ 10 mIU/mL) were higher in the CpG adjuvanted group (94% vs. 69%) at week 4. GMC levels were 15-fold higher at 4 week (243 vs. 16 mIU/mL; p &lt; 0.001). Difference between groups diminished with boosting but persisted through week 32 (863 vs. 439 mIU/mL; p = 0.038).</td>
<td>[139]</td>
</tr>
<tr>
<td>NA</td>
<td>HBV</td>
<td>Engerix-B® (20 μg HBsAg) + CpG 7909 (0.125, 0.5, 1 mg)</td>
<td>IM</td>
<td>II</td>
<td>04/99–06/01</td>
<td>Inclusion of CpG accelerated the development of protective anti-HBsAg Ab titers (p &lt; 0.001) vs. vaccine alone. The 0.5 mg dose of CpG 7909 was most effective (2-fold higher than 0.125 or 1.0 mg, p &lt; 0.05).</td>
<td>[61]</td>
</tr>
<tr>
<td>NA</td>
<td>HBV in HIV-infected adults</td>
<td>Engerix-B® (40 μg HBsAg) + CpG 7909 (0.5, 1 mg)</td>
<td>IM</td>
<td>III</td>
<td>NA</td>
<td>Inclusion of CpG significantly increased anti-HBsAg Ab titers from 6 to 48 weeks. T helper cells showed higher proliferative response to HBsAg ex vivo at week 8 (p = 0.042) and 48 (p = 0.024). No changes in CD4 or CD8 T cell subsets through 5 years of follow up. The CpG group retained seroprotective Anti-ABsAg titers at 60 months (80% vs. 40%, p = 0.004).</td>
<td>[90–92]</td>
</tr>
<tr>
<td>NA</td>
<td>Anthrax</td>
<td>Bio-Thrax® (0.5 mL), ±CpG 7909 (1 mg)</td>
<td>IM</td>
<td>I</td>
<td>NA</td>
<td>BioThrax® + CpG 7909 induced 6 fold higher IgG anti-PA Ab titers (1465 μg/mL vs. 232 μg/mL, p &lt; 0.001) that peaked earlier (day 21 vs. 40, p &lt; 0.001) vs. vaccine alone. Toxin neutralizing capacity was 8.8 fold higher (p &lt; 0.001) and also developed earlier (day 22 vs. 46, p &lt; 0.001).</td>
<td>[53]</td>
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<tr>
<td>Trial no.</td>
<td>Infection</td>
<td>Treatment</td>
<td>Route</td>
<td>Phase</td>
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<td>Results</td>
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<tr>
<td>NCT01263691</td>
<td>Anthrax</td>
<td>Bio-Thrax® (0.5 mL), AV7909: AVA (0.25–0.5 mL) + CpG 7909 (0.25–0.5 mg)</td>
<td>IM</td>
<td>I</td>
<td>NA</td>
<td>Inclusion of any dose of AV 7909 yielded 2-fold higher GMT in TNA NF50 (50% neutralization factor). CpG accelerated reaching peak titer (day 28 vs. 35).</td>
<td>[54]</td>
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</table>
Table 2
CpG ODN as adjuvant in tumor vaccine trials.

<table>
<thead>
<tr>
<th>Trial no.</th>
<th>Type of cancer</th>
<th>Treatment</th>
<th>Route</th>
<th>Phase</th>
<th>Date</th>
<th>Results</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>NA</td>
<td>Malignant melanoma</td>
<td>2/Melan-A (100 μg), Montanide® ISA-51 (300 μg), +CpG 7909 (500 μg)</td>
<td>SQ</td>
<td>I</td>
<td>NA</td>
<td>CpG group: more rapid (after 2 vs. 4 vaccinations) and 8-fold higher Melan-A-specific CD8 T cell response compared to the non-CpG group (1.15 ± 0.93% vs. 0.13 ± 0.11%, p &lt; 0.01). Response rate was 100% after 4 vaccinations vs. 50% in controls. Clinical response: one patient (12%) remained disease free, one patient SD, 6 patients (75%) PD.</td>
<td></td>
</tr>
<tr>
<td>NCT0085189</td>
<td>Malignant melanoma stage III–IV</td>
<td>NY-ESO-1 (600 μg), Montanide® ISA-51 (3 mL), +CpG 7909 (2 mg)</td>
<td>SQ</td>
<td>II</td>
<td>05/04–09/07</td>
<td>GpG group: 100% of patients (n = 3) developed NY-ESO-1 specific CD8 T cells which increased 3–10 fold after 6 vaccinations compared to pre-vaccination status. CpG alone (without NY-ESO-1) had no effect. Clinical outcome: 1 (12%) patient had SD for 6 months then progressed as did all other patients.</td>
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<tr>
<td>NCT00306514, 00306566, 00306553</td>
<td>Malignant melanoma stage II–IV</td>
<td>CYT004-MelQbG10 (1 mg), n = 22 in 4 groups, 6 or 11 vaccinations, (wkly or daily 5x)</td>
<td>SQ/ID</td>
<td>I/II</td>
<td>2006</td>
<td>Significant increase in Ag-specific T cells in 14/22 patients (63%) compared to pre-vaccination status (no differences between groups I-IV). T cells produced TNF-α, IFN-γ and IL-2 and up-regulated expression of surface antigens LAMP-1 and CD107a. Significantly more memory T cells vs. seen patients vaccinated with Melan-A peptide plus IFA (p &lt; 0.05) from study [102]. Clinical response: 7 (31%) patients were free of disease, 1 patient (4%) PR, 11 patients (50%) PD, 1 patient (4%) SD, 2 (9%) patients died.</td>
<td></td>
</tr>
<tr>
<td>NCT00651703</td>
<td>Malignant melanoma stage III–IV</td>
<td>CYT004-MelQbG10, Montanide® ISA-51, Imiquimod 5%, n = 21 in 4 groups, 3x wkly, 3x monthly</td>
<td>SQ/ID/IN</td>
<td>IIa</td>
<td>04/08–07/10</td>
<td>76% of patients responded to MelQbG10 vaccination by a &gt;2 fold increase in Ag-specific T cells. Clinical response: 5 patients (23.8%) had SD, 9 patients (42%) PD, 5 (23%) patients without evidence of disease, 2 (9%) patients died.</td>
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<tr>
<td>NCT00112229</td>
<td>Malignant melanoma stage III–IV</td>
<td>Melan-A/Mart-1, Tyrosidase (YMD), Montanide® ISA-51 ± CpG 7909, n = 24 in 2 groups monthly</td>
<td>SQ</td>
<td>I</td>
<td>04/03–06/12</td>
<td>CpG group: Melan-A/MART-1 specific T cell frequencies were 10-fold higher (p &lt; 0.01) and produced higher levels of IFN-γ, TNF-α and IL-2 (p &lt; 0.01) compared to non-CpG group.</td>
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<tr>
<td>Trial no.</td>
<td>Type of cancer</td>
<td>Treatment</td>
<td>Route</td>
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<tr>
<td>NA</td>
<td>Malignant melanoma stage III–IV</td>
<td>MART-1, gp100, tyrosinase peptides, Montanide® ISA-51, + CpG 7909 (600 μg), GM-CSF (80 μg)</td>
<td>SQ</td>
<td>I</td>
<td>01/09–12/10</td>
<td>2 (9.5%) patients had PR. 8 (38%) patients with SD (2–7 months, 50% of them with positive Elispot ≥10 spots). 20 (95%) patients had PD in follow up; median PFS 1.87 months, median OS 13.4 months.</td>
<td>[116]</td>
</tr>
<tr>
<td>NA</td>
<td>Multiple types (melanoma, breast cancer, ovarian cancer, sarcoma) stages I–IV</td>
<td>rNY-ESO-1 (100, 400 μg), Montanide® ISA-51, + CpG 7909 (2.5 mg), n = 18 in 2 groups 4 × 3 week interval</td>
<td>SQ</td>
<td>NA</td>
<td>NA</td>
<td>94% of patients developed CD4 T cell responses and 50% CD8 T cell responses. IgG Ab titer of 24 ± 10 in those vaccinated with 100 μg NY-ESO-1; 42 ± 29 in the 400 μg NY-ESO-1 group (preferentially IgG1 and IgG3).</td>
<td>[141]</td>
</tr>
<tr>
<td>NCT00199836</td>
<td>Multiple types (melanoma, NSCLC, breast cancer, ovarian cancer, sarcoma) stage III–IV</td>
<td>NY-ESO-1b (100 μg), Montanide® ISA-51 (0.5 mL), +CpG 7909 (1 mg) n = 14 one group 4 × 3 week interval</td>
<td>SQ</td>
<td>I</td>
<td>09/03–12/05</td>
<td>Induction of Ag-specific CD8 T cells against NY-ESO-1 in 9 of 14 patients (63%). Clinical response: 2 patients (14%) showed no evidence of disease, 9 patients (64%) had PD, 3 patients (21%) SD.</td>
<td>[142]</td>
</tr>
<tr>
<td>UMIN 000002771</td>
<td>Multiple types (colorectal, pancreatic, rectal, lung, cervical cancer, epitheloid cancer and papilla cancer)</td>
<td>WT-1, GM-CSF (100 μg), Montanide® ISA-51, ±CpG7909 (100 μg) n = 28 in 2 groups 8x weekly</td>
<td>ID</td>
<td>I</td>
<td>01/10–11/10</td>
<td>Clinical response: 60% of patients treated with WT-1 + Montanide® ISA-51 + CpG 7909 showed SD, 40% PD. (WT-1 only: 20% SD, 80% PD; WT1 + GM-CSF: 25% SD, 75% PD)</td>
<td>[118]</td>
</tr>
<tr>
<td>NCT00669292</td>
<td>Esophageal cancer</td>
<td>URLC10-117 peptide (1 mg), TTK-567 peptide (1 mg), Montanide® ISA-51, + CpG 7909 (0.02, 0.1 mg/kg) n = 9 in 3 groups days: 1, 8, 15, 22</td>
<td>SQ</td>
<td>I/II</td>
<td>11/06–11/09</td>
<td>Serum levels of IFN-α and 2–5 AS increased with increasing dose of CpG 7909. Increase in IFN-α producing Ag-specific CD8 T cell responses in 33% (TTK-567) or 66% (LY6K-177) of the patients. Clinical response: no partial or complete responses, 33% of patients received 0.02 mg/kg CpG 7909 and 66% of patients received 0.1 mg/kg CpG 7909 showed SD.</td>
<td>[143]</td>
</tr>
<tr>
<td>NCT00299728</td>
<td>Prostate cancer</td>
<td>NY-ESO-1 (100 μg), +CpG 7909 (2.5 mg) n = 15 one group 4 × 3 week interval</td>
<td>ID</td>
<td>I</td>
<td>03/06–12/06</td>
<td>9 patients (60%) had NY-ESO-1 specific CD4 T cells by week 7–28 (mean 14) and 6 patients (40%) had NY-ESO-1 specific CD8 T cells by week 10–28 (mean 14). 2 patients (13%) developed anti-CpG Ab (1:25,000, 1:6,400). Clinical response: 2 patients (13%) showed no evidence of disease, 8 patients (53%) showed SD and 3 (20%) PD, no PR or CR was seen after 4 vaccinations.</td>
<td>[128]</td>
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</tbody>
</table>