

For reprint orders, please contact:
reprints@future-drugs.com



Immunostimulatory DNA as a vaccine adjuvant

Debbie Higgins[†], Jason D Marshall, Paula Traquina, Gary Van Nest and Brian D Livingston

Immunostimulatory DNA containing unmethylated CpG motifs is recognized by Toll-like receptor 9, resulting in the activation of innate immune responses that subsequently amplify the adaptive-immune response. Advances in the characterization of Toll-like receptor 9 signaling have identified immunostimulatory sequences (ISS) with distinct biological activities. Numerous animal models have demonstrated that synthetic ISS are effective adjuvants that enhance both humoral and cellular immune responses in diverse indications, ranging from infectious disease to cancer and allergy. An added benefit supporting the use of ISS as a vaccine adjuvant is that the specific activation of a pathway critical to the regulation of the immune response results in minimal toxicity. To date, clinical testing has largely affirmed the potency and safety of ISS-adjuvanted vaccines.

Expert Rev. Vaccines 6(5), 747–759 (2007)

Immunostimulatory sequences

When a host organism encounters a viral or bacterial pathogen, recognition is achieved primarily through early sentinels called pattern recognition receptors (PRRs), molecules that bind with high avidity to pathogen-associated molecular patterns (PAMPs), including lipid, carbohydrate, peptide and nucleic acid structures. One of the best-studied PRR families is the Toll-like receptor (TLR) family, transmembrane signaling molecules that play a key role in the initiation of innate immune responses and also influence the later and more antigen-specific adaptive immune response (reviewed in [1]). Most TLRs are expressed on the cells surface, but a subset is localized to the endosomal/lysosomal compartment of the cell, into which foreign pathogens can be endocytosed and enzymatically degraded and microbial nucleic acids can be presented as short RNA or DNA segments, the ligands for this TLR subset. The most prominent and well-characterized TLR within this subset is TLR9, which shows a restricted and species-specific pattern of cellular distribution. In rats and mice, TLR9 is widely expressed on nearly all antigen-presenting cells (APCs), including B cells, dendritic cells (DCs), and the

myeloid-derived lineage comprising monocytes, macrophages and myeloid dendritic cells (MDCs) [2]. In humans and primates, TLR9 distribution is primarily limited to B cells and a specific subset of DCs, plasmacytoid dendritic cells (PDCs) [3,4]. TLR9 protein is expressed in the endoplasmic reticulum in resting cells but transfers to the early- and late endosomal compartments after the cell encounters the appropriate PAMP, which is unmethylated CpG-containing DNA [5]. A CpG dinucleotide is an absolute requirement for TLR9 activation, although flanking nucleotides can also play a role, both qualitatively and quantitatively, in the response. TLR9 ligands can include DNA from DNA viruses, such as herpes simplex virus-1 and -2 [6] and murine cytomegalovirus [7]; bacterial DNA from microbes, such as *Streptococcus pneumoniae* [8], *Propionibacterium acnes* [9] and *Mycobacterium tuberculosis* [10]; plasmid DNA [11]; or short, synthetic oligodeoxynucleotide (ODN) sequences, engineered with one or more CpG motifs. These CpG-containing ODNs are referred to as immunostimulatory sequences (ISS) and have become the basis for vaccine development for a wide range of applications because of their formidable immune-activating properties.

CONTENTS

Immunostimulatory sequences

ISS as a vaccine adjuvant: results from preclinical studies

ISS as a vaccine adjuvant: results from toxicology & safety studies

ISS as a vaccine adjuvant: results from clinical studies

Expert commentary & five-year view

Financial & competing interests disclosure

Key issues

References

Affiliations

[†] Author for correspondence
Dynavax Technologies,
2929 Seventh Street, Suite 100,
Berkeley, CA 94710, USA
Tel.: +1 510 665 7229
Fax: +1 510 848 1327
dhiggins@dynavax.com

KEYWORDS:
adjuvant, cell-mediated immunity, humoral immunity, immunomodulation, immunostimulatory DNA, Toll-like receptor 9, vaccine

Three classes of ISS ODNs: CpG-A, CpG-B & CpG-C

Extensive manipulation of the sequence, composition and structure of ISS ODNs has resulted in the definition of three major classes that differ not only physically and chemically but also in the *in vitro* biological activities they generate. The first ISS ODNs were derived from plasmid DNA sequences and were generally engineered to have a phosphorothioate (PS) DNA backbone (for increased stability), a length of 12–30 nts, and one or more interior CpG dinucleotides that may be flanked by a variety of base combinations. These make up the ‘B’ class of ISS, or ‘CpG-B’, and exhibit a wide variety of immunostimulatory activities *in vitro*, including the activation of B cells and PDCs [12] and, in rodents, monocytes and MDCs as well. CpG-B activates B cells to express CD69, CD80, CD40 and MHC class II, to secrete IL-6 and IL-10, and to proliferate [13–16]. PDC activation by CpG-B is characterized by an upregulation of CD80, CD86, CD40, CD54 and MHC class II expression [17–19], induction of TNF- α , IL-6 and IL-12 [20,21], repression of apoptotic signals and differentiation to a mature DC stage in which antigen processing and presentation are engaged [22]. PDCs are best known as prodigious synthesizers of IFN- α ; however, CpG-B is relatively weak in this activity, eliciting little IFN- α induction from either human peripheral blood mononuclear cells (PBMCs) or purified PDCs [19].

Another class of ISS was created by the addition of PS polyguanosine sequences to the 5′ and/or 3′ ends of short phosphodiester CpG-containing ODNs. Since poly-G sequences can form G-tetrad structures through guanine–guanine Hoogsteen base-pairing, these ODNs, known as ‘CpG-A’, have been shown by size-exclusion chromatography to be in the form of high-molecular-weight aggregates, composed of approximately 200–500 ODNs [23]. The aggregation enabled by poly-G motifs leads to enhanced uptake of large numbers of CpG-A ODNs simultaneously, compared with the less efficient kinetics

of CpG-B monomer uptake. CpG-A displays a functional phenotype that is very distinct from CpG-B. Its main attribute is the induction of very high levels of IFN- α from human PBMCs or PDCs, 100–1000-fold higher than those elicited by CpG-B [24]. CpG-A is also effective at eliciting substantial IFN- α expression from mouse and monkey PDCs [25–27] and this activity is dependent on the high-molecular-weight structure of aggregated CpG-A. Interference with the formation of G-tetrads by substitutions within the poly-G sequence severely diminishes the IFN- α -inducing capacity of CpG-A [28]. Interestingly, CpG-A shows little or no ability to activate B cells and far outclassed by CpG-B in this respect [14]. In addition, CpG-A is a poor inducer of PDC maturation and is also weaker in the induction of TNF- α and IL-6 [21,28]. In fact, induction of high levels of IFN- α (and consequently its downstream effects, such as natural killer [NK] cell activation and IFN- γ synthesis) appears to be nearly the only direct ISS function that CpG-A reliably exerts *in vitro* [29,30]. Thus, the functional repertoires of the CpG-A and CpG-B classes appear to be almost mutually exclusive and can be easily remembered through the mnemonic device of ‘A’ for potent IFN- α induction and ‘B’ for potent B cell activation (TABLE 1).

More recently, a third class of ODNs was engineered to have the functional properties of both the A and B classes of CpG and has been termed ‘CpG-C’. Similar to CpG-B, these ODN are PS sequences of 20–30 residues containing multiple interior CpG motifs, but they differ from CpG-B in the inclusion of two critical elements: one or more 5′-TCG motifs and a palindromic sequence of 12–20 nucleotides that usually follows (but may include) the 5′-TCG motif [16,28,31]. The palindromic sequence allows self-annealing to occur and both the monomer (hairpin) and duplex forms of CpG-C participate in the induction of high levels of IFN- α . The level of CpG-C-mediated IFN- α is usually not as great as that induced by CpG-A but is still generally 50–100-fold higher than that induced by

Table 1. Characteristics of classes of immunostimulatory sequences.

ISS class	Sequence and structural elements	Plasmacytoid dendritic cell IFN- α	Plasmacytoid dendritic cell maturation	B-cell activation
CpG-A	Phosphodiester CpG motif(s) flanked by phosphorothioate polyguanosines; forms higher-molecular-weight aggregates through G-tetrads	++++	±	±
CpG-B	CpG motifs; phosphorothioate monomers	±	+++	+++
CpG-C	5′-TCG and >12-nucleotide palindromic sequence; phosphorothioate monomers and duplexes	+++	+++	+++
CIC/immunomers	Short 7–10-base oligonucleotides linked by chemical compounds, such as C3, HEG	variable	variable	variable
Particle ISS	ISS associated with positively charged peptides or nanoparticles	++++	±	±

C3: Propanediol; CIC: Chimeric immunostimulatory compound; HEG: Hexaethylene glycol; ISS: Immunostimulatory sequences.

CpG-B [32]. In addition to IFN- α induction, CpG-C also has significant potency in all other *in vitro* assays of ISS activity, such as B-cell activation, PDC maturation and cytokine induction [16,28,31]. Therefore, CpG-C combines the activity profiles of both CpG-A and CpG-B, but accomplishes this without the difficult-to-characterize structural heterogeneity of CpG-A and, thus, is more suitable for clinical vaccine development.

In an effort to optimize ISS functional activities, some of the sequence elements from the three major classes of CpG have been used to engineer a distinct subgroup of ISS called either chimeric immunostimulatory compounds (CICs) [33] or immunomers [34,35]. These ODNs consist of two or more PS CpG-containing segments that are too short to have activity on their own (7–11 nucleotides) connected by non-nucleoside chemical linkers, such as propanediol (C3), butanediol (C4), triethylene glycol (TEG) or hexaethylene glycol (HEG) [33,34]. These segments can be arranged so that a linear CIC/immunomer can have two 5' ends, and a branched (Y-shaped), glycerol-anchored version can have 2, 3 or even more 5' ends, which is desirable since the 5'-TCG motif from the CpG-C class correlates with the promotion of elevated IFN-I synthesis. This type of engineering has yielded CICs/immunomers that can be 'programmed' for a specific ISS activity profile (e.g., high IFN- α induction and low B-cell activity, or high human activity and high mouse activity [33]).

Some investigators have reported on ODN-mediated immune-promoting activity that is independent of CpG motifs in the ODN sequence and, indeed, has been observed with ODNs that lack them. The activity generated by non-CpG ODNs is normally minor in magnitude compared with CpG-mediated activity, and is also usually relegated to B-cell activation [36–38]. Other ISS functions, such as IFN- α induction and NK activation, have consistently been found to be CpG dependent [36,38] and, therefore, it is clear that the presence of CpG motifs is critical for any and all induction of some ISS functions, while being only necessary for substantial and optimal induction of other ISS functions.

Enhancement of ISS activity by particle formation

Much of the interest focused on the development of ISS ODNs lies in their ability to promote very high levels of IFN- α secretion from PDCs, a feature that is virtually unique among biologies. The administration of IFN-I has shown efficacy in hepatitis B virus (HBV), hepatitis C virus (HCV), herpes simplex virus and human papilloma virus infections [39–41], yet the beneficial effects are often accompanied by adverse events and toxic effects. Therefore, a vaccine that induces endogenous secretion of IFN-I but avoids unnecessary systemic exposure to IFN- α is desirable. This has promoted the development of ISS configurations that are engineered specifically to optimize the IFN- α -inducing abilities of ISS. The aggregated nature of CpG-A that allows a high concentration of ISS 'epitopes' per particle has led to the further investigation of adapting ISS to particulate configurations. For example, ISS ODNs that are combined with the small polycationic peptide antibiotic polymyxin B (PMXB) will

form a heterogeneous continuum of particles (100–500 nm in diameter) that have been shown to greatly boost the IFN- α -inducing capacity of the ISS used [42]. The IFN- α activity of CpG-B is enhanced 50–100-fold, and that of CpG-C 5–10-fold, by complexation with PMXB [42]. On the other hand, other direct ISS activities, such as B-cell activation and PDC maturation, are not enhanced by an ISS/PMXB formulation [42], a profile of activities that mimics that of CpG-A, which also delivers ISS in particulate configuration. Type I IFN-boosting or Th1-enhancing effects have also been demonstrated by associating ISS with poly-L-lysine [43], protamine [44,45], HCV core peptides [46] or nuclear histone proteins [43], which, similarly to PMXB, are all polycationic and, thus, also likely to form complexes with negatively charged DNA ODNs that result in particles that present multiple ISS molecules simultaneously. Another method for incorporating ISS into particles is to associate them with prefabricated positively charged microparticles composed of poly(D,L-lactide-co-glycolide) [47–49] or polystyrene [23]. This method has also yielded greatly enhanced ISS activity *in vitro* [23] and *in vivo* [47,49].

The mechanism by which application of CpG ODNs in particulate form results in enhanced activity of the ISS is currently under investigation. Ingestion of microparticles by PDCs results in the absorption of a bolus of ISS into the endosomal compartment, and studies with fluoresceinated CpG ODNs show significantly higher ISS uptake in particulate versus monomeric form [48]. Thus, particles can increase the endosomal concentration of ISS ODNs. Another possibility is that the presentation of multiple ISS ODNs in the same structure results in 'cross-linking' of several TLR9 receptors in the endosome, thus causing increased signaling and an augmentation of downstream effects. However, the low endosomal pH (5.5) required by TLR9 for effective signaling by CpG [50] and degradation by proteases would make the maintenance of complex structures in the endosome unlikely. A more promising solution comes from the observation of Guiducci *et al.* that CpG-A, which forms particles and induces high levels of IFN- α , was found by confocal microscopy to compartmentalize to the early endosomes, whereas the monomeric and poor IFN- α -inducer CpG-B class sublocalized to late endosomes [51]. Interestingly, CpG-B/PMXB complexes, which induce much more IFN- α than monomeric CpG-B, also localized to the early endosomal compartment. This suggests that retaining ISS within the early endosome and preventing it from shuttling through to the late compartment will result in TLR9 signaling conducive to greatly augmented IFN- α production. Why early endosomal TLR9 and not late endosomal TLR9 is selective for this activity is unclear.

ISS as a vaccine adjuvant: results from preclinical studies ISS enhances immune responses to codelivered antigens & allergens

Over the last several years, more than 500 papers have been published on preclinical studies investigating the ability of ISS to enhance antigen-specific immune responses. It is beyond the

scope of this review to discuss every model system, since ISS is being evaluated as an adjuvant for a vast variety of vaccines against infectious disease (viral [52–54], bacterial [55,56], fungal [57,58]), cancer [59] and allergy (seasonal [60], environmental [61], food [62]), both for prophylactic and therapeutic purposes. These studies have led to important insights into the application of ISS as adjuvants for human vaccines. While ISS exhibit activity in a variety of species, the differences have been examined extensively between mouse and human systems, but less is known about ISS activity in other species [63–65]. Consequently, preclinical studies in murine models have dominated the field.

As reviewed earlier by Klinman [66], McCluskie [67] and Daubenerger [68], studies in animals have shown that ISS coadministered with protein or peptide antigens, polysaccharides conjugated to a protein carrier, live or killed virus, as well as autologous cellular and DC vaccines, increases antigen-specific B-cell responses, reduces B-cell apoptosis and enhances immunoglobulin class switching. These features result in the induction of faster, higher humoral responses with longer duration, greater affinity and also allow antigen-dose sparing.

ISS enhances vaccine responses in hyporesponsive populations, such as the elderly, HIV patients, dialysis patients or people with other immune-deficient conditions. Several published studies have demonstrated that ISS can provide significant enhancement of vaccine efficacy in preclinical models of immunodeficiency. Orangutans are naturally hyporesponsive to immunization with the commercial hepatitis B vaccine, Engerix-B™ (GlaxoSmith-Kline), containing hepatitis B surface antigen (HBsAg) and alum. However, the addition of ISS to the commercial vaccine resulted not only in high HBsAg-specific antibody responses but also increased the seroconversion rate [69]. Concomitant infection with other viruses can also reduce immune responses. In simian immunodeficiency virus (SIV)-infected rhesus macaques, who were also hyporesponsive to Engerix-B, the addition of ISS to the commercial vaccine induced a significant anti-HBsAg response that was inversely correlated to the SIV viral load at the time of vaccination [53].

Both ends of the age spectrum present significant obstacles for vaccine immunogenicity. The immune response of neonates to foreign pathogens is frequently insufficient owing to the immature nature of their immune systems [70]. Likewise, responses in the elderly are diminished owing to declining efficiency of humoral and cellular immunity associated with aging [71]. Studies in both newborn and elderly mouse models indicate that use of ISS as a vaccine adjuvant can enhance weak immune responses [55,72–74]. Polysaccharide–protein conjugate vaccines circumvent immature polysaccharide-specific humoral immunity in neonates by recruiting CD4⁺ T-cell help. However, immunosenescence in the elderly results in diminished T-helper responses, resulting in reduced antibody responses to the pneumococcal conjugate vaccine. Sen *et al.* have shown that the addition of ISS to a polysaccharide–protein conjugate restored responses to young-adult levels in an elderly mouse model [55].

Enhancing ISS activity

Formulation of the ISS and antigen, as well as the route of delivery, can impact vaccine potency dramatically. Delivery of ISS and antigen to the same APC significantly enhances vaccine immunogenicity. This can be accomplished by a variety of methods, including physical linking of the ISS oligonucleotide to the antigen [60,75–77], coinorporation into or onto particles [78], codelivery in liposomes [79–81] or increasing the number of CpG motifs in a plasmid construct [82]. Immunization with linked ISS and antigen preparations has been shown to result in greater uptake of soluble antigens, significant reduction in the amount of ISS required and induction of strong CD4⁺ and CD8⁺ T-cell-mediated immune responses [75,77,78,83,84]. ISS-linked antigens also have the ability to enhance responses to other coadministered antigens, owing to the Th1 cytokine milieu induced by the linked antigen-ISS material. As an example from our own work, coimmunization with soluble HIV gp160 and ragweed allergen Amb a 1 linked to ISS (Amb a–ISS) induces enhanced HIV gp160-specific IgG_{2a} and IFN- γ responses, reduced IL-5 responses in mice and is compatible with the ability of ISS to skew responses toward Th1 (FIGURE 1) (HIGGINS *ET AL.*, UNPUBLISHED DATA). We have also seen this effect with other antigens and conjugates. Thus, with a vaccine that includes a mixture of antigens, strong Th1 enhancement of immune responses to all the antigens can be accomplished by simply linking one of the antigens to ISS.

ISS activity can be enhanced by coformulation with other adjuvants or delivery vehicles. Several studies in animal models using ISS combined with alum [52,53,56,85,86], oil emulsions (Montanide™ [SEPPIC] [87,88] and MF59 [89]), micro- and nanoparticles [42,47,49,90], as well as liposomes [87,91], have shown that combinations can synergistically enhance immune responses. ISS/PMXB nanoparticle formulations have been shown to enhance and accelerate responses to HBsAg in murine and primate models compared with animals immunized with HBsAg plus ISS [42]. ISS has been shown to enhance responses to Engerix-B, containing HBsAg bound to alum [53,85], as well as the licensed anthrax vaccine, BioThrax® (Emergent Biosolutions, MD, USA), a sterile culture filtrate from avirulent *Bacillus anthracis* containing alum [56]. Challenge studies in mice evaluating a protein-based smallpox vaccine combined with both ISS and alum showed complete protection in terms of survival, minimal weight change and significant reduction in lung and spleen viral titers. Inclusion of both adjuvants was key since control immunizations with the viral proteins plus either alum or ISS alone provided no protection [52].

ISS is active as a vaccine adjuvant using a variety of administration routes. Many studies have shown that ISS enhances systemic vaccine responses when delivered via parenteral routes (intramuscular, subcutaneous and intraperitoneal [53,54,56,92,93]). Studies have also addressed targeting immune responses to relevant locations where infection occurs, such as respiratory, gastrointestinal or urogenital mucosa [72,94,95]. Jiang *et al.* showed that intranasal delivery of HIV-1 antigen with ISS induced local immune responses in the genital tract of mice, including HIV-specific proliferation, IFN- γ , β -chemokines and CD8⁺

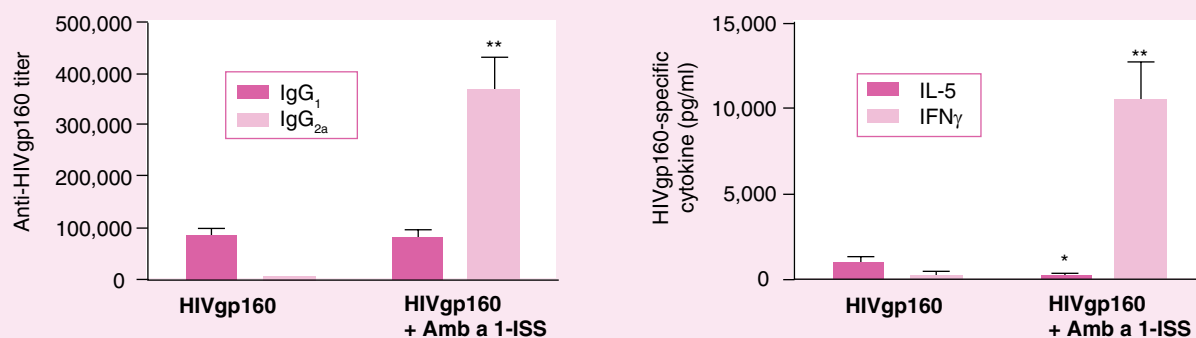


Figure 1. Antigen-ISS conjugates enhance responses to codelivered antigens. BALB/c mice ($n = 10$ per group) were immunized twice at weeks 0 and 2 with HIV gp160 ($5 \mu\text{g}$) \pm Amb a 1-ISS conjugate ($10 \mu\text{g}$). HIV gp160-specific antibody titers of sera were determined by ELISA at weeks 2 and 4. Antibody responses after the first immunization mirror the higher responses seen after the second (data not shown). Splenocytes were harvested at week 6, and cultures restimulated with HIV gp160 to determine IFN- γ and IL-5 cytokine responses. Codelivery of HIV gp160 with the Amb a 1-ISS conjugate enhanced HIV gp160-specific Th1 responses (mean \pm standard deviation, * $p < 0.005$, ** $p < 0.001$) compared with mice immunized with HIV gp160 alone. ISS: immunostimulatory sequence

T cells. Mice challenged intravaginally with recombinant vaccinia viruses expressing HIV-1 gag were protected and cross-clade protection was also observed [95].

ISS can modify antigen-specific allergic responses

Furthermore, ISS modulates the antigen-specific response from Th2 toward Th1 in a variety of animal models [60,87,93,96,97], important features for vaccine approaches where induction of cell-mediated immunity or reprogramming of inappropriate immune responses are required. ISS can be used in an allergen-specific vaccine format to reprogram an inappropriate allergic Th2 response toward a noninflammatory, nonallergic Th1 response. Studies in mice immunized with Amb a 1, the major allergen in ragweed, linked to ISS have shown that allergen-specific responses are modified from IgG₁ to IgG_{2a}, and from IL-5 to IFN- γ compared with animals immunized with antigen alone [84]. Similar results were demonstrated with ovalbumin (OVA)-ISS conjugates in mouse allergy models [75]. Furthermore, antibody recognition can be blocked with the conjugated material, presumably by linking ISS, at or near, B-cell epitopes while still inducing T-cell responses, increasing IFN- γ and reducing IL-5 [60]. This ability to block B-cell epitopes can serve to reduce the allergenicity of the vaccine material to avoid IgE recognition [60]. Studies in both ragweed and OVA allergic mouse models have shown that immunization with the antigen-ISS vaccine was able to reverse established Th2-driven airway responses [75,98], suppressing an ongoing eosinophilic inflammation and antigen-induced airway hyper-reactivity.

ISS as a vaccine adjuvant: results from toxicology & safety studies

The safety profile for PS ODNs has been characterized in numerous reports over the last decade and the effects observed in these studies in rodents and nonhuman primates show consistent

class effects of PS ODNs [99–103]. Two key features associated with the toxicity profile of all PS ODNs are their polyanionic character and the degree of immune stimulation. While the patterns of toxicity may be similar between PS ODNs independent of sequence, some differences are related to the ISS potency and species differences, with rodents being more sensitive to PS ODN class effects than primates. The immune toxicities for PS ODN are characterized by dose-related increases in B cells in lymphoid organs (in rodents and nonhuman primates), lymphoid hyperplasia, with associated splenomegaly and enlarged lymph nodes (in rodents), and diffuse mixed mononuclear cell infiltrates notable in the injection site of all species, as well as in the liver and kidney (in rodents). Effects on hematopoiesis in rodents administered with high-dose PS ODN repeatedly, include reduction in circulating platelets, increases in monocytes, extramedullary hematopoiesis in spleen and liver, and a slight anemia [101] (associated with IFN- γ -mediated suppression of erythropoiesis in CpG-treated mice [104]). In monkeys, effects on hematopoiesis are characterized by transient acute reductions in platelet counts (noted during infusion) and increases in clotting time (as measured by concentration-dependent prolongation of activated partial thromboplastin times) [101]. In most circumstances the effects observed in these studies were reversible. For ISS ODNs, by nature of their intended pharmacology, the immune toxicities elicited in rodents and primates appear to be more pronounced and the dose-responses shifted significantly lower than the effects elicited by other non-ISS ODNs (e.g., antisense compounds).

While there is a broad literature database for non-ISS PS ODNs, published reports on the toxicology of ISS for vaccine applications is very limited. Fever, and in some cases a mild increase in circulating neutrophils and elevated serum haptoglobin, was reported in mice, cattle and sheep treated with ISS [105–107]. ISS was a safe mucosal adjuvant with no inflammation or tissue damage observed locally or systemically

after nasal administration to mice [108]. In addition, combining ISS with other adjuvants, including aluminum hydroxide, was not found to enhance tissue reactions in rabbits and chickens [109,110].

A large number of good laboratory practice-compliant toxicity studies have been performed with 1018 ISS either alone or formulated with HBsAg or ragweed allergen (TRAQUINA *ET AL.*, UNPUBLISHED DATA). These studies have demonstrated that, in rodents and nonhuman primates, there were no clinically significant toxicities with ISS doses up to 12.5 mg/kg. As expected, the toxic effects observed in rodents and nonhuman primates were immune related and consisted of histopathologic alterations observed at the injection site and in spleen and lymph nodes that correlated with modest increased tissue weight. These effects were consistent with the known class-effects of PS ODN but were somewhat exaggerated owing to the potent immunostimulatory activity of ISS. A multigenerational reproductive toxicity study in female rats treated with seven doses of 1018 ISS conjugated to ragweed allergen administered over the pre-mating, mating, gestation and lactation phases of the study, indicate that there were no effects on clinical parameters, pregnancy rates, gestation lengths and parturition, and no macroscopic or microscopic findings at necropsy of the dams (TRAQUINA *ET AL.*, UNPUBLISHED DATA). Maternal treatment with 1018 ISS did not have any effect on the pup's survival rate, clinical condition, physical and locomotor development or learning and memory responses post-weaning. In addition, results of genetic toxicity studies indicate that 1018 ISS is not mutagenic or clastogenic. Importantly, safety studies in mice and baboons immunized with 1018 ISS vaccine formulations have shown no production of dsDNA antibodies.

While ISS is generally well tolerated when utilized as a vaccine adjuvant, adverse effects have been reported in specialized high-dose studies. Daily administration of ISS for more than 7 days has been associated with adverse effects, such as impaired adaptive B-cell immune responses, compromised morphology of lymphoid organs and induced systemic toxicity including peritonitis, hepatotoxicity and splenomegaly [111]. Treatment of pregnant mice with very high doses of ISS (approximately 15 mg/kg), resulted in increased fetal resorptions and cranio/facial/limb defects [112]. It should be noted that 15 mg/kg corresponds to 30-fold higher than the maximum dose administered to humans in nonvaccine clinical trials and is more than 300-fold higher than the ISS doses that have been used in vaccine clinical trials to date.

Overall, studies with ISS have demonstrated that these compounds have low toxicity and good tolerability. Effects detected in toxicity studies were typically the result of immunostimulation and were seen at high doses. The overall safety of ISS, coupled with its potent preclinical adjuvant effects, have paved the way for clinical testing of ISS adjuvants.

ISS as a vaccine adjuvant: results from clinical studies

The potent adjuvant activity of immunostimulatory oligonucleotides observed in a variety of preclinical models has prompted human clinical testing of the technology for a

number of indications. In the last 5 years, ISS has been tested as a vaccine adjuvant in over a dozen clinical trials in diverse fields including infectious disease, cancer and allergy; in some instances, the development has proceeded as far as Phase III testing (TABLE 2). Coincidentally, the most extensive clinical testing has been performed with prophylactic hepatitis B vaccination where two related but independent vaccine strategies have been applied to investigate how ISS can be used to enhance immunogenicity. A prophylactic HBV vaccine utilizing ISS as its sole adjuvant has been extensively tested in clinical trials [113,114]. This vaccine, referred to as HEPLISAV™ (Dynavax, CA, USA), contains HBsAg combined with 3 mg of 1018 ISS. Vaccination with HEPLISAV promotes faster seroprotection compared with the commercially approved HBV vaccine, Engerix-B. In Phase II clinical trials, nearly 80% of ISS-adjuvanted vaccine recipients were seroprotected after a single immunization compared with 12% of the subjects vaccinated with the alum-adjuvanted vaccine. One week after a second immunization, 100% of the subjects receiving the ISS-adjuvanted vaccine demonstrated protective titers compared with 18% of subjects receiving Engerix-B [114]. Phase III trials of HEPLISAV have shown that ISS significantly enhances the responses in largely hyporesponsive older patient populations; in subjects aged 40–70 years, a 97% seroconversion frequency was observed after two immunizations with the ISS-adjuvanted vaccine compared with a 23% seroconversion rate achieved with the approved alum-adjuvanted vaccine (FIGURE 2) (MARTINS *ET AL.*, UNPUBLISHED DATA). Importantly, administration of ISS as a vaccine adjuvant has been demonstrated to be safe and well tolerated; collectively in over 400 HEPLISAV vaccinees, from Phase I through Phase III clinical trials, there have been no serious vaccine-related adverse events reported. Although the use of ISS as an adjuvant is associated with a higher frequency of injection site tenderness, the severity is generally mild and transient. The frequency and severity of systemic adverse events were not different between HEPLISAV and the licensed vaccine comparator.

Another approach has been to add ISS (CpG 7909) to Engerix-B, consisting of recombinant HBV surface antigen adsorbed to alum. As observed in the aforementioned studies, in healthy adults the inclusion of ISS was found to result in faster seroconversion and increase the magnitude of the HBsAg-specific antibody titers. Approximately 50% of subjects vaccinated with Engerix-B combined with either 0.5 or 1 mg of oligonucleotide exhibited seroprotective titers after a single immunization, whereas in the absence of ISS there were no detectable anti-HBsAg responses [115]. Interestingly, the presence of ISS enhanced the affinity maturation process increasing the pool of high-avidity antibodies [116]. The ability of ISS to adjuvant Engerix-B was further demonstrated in a subsequent clinical trial in antiretroviral-treated HIV-infected adults. Similar results were observed in this difficult-to-treat patient population, although in this patient population the induction of HBsAg antibody titers required two immunizations [117]. While the relative immunogenicity of the respective approaches to adjuvanting HBV vaccines is difficult to quantitate owing to

Table 2. Clinical development of immunostimulatory oligodeoxynucleotide sequences as vaccine adjuvants.

Indication	Vaccine antigen	Status	Ref.
HBV	HBsAg (HEPLISAV™)	Phase III	[113,114]
	HBsAg (Engerix-B®)	Phase I/II	[115,117]
Influenza	Trivalent influenza vaccine (Fluarix®)	Phase I	[118]
Anthrax	Culture filtrate <i>B. anthracis</i> (BioThrax™)	Phase I	[201]
Malaria	AMA1-C1 and MSP1-42	Phase I	[202–204]
HIV	gp120-depleted HIV-1 (Remune®)	Phase I	[205]
Melanoma	MART-1 peptide	Phase I	[119,120,206–208]
NSCLC	NY-ESO-1	Phase I	[208]
Allergic rhinitis	Ragweed Amb a 1 (TOLAMBA™)	Phase II	[122–124]
	Dust mite allergen extract (CYT003-QbG10)	Phase II	[208]

NSCLC: Non-small-cell lung cancer; HBsAg: Hepatitis B surface antigen.

differences in formulations and dosing regimens, qualitatively the collective data clearly illustrates the ability of ISS to enhance HBV vaccine immunogenicity.

ISS is also being utilized to adjuvant a variety of other infectious disease vaccines. In a study reported by Cooper *et al.*, the addition of immunostimulatory oligonucleotide (CpG 7909) was found to have a modest effect on the immunogenicity of standard influenza vaccine [118]. Given the relatively high immunogenicity of influenza vaccine, the effects of ISS adjuvant may be difficult to detect. Preliminary results from ISS-adjuvanted (CpG 7909) clinical trials in anthrax and malaria have been more compelling, although much of this work has not yet been published. The widespread applicability of using ISS to increase the potency of infectious disease vaccines should be better appreciated as these trials are interpreted.

In recent years, considerable effort has been devoted to the development of therapeutic cancer vaccines. The cloning of tumor-specific antigens has enabled vaccine strategies designed to induce cellular immune responses that could potentially eliminate tumors by killing cancerous cells. Utilizing ISS as an adjuvant in this setting has the potential to promote tumor regression either directly through the activity of cytokines such as IFN- α , or indirectly by increasing the effectiveness of PDCs to induce T-cell responses capable of recognizing transformed cells expressing the tumor-specific antigens. The effectiveness of this vaccine strategy has been evaluated in melanoma patients. Subjects receiving four subcutaneous immunizations with a MART-1 peptide combined with ISS (CpG 7909) and incomplete Freund's adjuvant had, on average, a tenfold increase in the frequency of MART-1-specific circulating T-cell responses compared with subjects immunized with the same vaccine without ISS [119]. In some individuals, the magnitude of the immune response exceeded 1% of the CD8⁺ T-cell population in PBMC. Subsequent characterization has found that the antigen-specific T cells in the periphery display greater effector functions, as

measured by IFN- γ and granzyme B expression, than the tumor-infiltrating T cells [120]. Recently, a vaccine based on the cancer antigen NY-ESO-1 in a formulation containing CpG 7909 and Montanide ISA-51, was reported to induce antigen-specific antibody and T-cell responses [121]. Collectively, these results illustrate the capacity of ISS to enhance cancer vaccine responses. Ongoing cancer vaccine trials may lead to further insight into utilizing ISS as a sole adjuvant, as well as, perhaps, defining the immune correlates that lead to either tumor regression or increased survival.

Allergy is another field where ISS has been applied for therapeutic vaccination. As opposed to traditional vaccine approaches designed to induce a primary immune response, in allergy the desired effect of vaccination is to redirect a highly active Th2-biased allergen-specific immune response. ISS is particularly well-suited for use as an allergic immunotherapeutic vaccine adjuvant, since TLR9 activation suppresses Th2-type responses and simultaneously promotes Th1-type responses, which are typically associated with successful conventional allergy therapy. Moreover, by delivering ISS chemically conjugated to the allergen, there is the added benefit of blocking IgE binding to the protein, which correspondingly reduces the mast-cell degranulation that occurs during the immediate phase of allergen reaction. A ragweed-allergy immunotherapeutic vaccine consisting of Amb a 1, linked to 1018 ISS, a compound referred to TOLAMBA™ (Dynavax, CA, USA), has been tested extensively in a number of clinical trials. Nasal biopsies of ragweed-allergic subjects immunized with TOLAMBA found that the treated group had significantly lower numbers of IL-4-, IL-5-, and IL-13-producing T cells, and higher numbers of IFN- γ -producing T cells than the placebo group following allergen challenge [122]. Similarly, ragweed-allergic subjects immunized with TOLAMBA demonstrated a systemic ragweed-specific redirection of Th2 responses to Th1 responses in PBMC cultures [123]. Additional

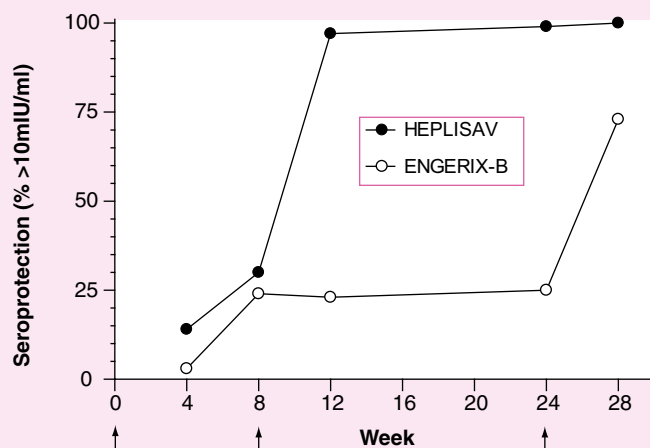


Figure 2. Immunostimulatory sequences-adjuvanted prophylactic HBV vaccine significantly enhances seroprotection in adults aged 40–70 years.

Subjects were immunized with the immunostimulatory sequences-adjuvanted HBV vaccine, HEPLISAV™, or the approved alum-adjuvanted ENGERIX-B® at weeks 0, 8 and 24 or 0, 4 and 24, respectively, as indicated by the arrows. Percentage of subjects having seroprotective anti-hepatitis B surface antigen antibody titers ≥ 10 mlU/ml is illustrated.

field-study clinical trials have demonstrated that TOLAMBA vaccination reduced hay fever symptoms significantly during the ragweed season compared with placebo recipients [124]. The peak season daily nasal symptom scores were statistically significantly reduced with a similar treatment effect observed for the full season. Although the patients received no further treatments in the second ragweed season, the improvements in clinical outcomes were maintained.

Expert commentary & five-year view

The discovery that TLR9, a key receptor critical in regulating innate and adaptive immune responses, can be activated specifically by comparatively small synthetic oligonucleotides has opened new avenues for the development of adjuvants with minimal toxicity and increased potency. The elucidation of the differential activity of various ISS classes raises the possibility of selectively utilizing tailored ISS sequences to engineer the immune response, be it heightened antibody production or increased T-cell responses, which are optimally suited to induce effector responses that will have the most beneficial impact on a particular disease indication. Similarly, as patterns of TLR9 expression and signal pathways have been characterized, the factors that influence the use of ISS as vaccine adjuvants have been better appreciated. Formulation strategies that alter the sub-cellular localization of ISS have been identified that increase ISS activity, and delivery of ISS and the antigen to the same cell can significantly enhance vaccine immunogenicity. ISS can also act synergistically with other adjuvants to increase immunogenicity.

Preclinical animal models have been an invaluable tool in determining the extent that ISS can be applied as a vaccine adjuvant. Numerous studies in a variety of animal species and disease indications have demonstrated that ISS can function as an adjuvant in traditional prophylactic vaccine strategies, as well as in therapeutic vaccines. These studies have provided important insights regarding the route of administration and have shown that ISS can have a broad immunological impact on the response to ISS conjugated, as well as codelivered antigens. These preclinical studies have also demonstrated that the inherent Th1-inducing properties of ISS can be used to redirect harmful immune responses, as in the case of an allergy. From a strictly toxicological perspective, the work done in preclinical studies have confirmed that ISS is safe and does not result in systemic effects.

The overall clinical experience to date using ISS as a vaccine adjuvant has confirmed many of the preclinical observations

previously published. ISS does appear to be a potent adjuvant that can significantly enhance antibody responses to vaccines in humans. ISS is able to redirect human allergic responses from Th2 to Th1 and lead to clinical efficacy in allergy. In addition, ISS appears to be quite safe and well tolerated in clinical applications. These early clinical studies have demonstrated the proof of concept and have paved the way for the much broader development of ISS-adjuvanted vaccines. In the coming 5 years, it is reasonable to expect that:

- Detailed understanding of TLR9 pathways and agonists will result in the identification of more diverse and potent ISS and formulation strategies
- Preclinical models will define the extent that ISS can be utilized in different vaccines
- Combined toxicology and clinical data will establish the safety of ISS as a vaccine adjuvant
- Clinical testing of ISS-adjuvanted vaccines will continue to escalate and several such products should be approved for human use

Financial & competing interests disclosure

All authors are employed by Dynavax Technologies, CA, USA and own company stock and options. 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.

No writing assistance was utilized in the production of this manuscript.

Key issues

- Three classes of immunostimulatory sequences (ISS), CpG-A, CpG-B and CpG-C, interacting through Toll-like receptor 9, play a key role in the initiation of innate immune responses and influence later antigen-specific adaptive immune responses.
- As a vaccine adjuvant, ISS can significantly enhance antigen-specific humoral and cellular immune responses, modifying responses in a Th1 direction as a hallmark feature.
- ISS adjuvant activity can be enhanced by delivery of ISS and antigen to the same antigen-presenting cell, as well as by coformulation with other adjuvants or delivery vehicles.
- ISS can significantly enhance vaccine responses in hyporesponsive animals or humans.
- ISS can modify antigen-specific allergic responses, and allergen-ISS conjugates have shown efficacy in the clinic.
- ISS shows low toxicity and good tolerability in animal studies and human trials.

References

Papers of special note have been highlighted as:

• of interest

•• of considerable interest

- Trinchieri G, Sher A. Cooperation of Toll-like receptor signals in innate immune defence. *Nat. Rev. Immunol.* 7(3), 179–190 (2007).
- Boonstra A, Rajsbaum R, Holman M *et al.* Macrophages and myeloid dendritic cells, but not plasmacytoid dendritic cells, produce IL-10 in response to MyD88- and TRIF-dependent TLR signals, and TLR-independent signals. *J. Immunol.* 177(11), 7551–7558 (2006).
- Hornung V, Rothenfusser S, Britsch S *et al.* Quantitative expression of Toll-like receptor 1–10 mRNA in cellular subsets of human peripheral blood mononuclear cells and sensitivity to CpG oligodeoxynucleotides. *J. Immunol.* 168(9), 4531–4537 (2002).
- Kadowaki N, Ho S, Antonenko S *et al.* Subsets of human dendritic cell precursors express different Toll-like receptors and respond to different microbial antigens. *J. Exp. Med.* 194(6), 863–869 (2001).
- Latz E, Schoenemeyer A, Visintin A *et al.* TLR9 signals after translocating from the ER to CpG DNA in the lysosome. *Nat. Immunol.* 5(2), 190–198 (2004).
- Lund J, Sato A, Akira S, Medzhitov R, Iwasaki A. Toll-like receptor 9-mediated recognition of herpes simplex virus-2 by plasmacytoid dendritic cells. *J. Exp. Med.* 198(3), 513–520 (2003).
- Krug A, French AR, Barchet W *et al.* TLR9-dependent recognition of MCMV by IPC and DC generates coordinated cytokine responses that activate antiviral NK cell function. *Immunity* 21(1), 107–119 (2004).
- Mogensen TH, Paludan SR, Kilian M, Ostergaard L. Live *Streptococcus pneumoniae*, *Haemophilus influenzae*, and *Neisseria meningitidis* activate the inflammatory response through Toll-like receptors 2, 4, and 9 in species-specific patterns. *J. Leukoc. Biol.* 80(2), 267–277 (2006).
- Kalis C, Gumenscheimer M, Freudenberg N *et al.* Requirement for TLR9 in the immunomodulatory activity of *Propionibacterium acnes*. *J. Immunol.* 174(7), 4295–4300 (2005).
- von Meyenn F, Schaefer M, Weighardt H *et al.* Toll-like receptor 9 contributes to recognition of *Mycobacterium bovis* Bacillus Calmette-Guerin by Flt3-ligand generated dendritic cells. *Immunobiology* 211(6–8), 557–565 (2006).
- Zelenay S, Elias F, Flo J. Immunostimulatory effects of plasmid DNA and synthetic oligodeoxynucleotides. *Eur. J. Immunol.* 33(5), 1382–1392 (2003).
- Verthelyi D, Ishii KJ, Gursel M, Takeshita F, Klinman DM. Human peripheral blood cells differentially recognize and respond to two distinct CPG motifs. *J. Immunol.* 166(4), 2372–2377 (2001).
- Hartmann G, Krieg AM. Mechanism and function of a newly identified CpG DNA motif in human primary B cells. *J. Immunol.* 164(2), 944–953 (2000).
- Gursel M, Verthelyi D, Gursel I, Ishii KJ, Klinman DM. Differential and competitive activation of human immune cells by distinct classes of CpG oligodeoxynucleotide. *J. Leukoc. Biol.* 71(5), 813–820 (2002).
- Wagner M, Poeck H, Jahrsdoerfer B *et al.* IL-12p70-dependent Th1 induction by human B cells requires combined activation with CD40 ligand and CpG DNA. *J. Immunol.* 172(2), 954–963 (2004).
- Vollmer J, Weeratna R, Payette P *et al.* Characterization of three CpG oligodeoxynucleotide classes with distinct immunostimulatory activities. *Eur. J. Immunol.* 34(1), 251–262 (2004).
- Hartmann G, Weiner GJ, Krieg AM. CpG DNA: a potent signal for growth, activation, and maturation of human dendritic cells. *Proc. Natl Acad. Sci. USA* 96(16), 9305–9310 (1999).
- Jarrossay D, Napolitani G, Colonna M, Sallusto F, Lanzavecchia A. Specialization and complementarity in microbial molecule recognition by human myeloid and plasmacytoid dendritic cells. *Eur. J. Immunol.* 31(11), 3388–3393 (2001).
- Kerkmann M, Rothenfusser S, Hornung V *et al.* Activation with CpG-A and CpG-B oligonucleotides reveals two distinct regulatory pathways of type I IFN synthesis in human plasmacytoid dendritic cells. *J. Immunol.* 170(9), 4465–4474 (2003).
- Ashkar AA, Rosenthal KL. Toll-like receptor 9, CpG DNA and innate immunity. *Curr. Mol. Med.* 2(6), 545–556 (2002).
- Duramad O, Fearon KL, Chan JH *et al.* IL-10 regulates plasmacytoid dendritic cell response to CpG-Containing immunostimulatory sequences. *Blood* 102(13), 4487–4492 (2003).
- Maurer T, Heit A, Hochrein H *et al.* CpG-DNA aided cross-presentation of soluble antigens by dendritic cells. *Eur. J. Immunol.* 32(8), 2356–2364 (2002).
- Kerkmann M, Costa LT, Richter C *et al.* Spontaneous formation of nucleic acid-based nanoparticles is responsible for high interferon- α induction by CpG-A in plasmacytoid dendritic cells. *J. Biol. Chem.* 280(9), 8086–8093 (2005).
- Krug A, Rothenfusser S, Hornung V *et al.* Identification of CpG oligonucleotide sequences with high induction of IFN- α/β in plasmacytoid dendritic cells. *Eur. J. Immunol.* 31(7), 1254–2163 (2001).
- Abel K, Wang Y, Fritts L *et al.* Deoxycytidyl-deoxyguanosine oligonucleotide classes A, B, and C induce distinct cytokine gene expression patterns

- in rhesus monkey peripheral blood mononuclear cells and distinct a interferon responses in TLR9-expressing rhesus monkey plasmacytoid dendritic cells. *Clin. Diagn. Lab. Immunol.* 12(5), 606–621 (2005).
- 26 Asselin-Paturel C, Boonstra A, Dalod M *et al.* Mouse type I IFN-producing cells are immature APCs with plasmacytoid morphology. *Nat. Immunol.* 2(12), 1144–1150 (2001).
- 27 Krug A, Rothenfusser S, Selinger S *et al.* CpG-A oligonucleotides induce a monocyte-derived dendritic cell-like phenotype that preferentially activates CD8 T cells. *J. Immunol.* 170(7), 3468–3477 (2003).
- 28 Marshall JD, Fearon K, Abbate C *et al.* Identification of a novel CpG DNA class and motif that optimally stimulate B cell and plasmacytoid dendritic cell functions. *J. Leukoc. Biol.* 73(6), 781–792 (2003).
- 29 Rothenfusser S, Tuma E, Endres S, Hartmann G. Plasmacytoid dendritic cells: the key to CpG. *Hum. Immunol.* 63(12), 1111–1119 (2002).
- 30 Yamamoto S, Yamamoto T, Iho S, Tokunaga T. Activation of NK cell (human and mouse) by immunostimulatory DNA sequence. *Springer Semin. Immunopathol.* 22(1–2), 35–43 (2000).
- 31 Hartmann G, Battiany J, Poeck H *et al.* Rational design of new CpG oligonucleotides that combine B cell activation with high IFN- α induction in plasmacytoid dendritic cells. *Eur. J. Immunol.* 33(6), 1633–1641 (2003).
- 32 Jurk M, Schulte B, Kritzler A *et al.* C-class CpG ODN: sequence requirements and characterization of immunostimulatory activities on mRNA level. *Immunobiology* 209(1–2), 141–154 (2004).
- 33 Marshall JD, Hessel EM, Gregorio J *et al.* Novel chimeric immunomodulatory compounds containing short CpG oligodeoxyribonucleotides have differential activities in human cells. *Nucleic Acids Res.* 31(17), 5122–5133 (2003).
- 34 Yu D, Kandimalla ER, Bhagat L *et al.* ‘Immunomers’ – novel 3’-3’-linked CpG oligodeoxyribonucleotides as potent immunomodulatory agents. *Nucleic Acids Res.* 30(20), 4460–4469 (2002).
- 35 Yu D, Zhu FG, Bhagat L *et al.* Potent CpG oligonucleotides containing phosphodiester linkages: *in vitro* and *in vivo* immunostimulatory properties. *Biochem. Biophys. Res. Commun.* 297(1), 83–90 (2002).
- 36 Elias F, Flo J, Lopez RA *et al.* Strong cytosine-guanosine-independent immunostimulation in humans and other primates by synthetic oligodeoxynucleotides with PyNTTTTGT motifs. *J. Immunol.* 171(7), 3697–3704 (2003).
- 37 Liang H, Nishioka Y, Reich CF, Pisetsky DS, Lipsky PE. Activation of human B cells by phosphorothioate oligodeoxynucleotides. *J. Clin. Invest.* 98(5), 1119–1129 (1996).
- 38 Vollmer J, Janosch A, Laucht M *et al.* Highly immunostimulatory CpG-free oligodeoxynucleotides for activation of human leukocytes. *Antisense Nucleic Acid Drug Dev.* 12(3), 165–175 (2002).
- 39 Cheney IW, Lai VC, Zhong W *et al.* Comparative analysis of anti-hepatitis C virus activity and gene expression mediated by α , β , and γ interferons. *J. Virol.* 76(21), 11148–11154 (2002).
- 40 Noisakran S, Carr DJ. Type I interferons and herpes simplex virus infection: a naked DNA approach as a therapeutic option? *Immunol. Res.* 24(1), 1–11 (2001).
- 41 Sen E, McLaughlin-Drubin M, Meyers C. Efficacy of two commercial preparations of interferon- α on human papillomavirus replication. *Anticancer Res.* 25(2A), 1091–1100 (2005).
- 42 Marshall JD, Higgins D, Abbate C *et al.* Polymyxin B enhances ISS-mediated immune responses across multiple species. *Cell. Immunol.* 229(2), 93–105 (2004).
- 43 Pedersen GM, Johansen A, Olsen RL, Jorgensen JB. Stimulation of type I IFN activity in Atlantic salmon (*Salmo salar* L.) leukocytes: synergistic effects of cationic proteins and CpG ODN. *Fish Shellfish Immunol.* 20(4), 503–518 (2006).
- 44 Kerkmann M, Lochmann D, Weyermann J *et al.* Immunostimulatory properties of CpG-oligonucleotides are enhanced by the use of protamine nanoparticles. *Oligonucleotides* 16(4), 313–322 (2006).
- 45 Scheel B, Teufel R, Probst J *et al.* Toll-like receptor-dependent activation of several human blood cell types by protamine-condensed mRNA. *Eur. J. Immunol.* 35(5), 1557–1566 (2005).
- 46 Riedl P, Buschle M, Reimann J, Schirmbeck R. Binding immune-stimulating oligonucleotides to cationic peptides from viral core antigen enhances their potency as adjuvants. *Eur. J. Immunol.* 32(6), 1709–1716 (2002).
- 47 Diwan M, Tafaghodi M, Samuel J. Enhancement of immune responses by co-delivery of a CpG oligodeoxynucleotide and tetanus toxoid in biodegradable nanospheres. *J. Control. Release* 85(1–3), 247–262 (2002).
- 48 Fearon K, Marshall JD, Abbate C *et al.* A minimal human immunostimulatory CpG motif that potently induces IFN- γ and IFN- α production. *Eur. J. Immunol.* 33(8), 2114–2122 (2003).
- 49 Singh M, Ott G, Kazzaz J *et al.* Cationic microparticles are an effective delivery system for immune stimulatory cpG DNA. *Pharm. Res.* 18(10), 1476–1479 (2001).
- 50 Rutz M, Metzger J, Gellert T *et al.* Toll-like receptor 9 binds single-stranded CpG-DNA in a sequence- and pH-dependent manner. *Eur. J. Immunol.* 34(9), 2541–2550 (2004).
- 51 Guiducci C, Ott G, Chan JH *et al.* Properties regulating the nature of the plasmacytoid dendritic cell response to Toll-like receptor 9 activation. *J. Exp. Med.* 203(8), 1999–2008 (2006).
- Provides evidence that the intracellular compartment where immunostimulatory sequence (ISS) interactions occur may be responsible for the different activity of the ISS classes.
- 52 Xiao Y, Aldaz-Carroll L, Ortiz AM *et al.* A protein-based smallpox vaccine protects mice from vaccinia and ectromelia virus challenges when given as a prime and single boost. *Vaccine* 25(7), 1214–1224 (2007).
- 53 Verthelyi D, Wang VW, Lifson JD, Klinman DM. CpG oligodeoxynucleotides improve the response to hepatitis B immunization in healthy and SIV-infected rhesus macaques. *AIDS* 18(7), 1003–1008 (2004).
- 54 Chu JH, Chiang CC, Ng ML. Immunization of flavivirus West Nile recombinant envelope domain III protein induced specific immune response and protection against West Nile virus infection. *J. Immunol.* 178(5), 2699–2705 (2007).
- 55 Sen G, Chen Q, Snapper CM. Immunization of aged mice with a pneumococcal conjugate vaccine combined with an unmethylated CpG-Containing oligodeoxynucleotide restores defective immunoglobulin G antipolysaccharide responses and specific CD4⁺-T-cell priming to young adult levels. *Infect. Immun.* 74(4), 2177–2186 (2006).
- 56 Gu M, Hine PM, James Jackson W, Giri L, Nabors GS. Increased potency of BioThrax anthrax vaccine with the addition of the C-class CpG oligonucleotide adjuvant CPG 10109. *Vaccine* 25(3), 526–534 (2007).

- 57 Amaral CC, Garcia IP, Fernandes GF *et al.* Adjuvant effect of synthetic oligodeoxyribonucleotides (CpG-ODN) from the *Paracoccidioides brasiliensis* gp43 gene on the Th2-Th1 immunomodulation of experimental paracoccidioidomycosis. *Scand. J. Immunol.* 62(4), 325–333 (2005).
- 58 Bozza S, Gaziano R, Lipford GB *et al.* Vaccination of mice against invasive aspergillosis with recombinant *Aspergillus* proteins and CpG oligodeoxynucleotides as adjuvants. *Microbes Infect.* 4(13), 1281–1290 (2002).
- 59 Mukherjee P, Pathangey LB, Bradley JB *et al.* MUC1-specific immune therapy generates a strong anti-tumor response in a MUC1-tolerant colon cancer model. *Vaccine* 25(9), 1607–1618 (2007).
- 60 Higgins D, Rodriguez R, Milley R *et al.* Modulation of immunogenicity and allergenicity by controlling the number of immunostimulatory oligonucleotides linked to Amb a 1. *J. Allergy Clin. Immunol.* 118, 504–510 (2006).
- 61 Mo JH, Park SW, Rhee CS *et al.* Suppression of allergic response by CpG motif oligodeoxynucleotide-house-dust mite conjugate in animal model of allergic rhinitis. *Am. J. Rhinol.* 20(2), 212–218 (2006).
- 62 Pons L, Burks W. Novel treatments for food allergy. *Expert Opin. Investig. Drugs* 14(7), 829–834 (2005).
- 63 Carrington AC, Secombes CJ. A review of CpGs and their relevance to aquaculture. *Vet. Immunol. Immunopathol.* 112(3–4), 87–101 (2006).
- 64 Mutwiri G, Pontarollo R, Babiuk S *et al.* Biological activity of immunostimulatory CpG DNA motifs in domestic animals. *Vet. Immunol. Immunopathol.* 91(2), 89–103 (2003).
- 65 Rankin R, Pontarollo R, Ioannou X *et al.* CpG motif identification for veterinary and laboratory species demonstrates that sequence recognition is highly conserved. *Antisense Nucleic Acid Drug Dev.* 11(5), 333–340 (2001).
- 66 Klinman DM. CpG DNA as a vaccine adjuvant. *Expert Rev. Vaccines* 2(2), 305–315 (2003).
- 67 McCluskie MJ, Krieg AM. Enhancement of infectious disease vaccines through TLR9-dependent recognition of CpG DNA. *Curr. Top. Microbiol. Immunol.* 311, 155–178 (2006).
- 68 Daubenberger CA. TLR9 agonists as adjuvants for prophylactic and therapeutic vaccines. *Curr. Opin. Mol. Ther.* 9(1), 45–52 (2007).
- 69 Davis HL, Suparto, II, Weeratna RR *et al.* CpG DNA overcomes hyporesponsiveness to hepatitis B vaccine in orangutans. *Vaccine* 18(18), 1920–1924 (2000).
- 70 Siegrist CA. Neonatal and early life vaccinology. *Vaccine* 19(25–26), 3331–3346 (2001).
- 71 Ginaldi L, Loreto MF, Corsi MP, Modesti M, De Martinis M. Immunosenescence and infectious diseases. *Microbes Infect.* 3(10), 851–857 (2001).
- 72 Alignani D, Maletto B, Liscovsky M *et al.* Orally administered OVA/CpG-ODN induces specific mucosal and systemic immune response in young and aged mice. *J. Leukoc. Biol.* 77, 898–905 (2005).
- 73 Brazolot Millan CL, Weeratna R, Krieg AM, Siegrist CA, Davis HL. CpG DNA can induce strong Th1 humoral and cell-mediated immune responses against hepatitis B surface antigen in young mice. *Proc. Natl Acad. Sci. USA* 95(26), 15553–15558 (1998).
- 74 Weeratna RD, Brazolot Millan CL, McCluskie MJ, Davis HL. CpG ODN can re-direct the Th bias of established Th2 immune responses in adult and young mice. *FEMS Immunol. Med. Microbiol.* 32(1), 65–71 (2001).
- 75 Shirota H, Sano K, Kikuchi T, Tamura G, Shirato K. Regulation of murine airway eosinophilia and Th2 cells by antigen-conjugated CpG oligodeoxynucleotides as a novel antigen-specific immunomodulator. *J. Immunol.* 164(11), 5575–5582 (2000).
- Shows ISS-linked proteins can modulate allergic responses in mouse asthma models.
- 76 Hayashi M, Satou E, Ueki R *et al.* Resistance to influenza A virus infection by antigen-conjugated CpG oligonucleotides a novel antigen-specific immunomodulator. *Biochem. Biophys. Res. Commun.* 329(1), 230–236 (2005).
- 77 Heit A, Schmitz F, O’Keeffe M *et al.* Protective CD8 T cell immunity triggered by CpG–protein conjugates competes with the efficacy of live vaccines. *J. Immunol.* 174(7), 4373–4380 (2005).
- 78 Standley SM, Mende I, Goh SL *et al.* Incorporation of CpG oligonucleotide ligand into protein-loaded particle vaccines promotes antigen-specific CD8 T-cell immunity. *Bioconjug. Chem.* 18(1), 77–83 (2007).
- 79 Alcon V, Baca-Estrada M, Vega-Lopez M *et al.* Mucosal delivery of bacterial antigens and CpG oligonucleotides formulated in biphasic lipid vesicles in pigs. *AAPS J.* 7(3), E566–E571 (2005).
- 80 Engler OB, Schwendener RA, Dai WJ *et al.* A liposomal peptide vaccine inducing CD8⁺ T cells in HLA-A2.1 transgenic mice, which recognise human cells encoding hepatitis C virus (HCV) proteins. *Vaccine* 23(1), 58–68 (2004).
- 81 Jerome V, Graser A, Müller R, Kontermann RE, Konur A. Cytotoxic T lymphocytes responding to low dose TRP2 antigen are induced against B16 melanoma by liposome-encapsulated TRP2 peptide and CpG DNA adjuvant. *J. Immunother.* 29(3), 294–305 (2006).
- 82 Gram GJ, Fomsgaard A, Thorn M, Madsen SM, Glenting J. Immunological analysis of a *Lactococcus lactis*-based DNA vaccine expressing HIV gp120. *Genet. Vaccines Ther.* 5, 3 (2007).
- 83 Tighe H, Takabayashi K, Schwartz D *et al.* Conjugation of protein to immunostimulatory DNA results in a rapid, long-lasting and potent induction of cell-mediated and humoral immunity. *Eur. J. Immunol.* 30(7), 1939–1947 (2000).
- 84 Tighe H, Takabayashi K, Schwartz D *et al.* Conjugation of immunostimulatory DNA to the short ragweed allergen Amb a 1 enhances its immunogenicity and reduces its allergenicity. *J. Allergy Clin. Immunol.* 106(1 Pt 1), 124–134 (2000).
- ISS-linked antigen, resulting in delivery to the same cell, significantly enhances humoral and cell-mediated immune responses.
- 85 Davis HL, Weeratna R, Waldschmidt TJ *et al.* CpG DNA is a potent enhancer of specific immunity in mice immunized with recombinant hepatitis B surface antigen. *J. Immunol.* 160(2), 870–876 (1998).
- 86 Giuliani MM, Adu-Bobie J, Comanducci M *et al.* A universal vaccine for serogroup B meningococcus. *Proc. Natl Acad. Sci. USA* 103(29), 10834–10839 (2006).
- 87 Mansour M, Pohajdak B, Kast WM *et al.* Therapy of established B16-F10 melanoma tumors by a single vaccination of CTL/T helper peptides in VacciMax[®]. *J. Transl. Med.* 5, 20 (2007).
- 88 Jeamwattanalert P, Mahakunkijcharoen Y, Kittigul L *et al.* Long-lasting protective immune response to the 19-kilodalton carboxy-terminal fragment of *Plasmodium yoelii* merozoite surface protein 1 in mice. *Clin. Vaccine Immunol.* 14(4), 342–347 (2007).
- 89 Vajdy M, Selby M, Medina-Selby A *et al.* Hepatitis C virus polyprotein vaccine formulations capable of inducing broad antibody and cellular immune responses. *J. Gen. Virol.* 87(Pt 8), 2253–2262 (2006).

- 90 Xie H, Gursel I, Ivins BE *et al.* CpG oligodeoxynucleotides adsorbed onto polylactide-co-glycolide microparticles improve the immunogenicity and protective activity of the licensed anthrax vaccine. *Infect. Immun.* 73(2), 828–833 (2005).
- 91 de Jong S, Chikh G, Sekirov L *et al.* Encapsulation in liposomal nanoparticles enhances the immunostimulatory, adjuvant and anti-tumor activity of subcutaneously administered CpG ODN. *Cancer Immunother.* 56(8), 1251–1264 (2007).
- 92 Klinman DM. CpG oligonucleotides accelerate and boost the immune response elicited by AVA, the licensed anthrax vaccine. *Expert Rev. Vaccines* 5(3), 365–369 (2006).
- 93 Wille-Reece U, Flynn BJ, Lore K *et al.* Toll-like receptor agonists influence the magnitude and quality of memory T cell responses after prime–boost immunization in nonhuman primates. *J. Exp. Med.* 203(5), 1249–1258 (2006).
- 94 Cong Y, Jupelli M, Guentzel MN *et al.* Intranasal immunization with chlamydial protease-like activity factor and CpG deoxynucleotides enhances protective immunity against genital *Chlamydia muridarum* infection. *Vaccine* 25(19), 3773–3780 (2007).
- 95 Jiang JQ, Patrick A, Moss RB, Rosenthal KL. CD8+ T-cell-mediated cross-clade protection in the genital tract following intranasal immunization with inactivated human immunodeficiency virus antigen plus CpG oligodeoxynucleotides. *J. Virol.* 79(1), 393–400 (2005).
- 96 Zhang L, Tian X, Zhou F. CpG oligodeoxynucleotides augment the immune responses of piglets to swine *Pasteurella multocida* living vaccine *in vivo*. *Res. Vet. Sci.* 83(2), 171–181 (2007).
- 97 Belyakov IM, Isakov D, Zhu Q *et al.* Enhancement of CD8+ T cell immunity in the lung by CpG oligodeoxynucleotides increases protective efficacy of a modified vaccinia Ankara vaccine against lethal poxvirus infection even in a CD4-deficient host. *J. Immunol.* 177(9), 6336–6343 (2006).
- 98 Santeliz JV, Van Nest G, Traquina P, Larsen E, Wills-Karp M. A 1-linked CpG oligodeoxynucleotides reverse established airway hyperresponsiveness in a murine model of asthma. *J. Allergy Clin. Immunol.* 109, 455–462 (2002).
- Shows ISS-linked proteins can modulate allergic responses in mouse asthma models.
- 99 Henry SP, Bolte H, Auletta C, Kornbrust DJ. Evaluation of the toxicity of ISIS 2302, a phosphorothioate oligonucleotide, in a four-week study in cynomolgus monkeys. *Toxicology* 120(2), 145–155 (1997).
- Illustrates toxicities of antisense phosphorothioate (PS) oligodeoxynucleotide (ODN) after repeat dose in monkeys.
- 100 Levin AA. A review of the issues in the pharmacokinetics and toxicology of phosphorothioate antisense oligonucleotides. *Biochim. Biophys. Acta* 1489(1), 69–84 (1999).
- Reviews the pharmacokinetic and toxicity issues of PS ODN.
- 101 Levin AA, Monteith DK, Leeds JM *et al.* Toxicity of oligonucleotide therapeutic agents. In: *Handbook of Experimental Pharmacology*. Crooke ST (Ed.). Springer-Verlag, Berlin, Germany 131, 169–215 (1998).
- Reviews the species differences and the organ systems associated with the toxicities of PS ODN therapeutic agents.
- 102 Monteith DK, Henry SP, Howard RB *et al.* Immune stimulation – a class effect of phosphorothioate oligodeoxynucleotides in rodents. *Anticancer Drug Des.* 12(5), 421–432 (1997).
- Illustrates toxicities of PS ODN in rodents associated with immune potency and CpG motif.
- 103 Monteith DK, Levin AA. Synthetic oligonucleotides: the development of antisense therapeutics. *Toxicol. Pathol.* 27(1), 8–13 (1999).
- 104 Thawani N, Tam M, Chang KH, Stevenson MM. Interferon- γ mediates suppression of erythropoiesis but not reduced red cell survival following CpG-ODN administration *in vivo*. *Exp. Hematol.* 34(11), 1451–1461 (2006).
- 105 Kozak W, Wrotek S, Kozak A. Pyrogenicity of CpG-DNA in mice: role of interleukin-6, cyclooxygenases, and nuclear factor- κ B. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 290(4), R871–R880 (2006).
- 106 Nichani AK, Dar MA, Krieg AM *et al.* Systemic innate immune responses following intrapulmonary delivery of CpG oligodeoxynucleotides in sheep. *Vet. Immunol. Immunopathol.* 115(3–4), 357–368 (2007).
- 107 Nichani AK, Mena A, Popowych Y *et al.* *In vivo* immunostimulatory effects of CpG oligodeoxynucleotide in cattle and sheep. *Vet. Immunol. Immunopathol.* 98(1–2), 17–29 (2004).
- 108 Kodama S, Abe N, Hirano T, Suzuki M. Safety and efficacy of nasal application of CpG oligodeoxynucleotide as a mucosal adjuvant. *Laryngoscope* 116(2), 331–335 (2006).
- 109 Ioannou XP, Gomis SM, Hecker R, Babiuk LA, van Drunen Littell-van den Hurk S. Safety and efficacy of CpG-containing oligodeoxynucleotides as immunological adjuvants in rabbits. *Vaccine* 21(27–30), 4368–4372 (2003).
- 110 Vleugels B, Ververken C, Goddeeris BM. Stimulatory effect of CpG sequences on humoral response in chickens. *Poult. Sci.* 81(9), 1317–1321 (2002).
- 111 Heikenwalder M, Polymenidou M, Junt T *et al.* Lymphoid follicle destruction and immunosuppression after repeated CpG oligodeoxynucleotide administration. *Nat. Med.* 10(2), 187–192 (2004).
- 112 Prater MR, Johnson VJ, Germolec DR, Luster MI, Holladay SD. Maternal treatment with a high dose of CpG ODN during gestation alters fetal craniofacial and distal limb development in C57BL/6 mice. *Vaccine* 24(3), 263–271 (2006).
- 113 Halperin SA, Van Nest G, Smith B *et al.* A Phase I study of the safety and immunogenicity of recombinant hepatitis B surface antigen co-administered with an immunostimulatory phosphorothioate oligonucleotide adjuvant. *Vaccine* 21(19–20), 2461–2467 (2003).
- 114 Halperin S, Dobson S, McNeil S *et al.* Comparison of the safety and immunogenicity of hepatitis B virus surface antigen co-administered with an immunostimulatory phosphorothioate oligonucleotide and a licensed hepatitis B vaccine in healthy young adults. *Vaccine* 24, 20–26 (2006).
- ISS alone can adjuvant hepatitis B surface antigen.
- 115 Cooper CL, Davis HL, Morris ML *et al.* CPG 7909, an immunostimulatory TLR9 agonist oligodeoxynucleotide, as adjuvant to Engerix-B HBV vaccine in healthy adults: a double-blind Phase I/II study. *J. Clin. Immunol.* 24(6), 693–701 (2004).
- 116 Siegrist CA, Pihlgren M, Tougne C *et al.* Co-administration of CpG oligonucleotides enhances the late affinity maturation process of human anti-hepatitis B vaccine response. *Vaccine* 23(5), 615–622 (2004).

- Provides evidence that ISS-containing vaccines may induce antibodies with higher antigen affinity.
 - 117 Cooper CL, Davis HL, Angel JB *et al.* CPG 7909 adjuvant improves hepatitis B virus vaccine seroprotection in antiretroviral-treated HIV-infected adults. *AIDS* 19(14), 1473–1479 (2005).
 - 118 Cooper CL, Davis HL, Morris ML *et al.* Safety and immunogenicity of CPG 7909 injection as an adjuvant to Fluarix influenza vaccine. *Vaccine* 22(23–24), 3136–3143 (2004).
 - 119 Speiser DE, Lienard D, Rufer N *et al.* Rapid and strong human CD8⁺ T cell responses to vaccination with peptide, IFA, and CpG oligodeoxynucleotide 7909. *J. Clin. Invest.* 115(3), 739–746 (2005).
 - ISS can be utilized to increase the activity of therapeutic cancer vaccines.
 - 120 Appay V, Jandus C, Voelter V *et al.* New generation vaccine induces effective melanoma-specific CD8⁺ T cells in the circulation but not in the tumor site. *J. Immunol.* 177(3), 1670–1678 (2006).
 - Characterizes T cells induced by a therapeutic cancer vaccine adjuvanted with ISS.
 - 121 Valmori D, Souleimanian NE, Tosello V *et al.* Vaccination with NY-ESO-1 protein and CpG in Montanide induces integrated antibody/Th1 responses and CD8 T cells through cross-priming. *Proc. Natl Acad. Sci. USA* 104(21), 8947–8952 (2007).
 - 122 Tulic MK, Fiset PO, Christodouloupoulos P *et al.* Amb a 1-immunostimulatory oligodeoxynucleotide conjugate immunotherapy decreases the nasal inflammatory response. *J. Allergy Clin. Immunol.* 113(2), 235–241 (2004).
 - 123 Simons FE, Shikishima Y, Van Nest G, Eiden JJ, HayGlass KT. Selective immune redirection in humans with ragweed allergy by injecting Amb a 1 linked to immunostimulatory DNA. *J. Allergy Clin. Immunol.* 113(6), 1144–1151 (2004).
 - Illustrates how T-cell responses are redirected following immunization with an allergen–ISS conjugate.
 - 124 Creticos PS, Schroeder JT, Hamilton RG *et al.* Immunotherapy with a ragweed–Toll-like receptor 9 agonist vaccine for allergic rhinitis. *N. Engl. J. Med.* 355(14), 1445–1455 (2006).
 - Treatment with allergen–ISS conjugates can improve clinical symptoms for seasonal allergic rhinitis.
- Websites**
- 201 Enhancement of the anthrax AVA vaccine with CpG ODN
<http://stinet.dtic.mil/oai/oai?&verb=getRecord&metadataPrefix=html&identifier=ADA455514>
 - 202 Safety of and immune response to a malaria vaccine (MPS1 42-C1) with or without CPG 7909 adjuvant
<http://clinicaltrials.gov/ct/show/NCT00320658?order=6>
 - 203 Phase I study of AMA1-C1/AlhydrogelTM + CPG 7909 malaria vaccine
<http://clinicaltrials.gov/ct/show/NCT00427167?order=1>
 - 204 Phase I study of safety and immunogenicity of AMA1-C1Alhydrogel + CPG 7909 vaccine for malaria
<http://clinicaltrials.gov/ct/show/NCT00414336?order=5>
 - 205 Immunization with the MAGE-3.A1 peptide mixed with the adjuvant CpG 7909 in patients with metastatic melanoma
<http://clinicaltrials.gov/ct/show/NCT00145145?order=4>
 - 206 Vaccine therapy in treating patients with recurrent Stage III or Stage IV melanoma that cannot be removed by surgery
<http://clinicaltrials.gov/ct/show/NCT00471471?order=9>
 - 207 Immunotherapy of HLA-A2 positive Stage III/IV melanoma patients
<http://clinicaltrials.gov/ct/show/NCT00112229?order=10>
 - 208 Cytos biotechnology – clinical trials
www.cytos.com/default3.asp?text=products_trials.asp&cbot=bot_products.htm
- Affiliations**
- Debbie Higgins, BA
Associate Director, Preclinical Research, Dynavax Technologies, 2929 Seventh Street, Suite 100, Berkeley, CA 94710, USA
Tel.: +1 510 665 7229
Fax: +1 510 848 1327
dhiggins@dynavax.com
 - Jason D Marshall, PhD
Senior Scientist, Preclinical Research, Dynavax Technologies, 2929 Seventh Street, Suite 100, Berkeley, CA 94710, USA
Tel.: +1 510 665 7259
Fax: +1 510 848 1327
jmarshall@dynavax.com
 - Paula Traquina, MPH
Senior Manager, Preclinical Programs, Dynavax Technologies, 2929 Seventh Street, Suite 100, Berkeley, CA 94710, USA
Tel.: +1 510 665 7230
Fax: +1 510 848 1327
ptraquina@dynavax.com
 - Gary Van Nest, PhD
Vice President, Preclinical Research, Dynavax Technologies, 2929 Seventh Street, Suite 100, Berkeley, CA 94710, USA
Tel.: +1 510 665 7232
Fax: +1 510 848 1327
gvannest@dynavax.com
 - Brian D Livingston, PhD
Director, Preclinical Research, Dynavax Technologies, 2929 Seventh Street, Suite 100, Berkeley, CA 94710, USA
Tel.: +1 510 665 7237
Fax: +1 510 848 1327
blivingston@dynavax.com