

Immunostimulatory DNA-based therapeutics for experimental and clinical allergy

Although allergen immunotherapy (IT) has been shown to modulate allergic hypersensitivities, its efficacy is limited. Recently, in various models of experimental allergy, a number of reagents which we have termed immunostimulatory DNA-based therapeutics have proven highly effective in both the prevention and reversal of Th2 mediated hypersensitivity states. These include immunization with gene vaccines, allergen mixed with immunostimulatory oligodeoxynucleotide (ISS-ODN), and physical allergen: ISS-conjugates (AIC), and immunomodulation with ISS-ODN alone. Results from our laboratory have shown that immunostimulatory DNA-based therapeutics may be effective for the reversal of allergic hypersensitivity states in humans and several clinical trials have already been initiated. This review will focus on our laboratory's experience with immunostimulatory DNA-based therapeutics in various murine models of allergy and their potential utility in the treatment of allergic patients.

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Key words: CpG motif, immunomodulation, immu-
nostimulatory DNA (ISS), immunotherapy, Th/Th2.

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Introduction

The prevalence of asthma and other allergic diseases has increased over the last two decades despite major advances in our understanding of their pathogenesis and an effective arsenal of medications for their treatment (1). One potential explanation for this trend is that with a declining incidence of many infectious diseases due to vaccination, the use of antibiotics, and other public health practices, a major source of Th1 immune provocation has been lost, with a consequent increase in the Th2 bias of immune responses toward environmental allergens (2). This theory, which has been referred to as the hygiene hypothesis, is supported by a number of recent studies (2, 3). While still unproven, the hygiene hypothesis offers an attractive explanation for current trends in the prevalence of allergic diseases and offers a provocative rationale for consideration of DNA-based therapeutics for the treatment of allergic diseases. The immunological activities of immunostimulatory DNA-based therapeutics are dependent on their content of specific immunostimulatory DNA sequences (CpG motifs) which are abundant in many microbial genomes but are rare in mammalian DNA. Therefore, immunostimulatory DNA-based therapeutics are likely to elicit immunity, in part by mimicry of natural infectious exposures. If the sterilization of the environment we live in proves to be a contributing factor in the rising prevalence of allergic diseases, as the hygiene hypothesis proposes, then immunostimulatory DNA-based therapeutics represent novel and rational approaches for their treatment.

The observation that plasmid gene vaccination induces a highly Th1 biased immune response and prevents the development of Th2 biased immunity was first published in 1996 (4, 5). Further studies have shown that gene vaccination prevents the development of Th2 biased immunity, and reverses pre-existing Th2 biased immune profiles (4). In the course of evaluating the immunogenicity of various gene vaccination vectors, we discovered that gene expression is but one of two factors that determine the immunogenicity of gene vaccination constructs (6). Work from several laboratories has determined that effective gene vaccination vectors additionally contain cryptic immunostimulatory DNA sequences (ISS-ODN or CpG motifs) which provide Th1 adjuvant activity for the immune responses that develop toward the gene product (6). Deletion of several of these ISS-ODNs from the vaccination plasmid attenuates the post-vaccination immune response without changing gene expression, and the immune response is restored by covaccination with a plasmid containing additional ISS-ODN.

Immunostimulatory DNA sequences were found to contain unmethylated CpG dinucleotides within a palindromic hexamer that followed the formula: 5'-purine-purine-CG-pyrimidine-pyrimidine-3' (e.g. 5'-GACGTC-3', 5'-AGCGCT-3' and 5'-AACGTT-3') (7). Interestingly, CpG dinucleotides generally occur at near the expected frequency (1 in 16) in many prokaryotic genomes but are much less frequent in eukaryotic DNA. In addition, less than 5% of the cytosines in prokaryotic CpG dinucleotides are methylated, whereas 70–90% of the CpG dinucleotides in eukaryotic

genomes contain an inactivating methylated cytosine (8). These observations have led to the proposal that the innate immune system has evolved the ability to detect CpG motifs found in bacterial genomes, as is the case with other microbial products such as LPS. This hypothesis is further supported by recent work which establishes that ISS enriched DNA and other microbial products are dependent on similar intracellular and extra-cellular signaling pathways for their activation of innate immunity (9, 10).

The antigen independent (innate) immune response induced by ISS-ODN is characterized by the production of IL-12, IL-18, IFNs, IL-6, and IL-10 (11–13). In addition, ISS-ODN induces the expression of a number of costimulatory molecules on the surface of B cells and APCs (14). In addition, ISS-ODN has recently been shown to increase IFN- γ receptor (CD119) and reduce IL-4 receptor (CD124) expression on human B cells (11). Finally, it has been demonstrated that ISS-ODN inhibits IL-4 dependent IgE synthesis from human B cells in an IL-12, IFN- α , IFN- γ , and IL-10 dependent manner (11). Functionally, ISS-ODN activates NK cells and APCs and induces B cell proliferation and antibody production. Vertebrate DNA, methylated bacterial DNA, and methylated ISS-ODN, by contrast, do not elicit an immune response.

Studies with ISS-ODN as a vaccine adjuvant have shown that it elicits a robust and multifaceted adaptive immune response to coadministered proteins, analogous to the immune response elicited by DNA vaccines (13, 15, 16). Furthermore, ISS-ODN has a long-lasting Th1 biasing adjuvant effect on antigens (and potentially allergens) encountered at the site of delivery. In fact, ISS-ODN delivery 1–7 days before antigen (prepriming) leads to immune responses of greater magnitude than those seen with antigen/ISS-ODN codelivery (15). This is likely to be due to ISS-ODN induced APC activation, which has been shown to last for up to 2 weeks in mice (14, 15). The robust and persistent immune activation and Th1 adjuvant activity elicited by ISS-ODN has prompted its study as an antiallergic vaccine adjuvant and immunomodulator for the prevention and reversal of Th2 biased (allergic) immune deviation, and the hypersensitivity responses which characterize asthma (17), allergic conjunctivitis (18), and anaphylaxis (19).

Immunostimulatory DNA-based therapeutics in models of allergic asthma

To evaluate the capacity of gene vaccination to prevent the late phase asthmatic response, we and others have utilized several rodent models. Using the OVA-induced murine model of asthma, gene vaccination with an OVA expression vector (pCMV-OVA) prior to sensitization significantly reduced eosinophilic airway

inflammation and systemic eosinophilia when compared to control mice vaccinated with a control plasmid lacking the gene insert or left unvaccinated (20, 21).

Experiments based on the Th1 adjuvant paradigm have further demonstrated that protein/ISS-ODN immunization protected against the development of an allergic immune profile with sensitization (13, 19). Much of the Th1 adjuvant activities of ISS-ODN are believed to be due to their activation of the innate immune system, and many of the cytokines produced in response to ISS-ODN (i.e. IL-12, IL-18, IFN- α , IFN- γ , IL-10) have known antiallergic activities. In particular, mice injected with ISS-ODN demonstrate elevated serum levels of IL-12 and IFN- γ for as long as 2 weeks or more (15). Because ISS-ODN generates a long-lasting antiallergic cytokine milieu in recipient mice, we have considered a novel paradigm for the application of ISS-ODN to the treatment of allergic disease. The hypothesis proposes that the cytokines produced in response to ISS-ODN might offer allergen independent protection to previously sensitized animals against subsequent allergen challenge (17, 18, 22, 23).

We initially evaluated the antiallergic immunomodulatory activity of ISS-ODN in the OVA-induced asthma model. ISS-ODN administration shortly before challenge of allergen sensitized mice reduced methacholine sensitivity by up to 70%. In addition, ISS-ODN delivery led to a dramatic reduction in lung parenchyma eosinophilic inflammation and eosinophilia in the BALF, blood, and bone marrow after allergen challenge. In fact, a single dose of ISS-ODN was as effective as 7 daily doses of dexamethasone (DXM) in protecting sensitized mice from eosinophilic inflammation of the airways with nebulized allergen challenge. Furthermore, ISS-ODN effectively attenuated the asthmatic challenge response when delivered either systemically or topically (i.e. with intratracheal or intranasal administration). Interestingly, both ISS-ODN and DXM mediated immunomodulation also inhibited allergen specific IL-5 production, while ISS-ODN further induced an IFN- γ response from splenocytes. Cytokine neutralization experiments have further shown that ISS-ODN mediated antiallergic immunomodulation in this asthma model is dependent on the production of IL-12, IFN- α/β , and IFN- γ (17).

Recently, we have conducted experiments to evaluate the longevity of ISS-ODN induced immunomodulation. Our findings demonstrate that in contrast to the long standing Th1 biased immunity elicited with DNA-based vaccination, the immunomodulatory activity of ISS-ODN in this asthma model is relatively short lived (22). A single delivery of ISS-ODN shortly before a primary allergen challenge led to a shift in the allergen specific cytokine response from a Th2 to a Th1 bias and a dramatic inhibition of the late phase asthmatic response. These mice were then protected from a second allergen challenge for an additional month.

However, 2 months after ISS-ODN delivery and primary allergen challenge the antigen specific immune and hypersensitivity responses to a second allergen challenge were similar to those of mice that did not receive ISS-ODN (22).

Taken together, these experiments on the immunomodulatory activity of ISS-ODN suggest that if used in clinical practice, regular ISS-ODN therapy could effectively prevent the late phase eosinophilic inflammation and bronchial hyperresponsiveness of asthma, while inducing a Th1 response to allergens upon natural exposure. Therefore, unlike DNA-based and other forms of vaccination, ISS-ODN immunomodulatory therapy could obviate the need to identify allergens in clinical practice. Furthermore, because of its effectiveness with topical application, ISS-ODN could be given by metered dose inhaler. Clearly, ISS-ODN immunomodulation represents a novel and promising approach for the treatment of allergic disease and an intellectual paradigm shift from the principles by which protein-based allergen immunotherapy (IT) is presently used for the treatment of allergic disease (23).

Immunostimulatory DNA-based therapeutics in models of allergic conjunctivitis

We have studied the immunomodulatory activity of ISS-ODN on early and late phase hypersensitivity responses in a murine model of ragweed (RW) pollen-induced allergic conjunctivitis. Ragweed sensitized mice were examined clinically for signs of an immediate hypersensitivity response 20 min after topical RW challenge. Eyes were graded for chemosis, conjunctival redness, lid edema, and tearing. Mice treated systemically or topically with ISS-ODN three days before RW challenge had significantly lower conjunctivitis scores than mutated (M)-ODN or PBS treated controls. Furthermore, codelivery of ISS-ODN with neutralizing anti-IL-12 monoclonal antibodies completely abolished the protective effects of ISS-ODN administration on the conjunctivitis score. When compared to DXM, ISS-ODN was equally effective in protecting against the immediate hypersensitivity response in this allergic conjunctivitis model (18).

To determine the late phase response, conjunctiva were prepared for histology 24 h after RW challenge to quantitate cellular inflammation. Topical and systemic ISS-ODN treatment 3 days prior to allergen challenge led to a marked attenuation of the late phase inflammatory response in the conjunctiva when compared to M-ODN therapy. In contrast, DXM treatment did not suppress late phase inflammation of the conjunctiva. Consistent with its effect on immediate hypersensitivity, neutralizing anti-IL-12 antibodies abolished the antiallergic activity of ISS-ODN on late phase conjunctival inflammation (18).

Administration of ISS-ODN to RW sensitized mice also induced high levels of antigen specific IFN- γ production from cultured lymphocytes, when compared to mice treated with M-ODN or DXM. This ISS-ODN induced antigen specific IFN- γ response was completely inhibited by codelivery with IL-12 mAb. In a separate series of experiments, RW sensitized and ISS-ODN or M-ODN treated mice were challenged on 2 separate occasions with RW, to determine their effects of IgE synthesis. Again, ISS-ODN treatment inhibited the RW specific IgE response significantly when compared to treatment with M-ODN (18).

Immunostimulatory DNA-based therapeutics in models of anaphylaxis

Since both gene and protein/ISS-ODN vaccinations have been shown to prevent subsequent Th2 biased immune responses, including IgE production, their efficacy in protecting mice from the development of anaphylactic hypersensitivity has been evaluated. Recognizing that the prevention of death and other severe outcomes remains the primary objective in treating patients with anaphylactic hypersensitivities, we developed a model of severe anaphylaxis in which death was the major outcome variable. In this model, mice were gene vaccinated with pACB-LacZ, which encodes β -galactosidase (β -gal) or empty plasmid vector (pACB) prior to sensitization with β -gal in alum and subsequent i.v. β -gal challenge. pACB-LacZ vaccination protected 5/15 mice from lethal anaphylaxis in 4 experiments, while 0/16 mice vaccinated with pACB survived challenge. Consistent with the survival data, plasma histamine levels 2 min post challenge were also significantly lower in pACB-LacZ vs. pACB vaccinated mice. Anaphylactic hypersensitivity is associated with Th2 biased immunity, and in humans is dependent on IgE production. Therefore, β -gal specific antibody and cytokine profiles of gene vaccinated/sensitized mice were further evaluated. IgE levels were on average 15-fold lower in pACB-LacZ vs. pACB vaccinated mice. In addition, pACB-LacZ vaccinated mice produced approximately 3-fold less IgG1 and 4-fold more antigen specific IgG2a than pACB vaccinated mice. Splenocytes from mice vaccinated with pACB-LacZ prior to sensitization further demonstrated a Th1 bias with significantly higher antigen specific IFN- γ and lower IL-4 and IL-5 responses than control mice (19).

As discussed previously, protein/ISS-ODN coimmunization and gene vaccination induce similar immune profiles. Therefore, the efficacy of β -gal/ISS-ODN vaccination in protecting against the development of anaphylactic hypersensitivity was also evaluated. Mice were vaccinated with β -gal and ISS-ODN, and then sensitized and i.v. challenged in a manner analogous to that described for the gene vaccination studies. One

immunization control group vaccinated with β -gal alone, the experimental equivalent of allergen extracts used in clinical practice. Five out of 12 β -gal/ISS-ODN vaccinated mice survived i.v. β -gal challenge vs. 0/36 control mice receiving β -gal or ISS-ODN, or β -gal and M-ODN. Post challenge histamine levels were also significantly lower in β -gal/ISS-ODN vaccinated vs. control mice. Like pACB-LacZ vaccinated mice, those immunized with β -gal and ISS-ODN demonstrated a 90% decrease in serum IgE postsensitization, when compared to controls. In addition, while β -gal/ISS-ODN vaccinated mice had similar IgG1 levels to control mice, IgG2a levels were 8-fold higher. Furthermore, splenocytes from β -gal/ISS-ODN vs. control vaccinated mice demonstrated a β -gal specific Th1 cytokine bias reflected in elevated IFN- γ and depressed IL-4 and IL-5 production upon culture with β -gal (19).

Allergen-ISS-conjugates (AIC)

ISS-ODN like any other natural DNA molecule has a highly negatively charged backbone. Because of this and its antiallergic Th1 adjuvant activity, we considered two potential consequences of physically linking ISS-ODN to allergens (AIC). The first was that AIC might be more immunogenic than a cocktail of allergen and ISS-ODN, as it would colocalize adjuvant and allergen in the microenvironment of responding cells (16, 24–26). The second was that AIC might be less allergenic than the allergen/ISS-ODN cocktail, as the ODNs coating the allergen could in principle create steric and/or electrostatic blockade of IgE binding epitopes (26, 27). These considerations have prompted investigations of the immunogenicity and allergenicity of AIC in rodents, nonhuman primates, and humans.

Recently, we have shown that injection of Ambal, the dominant ragweed (RW) allergen, chemically linked to ISS-ODN (Ambal-ISS-conjugate) led to robust Th1 biased responses against Ambal in mice, rabbits, and monkeys while Ambal alone induced a Th2 biased immune response or no immune response at all. In addition, the Ambal-ISS-conjugate induced immune response was significantly stronger than the immune response elicited after injection of Ambal mixed with ISS-ODN or plasmid encoding Ambal (26).

We have further evaluated the effectiveness of Ambal-ISS-conjugate in the reversal of allergic immune deviation. In these studies, a model of tertiary prevention was used to mimic the clinical scenario of patients receiving IT with allergens such as Ambal. A primary Th2 biased immune profile was induced by injection of mice with Ambal in alum. The mice then received IT with Ambal-ISS-conjugate, Ambal, or nothing. Finally, the mice were challenged with Ambal. The results of these studies clearly showed that

Ambal-ISS-conjugate vaccination reversed pre-existing Th2 biased immune deviation, and induced Th1 biased immunity. Furthermore, while sensitized mice receiving Ambal IT demonstrated a continued rise in serum IgE, mice receiving Ambal-ISS-conjugate IT did not. Finally, Ambal-ISS-conjugate IT was significantly more effective than Ambal IT for preventing the serum IgE spike seen with the final allergen challenge (26).

Additional unpublished experiments from this laboratory have shown that AIC vaccination is more effective than allergen/ISS-ODN coadministration in preventing Th2 biased immune deviation and the early phase hypersensitivity response in the model of anaphylaxis presented previously. Taken together, these studies with AIC demonstrate that it is more immunogenic than native antigens and antigen/ISS-ODN cocktails across species, and more effective in the prevention of allergic hypersensitivity responses.

We have also evaluated the allergenicity of Ambal-ISS-conjugates. Basophils from RW allergic donors were incubated with Ambal-ISS-conjugate and native Ambal and histamine release was determined by ELISA. These studies demonstrated that Ambal-ISS-conjugate elicited substantially less histamine release than native Ambal over a range of concentrations. Furthermore, the Ambal-mODN-conjugate (i.e. conjugated to a mutated, control ODN) also proved to be significantly less allergenic than native Ambal, confirming that steric hindrance and/or electrostatic blockade by ODN conjugation blocks IgE binding sites on the allergen in a sequence independent manner (26). The Ambal-ISS-conjugate has also been compared to RW extract in skin test end point titration studies with RW allergic patients. In the 6 patients tested, Ambal-ISS-conjugate was from 100 to 300-fold less allergenic than the RW extract presently used for IT in clinical practice. In addition to demonstrating that it is much less allergenic than native RW, this skin testing study demonstrated that Ambal-ISS-conjugate was well tolerated, and without significant toxicity in humans (27).

Discussion

Immunostimulatory DNA-based therapeutics have shown great promise in rodent models of allergic disease. Presently, four basic DNA based strategies have been utilized in the modulation of allergic hypersensitivities. These include gene vaccination, protein (allergen)/ISS vaccination, AIC vaccination and ISS-ODN immunomodulation. Gene and protein/ISS-ODN vaccination have proven highly effective in the induction of Th1 and reversal of Th2 biased immune profiles. As a consequence of preventing and reversing underlying allergic sensitivities, these immunostimula-

tory DNA-based vaccination strategies have been further shown to attenuate the immediate hypersensitivity response of anaphylaxis, and the late phase allergic response of asthma (19, 20). Recently, physical conjugates of allergen and ISS-ODN (AIC) have been developed. AIC have proven even more immunogenic than allergen/ISS-ODN cocktails in rodents and primates, and less allergenic than allergen extracts in *in vitro* studies with human basophils and in skin testing studies of allergic patients (16, 24, 26, 27). These attributes suggest that AIC may be even safer and more effective than allergen expressing plasmids and allergen mixed with ISS-ODN for IT in allergic patients. The antiallergic bioactivity of immunostimulatory DNA-based reagents has been well characterized within the context of the traditional vaccination paradigm, and the Th1 biased immune responses elicited have been shown to be long lasting, with antigen specific immunity being detectable up to one year or more after immunization (4, 13, 16, 19, 24, 26).

Allergen IT was initially described by Noon in 1911 (28). Since that time, the major limiting factor in the effectiveness of protein-based allergen IT has been the therapeutic index (therapeutic dose/allergenic dose) of the allergen extracts used to treat patients. However, results with AIC suggest that it represents a major breakthrough in the development of safer and more effective IT reagents, as conjugation of allergen to ISS-ODN is the first reported chemical modification that leads to a more immunogenic and less allergenic reagent than the original allergen extract from which it was developed. In fact, because of the encouraging results seen in mice, rabbits and monkeys, and AIC's favorable therapeutic index, clinical trials have already been initiated to evaluate the safety and efficacy of Ambal-ISS-conjugate IT in humans with RW allergies.

Alternatively, ISS-ODN has been shown to be an effective allergen independent immunomodulator, as sensitized mice treated with ISS-ODN alone have attenuated hypersensitivity responses to subsequent allergen challenge (17, 18, 22, 23, 29). In fact, ISS-ODN has proven to be as effective an immunomodulator as DXM in the prevention of the immediate phase hypersensitivity response of allergic conjuncti-

vitis, and more effective than DXM in the prevention of the late phase inflammatory responses seen in murine allergic conjunctivitis and asthma models (17, 18). Furthermore, like corticosteroids, allergen independent ISS-ODN delivery has been shown to inhibit allergen specific IL-5 production from Th2 sensitized mice. However, in contrast to corticosteroids, ISS-ODN immunomodulation also stimulates allergen specific IFN- γ production from previously Th2 sensitized mice (17, 18). In further consideration of the dichotomy between DNA vaccination and ISS-ODN mediated immunomodulation, we have found that in contrast to the long standing and antigen specific immune responses elicited with DNA-based vaccination, allergen nonspecific ISS-ODN induced immunomodulation is relatively short lived (22).

The principles of vaccination and immunomodulation represent two distinct yet interrelated strategies for the utilization of immunostimulatory DNA-based reagents in the treatment of allergic diseases. With DNA-based vaccination, a long lasting Th1 biased adaptive immune response develops (4, 6, 19, 24–26, 30). This Th1 biased immune profile both prevents and reverses allergen specific Th2 biased T and B cell responses, and consequently, animals vaccinated with DNA-based vaccines are protected from allergic hypersensitivity responses for an extended period of time. In contrast, ISS-ODN induced immunomodulation appears to depend on the antiallergic innate cytokine response which is generated by macrophages and other APCs upon exposure to ISS-ODN. It is likely that ISS-ODN induced immunomodulation requires repeated exposures to allergen in the context of ISS induced innate immune activation in order to imprint a Th1 bias on the adaptive immune system. These repeated exposures to allergen and ISS-ODN would serve to fully vaccinate mice and commit their antigen specific lymphocytes to a Th1 phenotype.

Acknowledgments

This work was supported in part by NIH Grants AI-40682 and AI-01490 from the NIAID and a grant from Dynavax Technologies Corporation.

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