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DNA-based immunotherapeutics for the treatment of allergic disease

Summary: Allergic diseases are a growing health concern in industrialized countries. Despite a number of effective therapeutic options for the prevention and treatment of the pathophysiologic responses which characterize allergic diseases, the induction of true allergen desensitization remains an elusive therapeutic goal. Only immunotherapy (IT) has been shown to have any effect on the underlying hypersensitivities which mediate allergic reactions, and traditional protein-based allergen IT has a limited scope of efficacy. However, a number of reagents collectively termed DNA-based immunotherapeutics have proven highly effective in both the prevention and reversal of Th2-mediated hypersensitivity states in mouse models of allergic disease. Four basic DNA-based immunotherapeutic modalities have been used for these studies. These include immunization with gene vaccines, allergen mixed with immunostimulatory oligodeoxynucleotide (ISS-ODN), and physical allergen-ISS-ODN conjugates (AIC), as well as immunomodulation with ISS-ODN alone. Results from many laboratories have generated guarded optimism that DNA-based immunotherapeutics 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 present understanding of the biological activities of DNA-based immunotherapeutics and their application to the treatment of allergic diseases.

Introduction

The prevalence of asthma and other allergic diseases has increased over the last two decades in the industrialized world, especially in young children (1–3). Allergic diseases affect as many as 30% of individuals in some countries, and in the United States the CDC reports that more than 17 million people currently suffer from asthma, about 7% of the population. In addition, 20% and 2% of the population suffer from allergic rhinitis and anaphylactic hypersensitivities, respectively (1). The economic cost of allergic diseases in developed countries is substantial, with asthma alone accounting for 1–2% of their direct and indirect medical expenditures. Indeed, in the United States, allergy-associated health costs are expected to exceed \$14 billion in the year 2000 (1). Because allergic diseases are so common, are increasing in prevalence,

and are expensive to treat, better strategies for their prevention and treatment remain a health care research priority.

Unfortunately, present trends in the prevalence of allergic diseases persist, despite major advances in our understanding of their pathogenesis and an effective arsenal of medications for their treatment. 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 (2–6). This theory, which has been referred to as the hygiene hypothesis, is supported by a number of studies, including two which demonstrate that PPD test converters and individuals infected with measles are less likely to demonstrate an allergic profile than individuals without these infectious exposures (5, 6). While still unproven, the hygiene hypothesis offers an attractive explanation for current trends in the prevalence of allergic diseases. Furthermore, the hygiene hypothesis provides a provocative rationale for consideration of DNA-based therapeutics for the treatment of allergic diseases. As will be reviewed below, the immunologic activities of DNA-based immunotherapeutics 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, 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 DNA-based immunotherapeutics represent novel and rational approaches for their treatment.

A number of medications have been successfully used in the prevention and treatment of the immediate and late-phase hypersensitivity responses which define the allergic diathesis. However, only protein-based allergen immunotherapy (IT) has proven effective in the reversal of the hypersensitivities which perpetuate the allergic phenotype (7–9). Unfortunately, the efficacy of traditional IT is limited. While over 90% of patients with anaphylactic hypersensitivities to stinging insects develop clinical tolerance with IT, only 30–50% of allergic rhinitis patients respond, and IT is even less effective for asthmatics (7, 8). The use of allergen IT for the treatment of food allergies is contraindicated due to the high risk of life-threatening allergic reactions (7, 10). Studies have shown that higher allergen extract doses improve the efficacy of IT in the reversal of allergic hypersensitivities (7–9). However, with higher allergen doses, the risk of life-threatening allergic

reactions to the IT injections increases significantly. Therefore, reagents with higher immunogenic and lower allergenic potentials than traditional allergen extracts would be attractive alternatives for use in IT. This review will focus on DNA-based immunotherapeutic strategies in terms of their immunogenicity, allergenicity, efficacy in animal models of allergic disease and potential applications in clinical practice.

A brief history of DNA-based immunotherapeutics

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 (11). Compared to mice injected with protein in alum, mice injected with plasmid encoding the gene product were shown to develop high IgG2a (interferon (IFN)- γ dependent) and low IgG1 and IgE (IL-4 dependent) responses. In addition, it was demonstrated that gene vaccination induces a Th1-biased cytokine profile characterized by high IFN- γ and low IL-5 production. Furthermore, gene vaccination was shown to prevent the development of Th2-biased immunity, and to reverse pre-existing Th2-biased immune profiles. Initial studies used experimental allergens such as β -galactosidase (β -gal) and ovalbumin (OVA) (11–13), and more recently have been replicated with clinically relevant allergens such as house dust mite, birch, latex, and peanut (14–18).

In the course of evaluating the immunogenicity of various gene vaccination vectors, it was discovered that gene expression is but one of two factors that determine the immunogenicity of gene vaccination constructs. 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 (19, 20). Deletion of several of these ISS-ODN from the vaccination plasmid attenuates the post-vaccination immune response without effecting gene expression, and the immune response is restored by co-vaccination with a plasmid containing additional ISS-ODN.

In retrospect, immunostimulatory DNA was first used as an adjuvant over 60 years ago. Whole mycobacterial extract mixed with paraffin oil constitutes Freund's famous adjuvant (21). However, mycobacterial DNA was shown to contribute to the adjuvant activity of this complex reagent only 16 years ago. Tokunaga and coworkers discovered that DNA purified from *Mycobacterium bovis* bacillus Calmette-Guerin (BCG) induced limited anti-tumor activity. This BCG DNA-mediated anti-tumor activity was shown to be the result of natural killer

(NK) cell activation (22) and IFN- α/β production (23) and was eliminated by DNase treatment. The 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') (24). 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 (25). These observations have led to the proposal that the immune system has evolved the ability to detect CpG motifs found in bacterial genomes as danger signals, stimulating an innate immune response in mammals in anticipation of impending microbial infection.

Studies with synthetic phosphorothioate ISS-ODN have shown that they elicit a robust and multifaceted innate immune response, analogous to the immune response elicited with bacterial DNA. This immune response is characterized by the production of interleukin (IL)-12, IL-18, IFNs ($\alpha/\beta/\gamma$), IL-6, and IL-10, (26–29). In addition, ISS-ODN induces the expression of a number of co-stimulatory molecules on the surface of B cells and APCs (30). 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 (30–35).

In a number of studies, ISS-ODN has further proven to be an effective Th1 and mucosal adjuvant, generating immune responses to co-administered protein antigens which mimic the immune responses seen after gene vaccination (28, 36–38). Additional studies have demonstrated that the Th1-biased immune profile elicited with protein/ISS-ODN vaccination is resistant to subsequent perturbation toward a Th2-biased phenotype (13, 28, 39). Furthermore, ISS-ODN has a long-lasting Th1-biasing adjuvant effect on antigens (and potentially allergens) encountered at the site of delivery (40, 41). In fact, ISS-ODN delivery 1–7 days before antigen (pre-priming) leads to immune responses of greater magnitude than those seen with antigen/ISS-ODN co-delivery (40). This is likely to be due to ISS-ODN-induced immunocyte activation, which has been shown to last for up to 2 weeks in mice (30, 40, 42). The robust and persistent immune activation and Th1 adjuvant activity elicited by ISS-ODN has prompted its study as an anti-allergic vaccine adjuvant and immunomodulator for the prevention and reversal of Th2-

biased (allergic) immune responses, and the allergen-specific hypersensitivity responses which characterize anaphylaxis, asthma, and allergic conjunctivitis. These studies will be reviewed below.

DNA-based vaccination in models of anaphylaxis

Anaphylactic reactions are systemic life-threatening allergic hypersensitivity responses which occur when preformed antibody encounters allergen, leading to cross-linking of Fc receptors on the surface of effector cells such as mast cells. This results in mast cell degranulation, the release of preformed mediators, and a characteristic systemic hypersensitivity response (43, 44). Anaphylaxis generally occurs within minutes of allergen exposure and is considered to be a definitive example of an immediate hypersensitivity response. While multiple pathways exist in mice, in humans allergen-specific anaphylaxis is thought to be mediated exclusively by IgE-triggered mast cell degranulation (43, 45–48). 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 (13, 18). Roy and colleagues demonstrated that gene vaccination can prevent moderate anaphylactic reactions to peanut allergen (Ara h 1) (18). These investigators orally vaccinated mice with chitan particles containing a plasmid encoding Ara h 1. Vaccinated mice were protected from both the development of a Th2-biased immune profile with sensitization and anaphylactic reactions upon challenge.

Recognizing that the prevention of death and other severe outcomes remains the primary objective in treating patients with anaphylactic hypersensitivities, we have investigated the efficacy of DNA-based vaccination in a model of severe anaphylaxis, in which death is the major outcome variable (13). As shown in Table 1, pACB-LacZ vaccination protected 5/15 mice from death in four experiments, while 0/16 mice vaccinated with a LacZ-deficient plasmid (pACB) survived challenge. Consistent with the outcomes of challenged mice, post-challenge plasma histamine levels were also significantly lower in pACB-LacZ versus pACB vaccinated mice (Table 1). 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. As seen in Fig. 1A, IgE levels were on average 15-fold lower in pACB-LacZ versus pACB vaccinated mice. In addition, pACB-LacZ vaccinated mice produced approximately 3-fold less

Table 1. Anaphylactic challenge outcomes of pACB-LacZ vaccinated mice

Vaccination	Survival	% Survival	Histamine
pACB-LacZ	5/15	33%*	7.4 ± 3 μM*
pACB	0/16	0%	41 ± 13 μM

C3H/HeJ mice received i.d. vaccinations with 50 μg of pACB-LacZ or pACB on 3 occasions 10 days apart. Twenty-five days after the last vaccination, mice were Th2 sensitized with a mixture of β-gal (100 μg), pertussis toxin (300 ng), and alum (1 mg) injected i.p. on 2 occasions 7 days apart. Mice then received an i.v. challenge with 150 μg of β-gal 3 weeks after their last i.p. sensitization. Plasma histamine levels were determined 2 min after challenge. p-values for survival were determined by Fisher's exact test, and p-values for histamine release were determined by an unpaired 2-tailed Student's t-test (* represents $p=0.018$ vs. control). Mice that died did so within 1 h of challenge. Surviving mice demonstrated signs of anaphylaxis within the first half hour after challenge but appeared healthy 1 h later.

Table 2. Anaphylactic challenge outcomes of β-gal/ISS-ODN vaccinated mice

Vaccination	Survival	% Survival	Histamine
β-gal+ISS-ODN	5/12	42%**	6.1 ± 2.7 μM*
ISS-ODN	0/12	0%	25.2 ± 11 μM
β-gal	0/12	0%	26 ± 6 μM
β-gal+M-ODN	0/12	0%	29 ± 7.8 μM

C3H/HeJ mice received i.d. vaccinations with ISS-ODN (10 μg) and β-gal (10 μg), ISS-ODN or β-gal alone or β-gal plus M-ODN (10 μg) on 3 occasions 10 days apart. Twenty-five days after the last vaccination, mice were Th2 sensitized with a mixture of β-gal (100 μg), pertussis toxin (300 ng), and alum (1 mg) injected i.p. on 2 occasions 7 days apart. Mice then received an i.v. challenge with 150 μg of β-gal 3 weeks after their last i.p. sensitization. Plasma histamine levels were determined 2 min after challenge. p-values for survival were determined by Fisher's exact test, and p-values for histamine release were determined by an unpaired 2-tailed Student's t-test (* represents $p=0.05$ vs. controls; ** represents $p=0.01$ vs. controls). Mice that died did so within 1 h of challenge. Surviving mice demonstrated signs of anaphylaxis within the first half hour after challenge but appeared healthy 1 h later.

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 (Fig. 1B).

As discussed previously, protein/ISS-ODN co-immunization 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 (13). Mice were vaccinated with β-gal and ISS-ODN, and then sensitized and i.v. challenged with the model allergen, in a manner analogous to that described for the gene vaccination studies. One immunization control group received β-gal alone, the experimental equivalent of

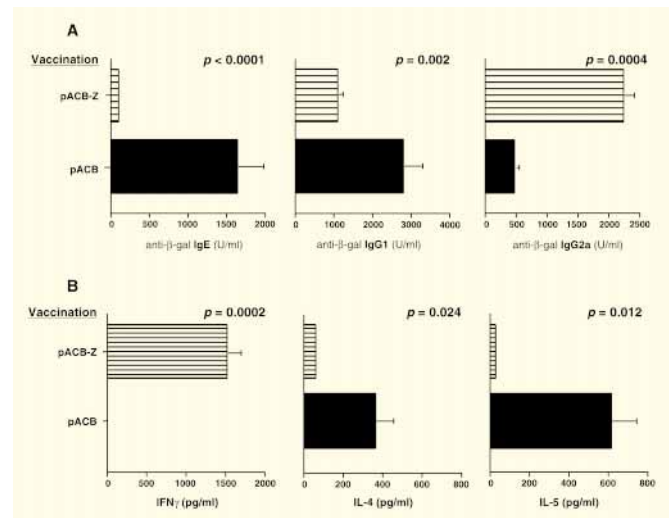


Fig. 1. Serum antibody and cytokine responses of pACB-LacZ vaccinated/Th2-sensitized mice. Mice were vaccinated, sensitized, and challenged as described in Table 1. Results represent mean values ± S.E. for 4 mice in each group, and are representative of 4 independent experiments. Reported p-values are between pACB-LacZ and pACB vaccinated mice by unpaired two-tailed Student's t-test. **A) Antigen-specific antibody levels.** One day prior to challenge serum was obtained for antibody determination by ELISA. **B) Antigen-specific cytokine responses.** After i.v. β-gal challenge, splenocytes were harvested and cultured with and without β-gal. Supernatants were harvested at 72 h for cytokine ELISA. Culture without β-gal led to negligible cytokine production (data not shown).

allergen extracts used in clinical practice. Other control mice were injected with ISS-ODN alone or β-gal plus a non-stimulatory mutated oligodeoxynucleotide (M-ODN) derived by base substitutions in the 2 CpG dinucleotides found within the parent ISS-ODN. As seen in Table 2, in three experiments 5/12 β-gal/ISS-ODN vaccinated mice survived i.v. β-gal challenge versus 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 post-sensitization when compared to controls (Fig. 2A). 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 an antigen-specific Th1 cytokine bias reflected in elevated IFN-γ and depressed IL-4 and IL-5 production upon culture with allergen (Fig. 2B).

The reasons why gene and protein/ISS-ODN vaccinations protected only 30–40% of sensitized mice from lethal anaphylactic reactions in these experiments have not been fully

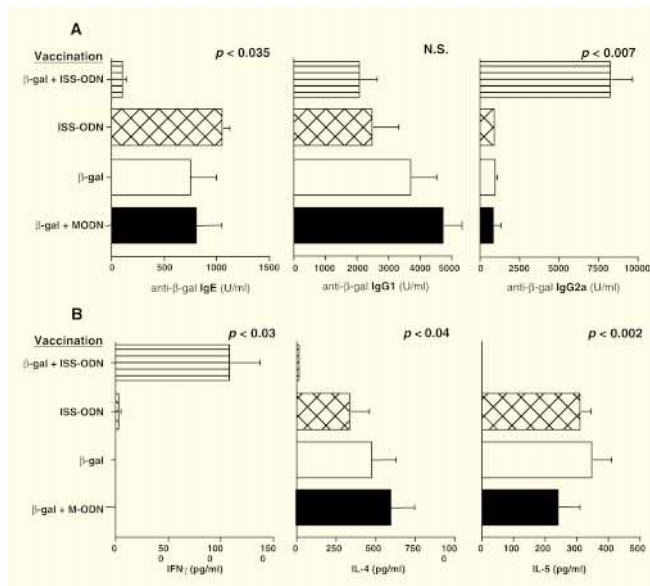


Fig. 2. Serum antibody and cytokine responses of β -gal/ISS-ODN-vaccinated/Th2-sensitized mice. Mice were vaccinated, sensitized, and challenged as described in Table 2. Results represent mean values $\pm$ S.E. for 4 mice in each group, and are representative of 3 independent experiments. Reported p-values are between β -gal/ISS-ODN and control vaccinated mice by unpaired two-tailed Student's t-test. N.S.=no significant difference. **A) Antigen-specific antibody levels.** One day prior to challenge serum was obtained for antibody determination by ELISA. **B) Antigen-specific cytokine responses.** After i.v. β -gal challenge, splenocytes were harvested and cultured with and without β -gal. Supernatants were harvested at 72 h for cytokine ELISA. Culture without β -gal led to negligible cytokine production (data not shown).

elucidated. It may be that the >90% reduction in allergen-specific serum IgE seen in DNA vaccinated vs. control mice is not adequate to protect from anaphylactic hypersensitivity. Alternatively, non-IgE-dependent pathways, including those acting through IgG isotypes, may play a role in the anaphylactic hypersensitivity responses seen in sensitized mice (45–48). In fact, Oettgen and colleagues have demonstrated that sensitized IgE knock-out and wild-type mice develop equivalent anaphylactic responses when challenged (48). Clearly, mouse models do not lend themselves to the study of IgE-dependent anaphylaxis. However, these murine studies do demonstrate that gene and protein/ISS-ODN vaccination provide significant though limited protection against the development of lethal anaphylactic hypersensitivity post-sensitization, and that IgE production and other parameters of the Th2-biased immune profile are attenuated. Of potentially greater clinical significance, DNA-based vaccination is shown to be significantly more effective than traditional protein-based allergen IT (β -gal alone) in protecting against the devel-

opment of lethal anaphylactic hypersensitivity and a Th2-biased immune profile with sensitization (13).

DNA-based vaccination in models of asthma

Allergic bronchial asthma is an inflammatory disease of the airways associated with bronchial hyperreactivity and episodic reversible airway obstruction (49, 50). While the pathogenesis of asthma is complex, Th2 cells are thought to play a pivotal role in the induction and maintenance of eosinophilic airway inflammation, and eosinophils are thought to play an important effector role in the development of the chronic bronchial hyperreactivity which underlies the asthmatic phenotype (51–54). As in anaphylaxis, mast cells armed with preformed antibody mediate an immediate phase response in asthmatics exposed to aeroallergen. However, the characteristic features of asthma, including eosinophilic airway inflammation and bronchial hyperreactivity, develop many hours to days after allergen exposure. Therefore the asthmatic response is considered to be in large part a late-phase allergic response (49, 50).

Studies presented previously have established that gene vaccination prevents the development of a Th2-biased immune profile with allergen sensitization and the immediate-phase hypersensitivity response of anaphylaxis with challenge (13, 18). To further evaluate the capacity of gene vaccination to prevent the late-phase asthmatic response, we and others have utilized rodent models of asthma (12, 14). Our group has utilized a model of OVA-induced asthma, in which mice were sensitized with OVA in alum and then received 2 OVA inhalation challenges, 5 days apart, and were sacrificed 24 h after the last OVA challenge. These mice demonstrated eosinophilic inflammation in bronchoalveolar lavage fluid (BALF) and in the lung parenchyma. In addition, eosinophilia was noted in the bone marrow of these mice. However, mice vaccinated with an OVA expression vector (pCMV-OVA) prior to sensitization demonstrated a significant reduction in eosinophilic airway inflammation and systemic eosinophilia when compared to control mice vaccinated with a control plasmid lacking the gene insert or left unvaccinated (Table 3) (12).

Hsu and colleagues have conducted similar gene vaccination experiments in a rat model of asthma using a clinically relevant house dust mite allergen (Der p 5). They found that gene vaccination prior to sensitization inhibited Der p 5 specific IgE synthesis, histamine release in BALF with aerosolized challenge and the subsequent development of airway hyperactivity to methacholine (14).

Like gene vaccination, ISS-ODN/protein vaccination has

Table 3. Effect of gene vaccination on pulmonary eosinophilic inflammation

Priming	Sensitization	Challenge	Eosinophil counts		
			Bone marrow	BALF	Lung
Saline	+	+	10.2±1.8	39.5±5.0	40.3±15.8
OVA-pDNA	+	+	3.1±0.7	6.2±1.8	12.0±1.4
pUC19	+	+	8.4±0.5	32.2±7.2	38.5±1.7
Naïve	—	—	3.7±0.5	0±0	0±0

BALB/c mice were i.d. immunized with OVA-pDNA (50 µg) or empty plasmid cassette (pUC19) and subsequently s.c. sensitized with OVA (25 µg) and alum (1 mg). Twenty-four hours after the second of 2 nebulized OVA challenges, spaced 5 days apart, bone marrow, lung, and BALF samples were collected. Eosinophil counts were performed by a “blinded” investigator and results represent mean counts±S.E. for 8 mice per group. Five hundred white blood cells were counted in the BALF of each mouse and ten random light microscope fields of bone marrow and lung sections were analyzed for determination of the eosinophils in these tissues. OVA-pDNA vaccination inhibited eosinophil infiltration in the bone marrow, bronchial airway lavage fluid (BALF) and the lung tissue (Lung) after inhalation challenge with OVA.

also proven effective for the prevention of the asthmatic phenotype. Injection of mice with *Schistosoma* eggs alone has been shown to elicit an inherently Th2-biased immune response, with a strong late-phase eosinophilic inflammatory infiltrate in the airways and bronchial hyperreactivity after nebulized allergen challenge. Kline et al. have demonstrated that injection of ISS-ODN with *Schistosoma* eggs led to attenuation of the Th2 bias of the subsequent immune response, and a blunted asthmatic response with nebulized allergen challenge (55). These studies and others have clearly demonstrated that gene and allergen/ISS-ODN vaccinations protect rodents against the late-phase allergic hypersensitivity response which characterizes asthma.

ISS-ODN-protein conjugates

Because of the relatively low level of clinical efficacy of traditional IT, there have been efforts over the years to improve its immunogenicity (i.e. improve its potential to elicit blocking antibody and anti-allergic T-cell responses). To date, success in this area has been limited. Increasing the dose of allergen used for IT has been shown to improve its immunogenicity (7–9). However, allergic symptoms experienced by patients receiving higher doses of allergen are generally more severe than those of untreated individuals during natural exposure, and occasionally these allergic reactions are life threatening (7, 8). Because dosing is a limiting factor in the safety and efficacy of traditional allergen extracts, reducing allergenicity has also been considered in the development of better allergen IT reagents. Unfortunately, efforts to generate hypoallergenic reagents have also been disappointing. Norman & Marsh have developed materials called “allergoids”, in

which allergens are selectively denatured by formalin (56). Lee & Sehon have used polyethylene glycol conjugation to block allergenic epitopes on the IT reagents (57). These and many other IT reagents with reduced allergenic potential have been produced. However, chemical modifications of allergens to reduce allergenicity have consistently led to reagents with a marked reduction in immunogenic (and therefore therapeutic) potential (7, 8, 56, 57).

As DNA, ISS-ODN has a highly negatively charged backbone. Because of this and its anti-allergic Th1 adjuvant activity, we considered two potential consequences of physically linking ISS-ODN to allergens. The first was that the new reagent might be more immunogenic than a cocktail of allergen and ISS-ODN, as it would co-localize adjuvant and allergen in the microenvironment of responding immunocytes. The second was that the allergen-ISS-ODN conjugate (AIC) might be less allergenic than the allergen/ISS-ODN cocktail, as the oligodeoxynucleotide coating the allergen could in principle create steric and/or electrostatic blockade of IgE-binding epitopes. These considerations have prompted investigations of the immunogenicity and allergenicity of AIC in rodents, non-human primates, and humans.

Studies have recently established the improved immunogenicity of AIC vs. antigen alone or antigen mixed with ISS-ODN (58–60). In particular, we have shown that injection of Amba1, the dominant ragweed (RW) allergen, chemically linked to ISS-ODN (Amba1-ISS-conjugate) led to robust Th1-biased responses against Amba1 in mice, rabbits, and monkeys, while Amba1 alone induced a Th2-biased immune response or no immune response at all (Fig. 3 and Table 4, rabbit and monkey data not shown) (59). In addition, the Amba1-ISS-conjugate-induced immune response was significantly

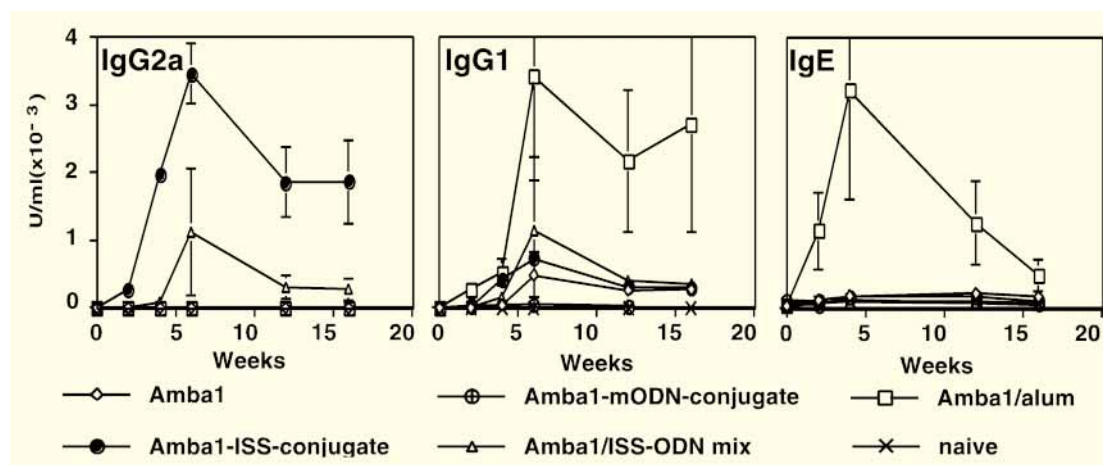


Fig. 3. Primary antibody response of Amba1-ISS-conjugate immunized mice. Female BALB/c mice were i.d. immunized with 10 μ g of Amba1, Amba1-ISS-conjugate (based on Amba1 content), or Amba1 mixed with 7.9 μ g ISS-ODN (equivalent of ISS-ODN in Amba1-ISS-conjugate) or Amba1-mODN-conjugate. Additional mice were sensitized with Amba1 (10 μ g) in alum (3 mg) i.p. to induce a strong

Th2 response. The mean $\pm$ S.E. of 4 mice per group is presented, and the results are representative of 4 experiments. By ANOVA analysis, Amba1-ISS-conjugate induced a significantly stronger IgG2a response than Amba1/ISS-ODN co-immunization ($p < 0.05$), and significantly weaker IgG1 and IgE responses than Amba1/alum sensitization ($p < 0.05$).

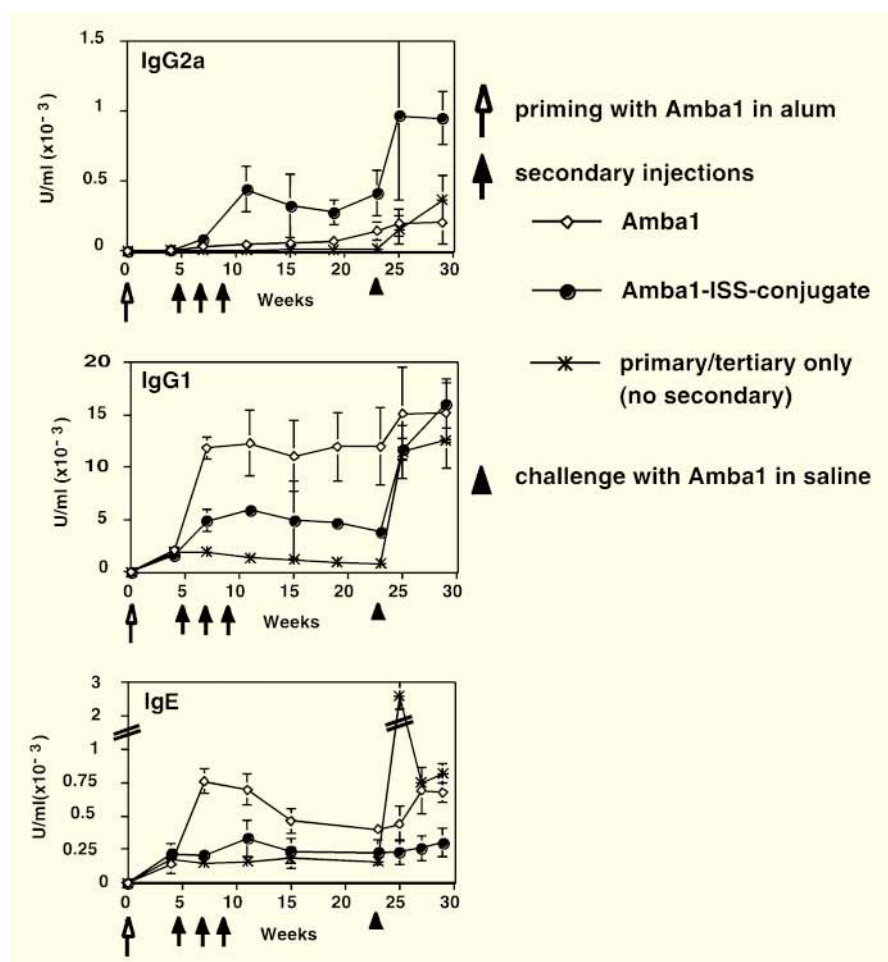


Fig. 4. Tertiary antibody response after Amba1/alum sensitization, Amba1-ISS-conjugate vaccination, and Amba1 challenge. Reagents were delivered as outlined in Fig. 3, at the time points indicated. The mean $\pm$ S.E. of 4 mice per group is presented, and the results are representative of 2 experiments. By ANOVA analysis, Amba1-ISS-conjugate-immunized mice had significantly lower IgE ($p < 0.05$) and higher IgG2a ($p < 0.05$) responses than either control group.

Table 4. Cytokine response after primary immunization with Amba1-ISS-conjugate

Vaccination	IFN- γ (pg/ml)	IL-5 (pg/ml)
None	<10	<10
Amba1	<10	630 $\pm$ 150
Amba1-ISS conjugate	8340 $\pm$ 2170*	<10*
Amba1-mODN-conjugate	<10	500 $\pm$ 150
Amba1/ISS-ODN mix	170 $\pm$ 110	610 $\pm$ 280
Amba1/alum	490 $\pm$ 240	4410 $\pm$ 880

Mice were immunized as outlined in Fig. 3 and spleens were harvested at week 16. Mean levels $\pm$ S.E. of 8 mice per group are presented. Amba1-ISS-conjugate induced a significantly stronger IFN- γ response than Amba1/ISS-ODN, and a significantly weaker IL-5 response than either Amba1/ISS-ODN mix co-immunization or Amba1/alum sensitization (* p <0.05 by ANOVA analysis).

Table 6. In vivo cytokine production induced by ISS-ODN

ISS-ODN	IL-12 (pg/ml)	IFN- γ (pg/ml)
Uninjected	<42	<14
Day (1)	4028 $\pm$ 1878	343 $\pm$ 83
Day (3)	2356 $\pm$ 464	312 $\pm$ 101
Day (7)	2034 $\pm$ 288	174 $\pm$ 36
Day (14)	763 $\pm$ 255	35 $\pm$ 71
M-ODN Day 7	<42	<14
LPS Day 7	<42	<14

At serial time points after i.d. injection of ISS-ODN (50 μ g) into BALB/c mice, serum was obtained and cytokine levels were analyzed by ELISA. Injection of LPS (10 μ g) and M-ODN (50 μ g) did not induce detectable cytokine in the serum at any of the time points. Results represent the mean $\pm$ SE for 3 mice in each time point.

Table 5. Tertiary antibody response after Amba1 in alum sensitization, Amba1-ISS-conjugate vaccination, and Amba1 in saline challenge

Priming	Secondary immunization	Sensitization	IFN- γ (pg/ml)	IL-5 (pg/ml)
None	None	None	<10	<10
Amba1/alum	Amba1	Amba1	602 $\pm$ 202	2380 $\pm$ 587*
Amba1/alum	Amba1-ISS-conjugate	Amba1	2318 $\pm$ 101*	2584 $\pm$ 506*
Amba1/alum	None	Amba1	1033 $\pm$ 133*	6276 $\pm$ 1332

Mice were immunized as outlined in Fig. 4 and spleens were harvested at week 20. Mean levels $\pm$ S.E. of 4 mice per group are presented. Secondary Amba1-ISS-conjugate immunization induced a significantly stronger IFN- γ response compared to Amba1 or no secondary immunization, and both Amba1-ISS-conjugate and Amba1 secondary immunized mice had significantly lower IL-5 responses than mice receiving no secondary immunization (* p <0.05 by ANOVA analysis).

stronger than the immune response elicited after injection of Amba1 mixed with ISS-ODN (Fig. 3 and Table 4 or plasmid encoding Amba1 (data not shown).

We have further evaluated the effectiveness of Amba1-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 Amba1. A primary Th2-biased immune profile was induced by injection of mice with Amba1 in alum. The mice then received IT with Amba1-ISS-conjugate, Amba1, or nothing. Finally, the mice were challenged with Amba1. The results of these studies clearly show that Amba1-ISS-conjugate vaccination reverses pre-existing Th2-biased immune deviation, and induces Th1-biased immunity (Fig. 4, Table 5). Furthermore, while sensitized mice receiving Amba1 IT demonstrated a continued rise in serum IgE, mice receiving Amba1-ISS-conjugate IT did not. Finally, Amba1-ISS-conjugate IT was

significantly more effective than Amba1 IT for preventing the serum IgE spike seen with the final allergen challenge.

Additional experiments from this laboratory have shown that AIC vaccination is more effective than allergen/ISS-ODN co-administration in preventing Th2-biased immune deviation and the early-phase hypersensitivity response in the model of anaphylaxis presented previously (A. Horner, unpublished observations). The effectiveness of AIC in inhibiting the late-phase asthmatic response has also recently been established by Shirota and colleagues (61). This group demonstrated that OVA-ISS-conjugate was 100-fold more efficient in preventing airway eosinophilia and bronchial hyperresponsiveness than either OVA alone or OVA mixed with ISS-ODN. 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.

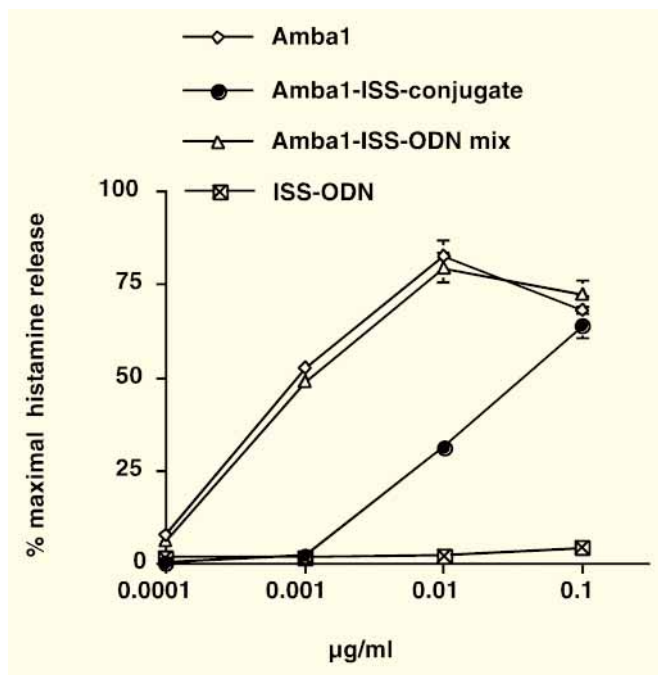


Fig. 5. Amba1-ISS-conjugate induced histamine release from the basophils of RW allergic subjects. Leukocytes from atopic donors were enriched by standard methods and incubated with Amba1, Amba1-ISS-conjugate, or Amba1 mixed with ISS-ODN (ISS-ODN added at the proportional molar dose found in Amba1-ISS-conjugate) at doses from 0.0001–0.1 µg/ml. The mean percentage of maximal histamine release $\pm$ S.E. from basophils of 4 different RW allergic donors is shown. On average, a 30-fold higher concentration of Amba1-ISS-conjugate vs. Amba1 was required to induce 50% maximal histamine release. Furthermore, the Amba1-mODN-conjugate also proved to be about 30 times less allergenic than native Amba1 (data not shown). This observation confirms that the steric hindrance and/or electrostatic blockade of oligodeoxynucleotide conjugation blocks IgE binding sites on the allergen, independent of its CpG content.

We have also evaluated the allergenicity of Amba1-ISS-conjugates. Basophils from RW allergic donors were incubated with Amba1-ISS-conjugate and native Amba1 over a range of concentrations and histamine release was determined by ELISA (59). These studies demonstrated that Amba1-ISS-conjugate elicited substantially less histamine release than native Amba1 over a range of concentrations (Fig. 5). Furthermore, the Amba1-mODN-conjugate proved to be significantly less allergenic than native Amba1 (data not shown). This observation confirms that the steric hindrance and/or electrostatic blockade of oligodeoxynucleotide conjugation blocks IgE-binding sites on the allergen, independently of its CpG content. The Amba1-ISS-conjugate has also been compared to RW extract in skin test end-point titration studies with RW allergic patients (62). In the six patients tested, Amba1-ISS-

conjugate was from 100- to 300-fold less allergenic than the RW extract presently used in clinical practice. In addition to demonstrating its decreased allergenicity, this skin testing study demonstrated that Amba1-ISS-conjugate was well tolerated, and without significant toxicity.

Allergen IT was initially described by Noon in 1911 (63). 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. These recent results suggest that AIC may be a major breakthrough for IT, 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 Amba1-ISS-conjugate IT in humans with RW allergies.

ISS-ODN as an immunomodulator in models of asthma

Since the first publication in 1997 (28), a number of laboratories have reported that ISS-ODN is a potent systemic and mucosal Th1 adjuvant (13, 36–38, 55, 59, 64, 65). In fact, ISS-ODN prepriming provides Th1 adjuvant activity for antigens encountered at the site of delivery for at least 14 days (40–42). Experiments based on the Th1 adjuvant paradigm have further demonstrated that protein/ISS-ODN immunization protects against the development of an allergic immune profile with sensitization and subsequent immediate-phase anaphylactic hypersensitivity and late-phase asthmatic hypersensitivity responses with challenge (13, 28, 55, 59). Much of the Th1 adjuvant and prepriming activities of ISS-ODN are believed to be due to its 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 anti-allergic activities in humans (A. Horner, manuscript submitted) (66, 67). 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 (Table 6) (40). Because ISS-ODN generates a long-lasting anti-allergic cytokine milieu in recipient mice, we and others 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.

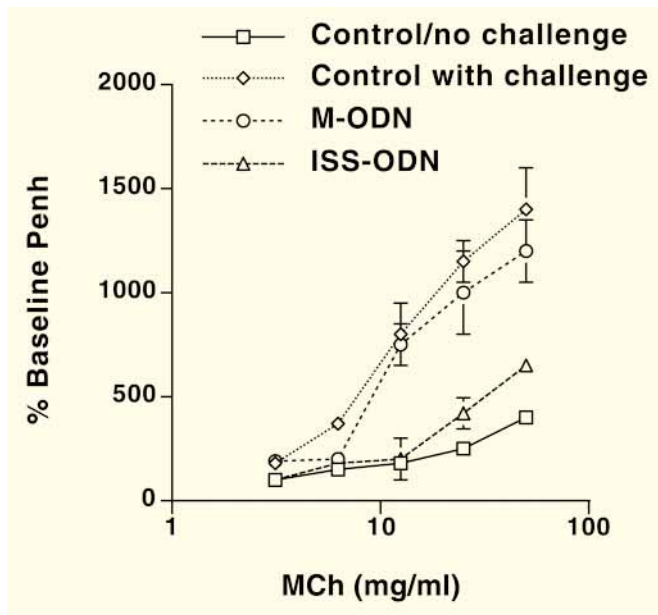


Fig. 6. The effect of ISS-ODN on methacholine (MCh) responsiveness. OVA-sensitized BALB/c mice (i.p. coinjection of 25 μ g OVA and 1 mg alum on days 0 and 10) received ISS-ODN (50 μ g) or M-ODN (50 μ g) i.p. 24 h before each of 3 daily OVA inhalation challenges, and MCh responsiveness was measured 48 h after the last challenge. Airway responsiveness to increasing concentrations of MCh (0–50 mg/ml) was assessed by barometric whole-body plethysmography, and Penh values were calculated. The mean $\pm$ S.E. for 12 mice in 3 experiments is expressed as a percentage of baseline Penh values observed after PBS exposure. Administration of ISS-ODN significantly reduced the increase in MCh responsiveness $69 \pm 9.9\%$ compared with M-ODN-treated mice ($p < 0.05$ by Tukey-Kramer HSD test).

We initially evaluated the anti-allergic immunomodulatory activity of ISS-ODN in an asthma model (68). Mice were sensitized with OVA and alum, and subsequently challenged with OVA delivered by nebulizer. Sensitized and challenged mice demonstrated eosinophilic inflammation of the airways, bronchial hyperresponsiveness, and an allergen-specific Th2-biased immune profile (i.e. high IL-5 and low IFN- γ production). However, ISS-ODN delivery either systemically (i.d.) or mucosally (mucosal data not shown) 1 day prior to nebulized allergen challenge had a dramatic inhibitory effect on these outcome parameters. ISS-ODN administration reduced methacholine sensitivity by up to 70% (Fig. 6). 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 (Figs. 7 and 8; data on blood and bone marrow not presented). 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 (Fig. 8A). Interestingly, both ISS-ODN- and corticosteroid-mediated immunomodulation also inhibited allergen-specific IL-5 production, while ISS-ODN further induced an IFN- γ response from splenocytes (Fig. 8B).

Sur and colleagues have conducted similar experiments to those outlined above, using RW (69). The results from this research group confirm that ISS-ODN delivered to sensitized mice independently of allergen provides protection against subsequent allergen challenge of the airways. In addition, this research group demonstrated that ISS-ODN immunomodulation inhibits Th2 cytokine production and the expansion of clones of allergen-specific IgE-expressing B cells. Recently, we have conducted experiments to evaluate the longevity of ISS-ODN-induced immunomodulation in our murine asthma model. Our unpublished 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. Cytokine neutralization experiments conducted by Sur and colleagues and by our own research group have further shown that ISS-ODN-mediated anti-allergic immunomodulation in these asthma models is dependent on the production of IL-12, IFN- α/β , and IFN- γ (68, 69).

Taken together, these experiments on the immunomodulatory activity of ISS-ODN suggest that if used in clinical practice, ISS-ODN 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 nasal spray or metered dose inhaler. Clearly, ISS-ODN immunomodulation represents a novel and promising approach to the treatment of allergic disease and an intellectual paradigm shift from the principles by which protein-based allergen IT is presently used for the treatment of allergic diseases.

ISS-ODN as an immunomodulator in a model of allergic conjunctivitis

Seasonal allergic conjunctivitis (SAC) is the most common allergic ocular disease (70, 71). It affects 15% of the population of the United States and contributes to up to 30% of school and workplace absences. The typical symptoms of SAC

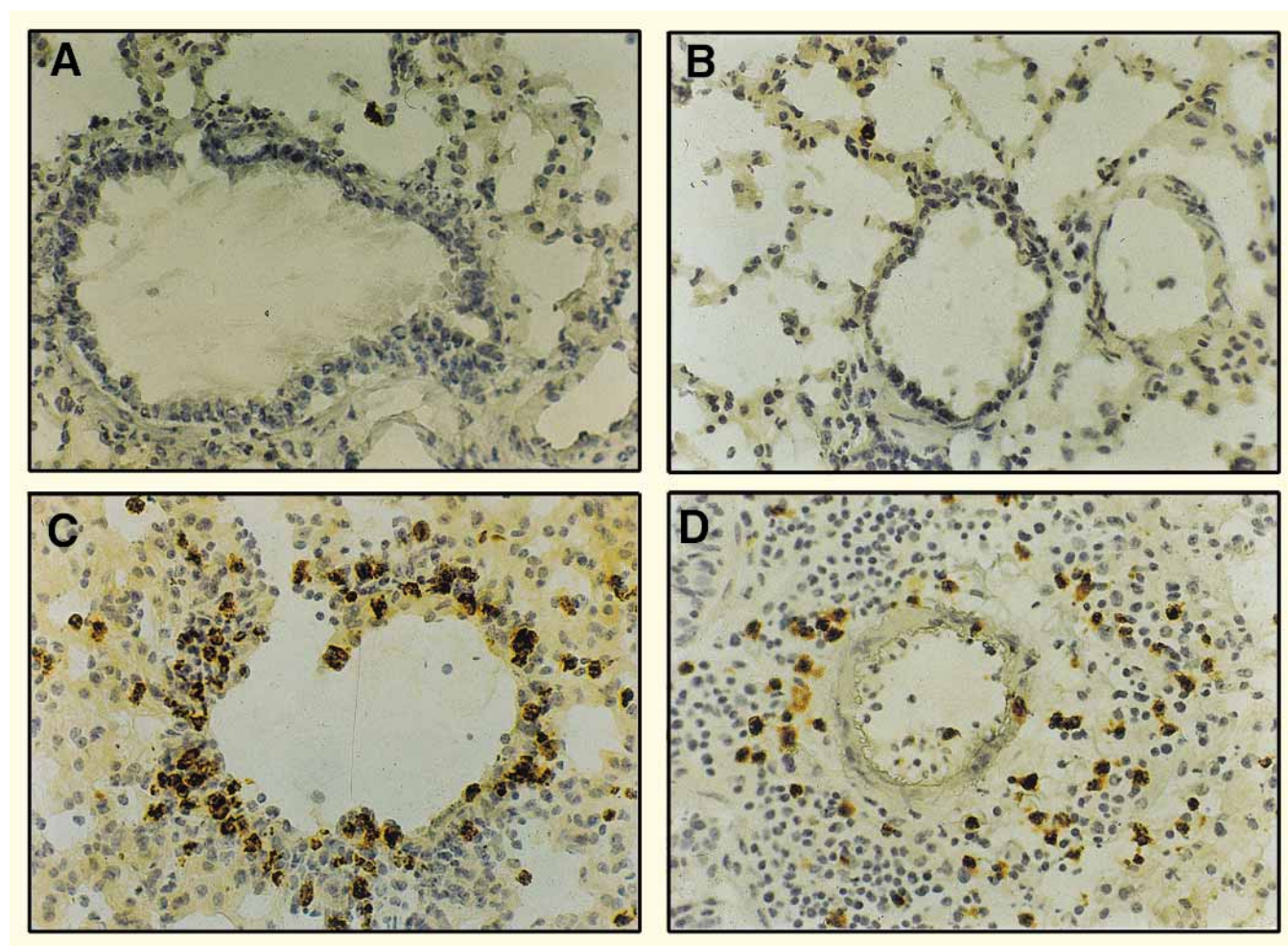


Fig. 7. The effect of ISS-ODN on eosinophilic inflammation of the lungs. OVA-sensitized BALB/c mice (s.c. coinjection of 25 μ g OVA and 1 mg alum on days 0, 7, 14, and 21) received ISS-ODN (100 μ g) or M-ODN (100 μ g) i.p. 24 h before the first of 2 OVA inhalation challenges (days 26 and 31). Lungs were cryosectioned and stained to detect eosinophil peroxidase using the peroxidase substrate DAB. Cells expressing eosinophil peroxidase develop a brown color (magnification

40 $\times$). Peribronchial pulmonary eosinophilia was inhibited significantly in ISS-ODN-treated (A) compared to M-ODN-treated mice (C). Similarly, lung parenchyma eosinophilia was reduced in ISS-ODN-treated (B) vs. M-ODN-treated mice (D). ISS-ODN also inhibited mononuclear cell inflammation (A and B) compared to M-ODN (C and D).

start within 30 min after exposure to allergen (immediate reaction) and include itching, eyelid swelling, conjunctival hyperemia, chemosis (conjunctival swelling), and mucoid discharge. These symptoms are caused principally by mast cell degranulation in response to IgE cross-linking via allergen deposited on the conjunctiva. Subsequent cellular infiltration occurs (late-phase reaction) in response to cytokine and chemokine production by local Th2 and other effector cells (70, 71).

In collaboration with the NIH/NEI, we have studied the immunomodulatory activity of ISS-ODN on early- and late-phase hypersensitivity responses in a murine model of RW

pollen-induced allergic conjunctivitis (72). 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 from 0 (no disease) to 12 (maximal disease). Mice treated systemically or topically with ISS-ODN 3 days before RW challenge had significantly lower conjunctivitis scores than M-ODN- or PBS-treated controls (Figs. 9 and 10A, data on topical application not shown). Co-delivery of ISS-ODN with anti-IL-12 mAb completely abolished the protective effects of ISS-ODN administration on the conjunctivitis score, while the corticosteroid DXM delivered 3 days before

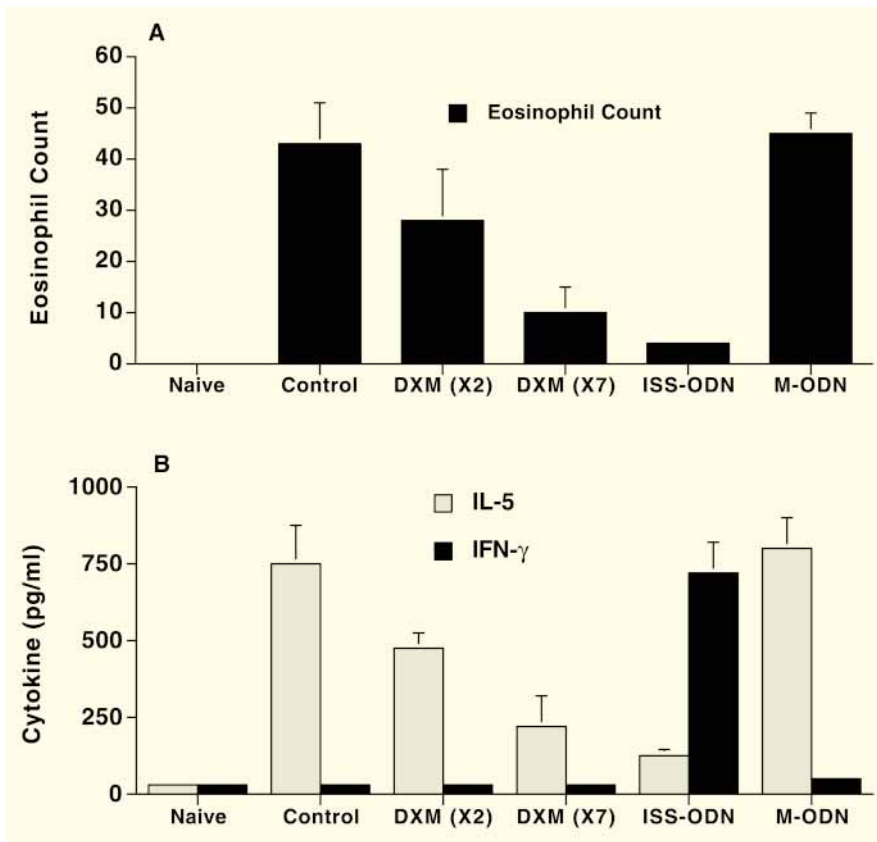


Fig. 8. The effect of ISS-ODN on BALF eosinophilia and cytokine responses.

BALB/c mice were sensitized and challenged with OVA as in Fig. 7. Some mice were given i.p. ISS-ODN (100 μ g) or M-ODN (100 μ g) as outlined in Fig. 7. Other mice received i.p. DXM (5 mg/kg/dose) on 2 or 7 consecutive days beginning the day before the first of the 2 OVA inhalation challenges. Means $\pm$ S.E. from 8 mice per group are presented in the bar graphs. p-values were determined by Student's t-tests (*represents a $p < 0.05$ versus the control group). **A) BALF eosinophilia.** Mice were sacrificed 24 h after the last OVA inhalation challenge, and the lungs were lavaged with 600 μ l of PBS. BALF samples were cytospun onto microscope slides and stained as outlined in Fig. 7. Results represent eosinophil percentages as a fraction of total nucleated cells. **B) Splenocyte cytokine production.** Splenocytes were isolated from mice 24 h after the last OVA inhalation challenge, and cultured at 5×10^6 cells/ml with or without OVA (10 μ g/ml). To determine OVA-specific cytokine responses, background cytokine levels were subtracted from levels generated in the presence of OVA.

allergen challenge also inhibited the immediate hypersensitivity response in this allergic conjunctivitis model.

Eyes were collected 24 h after topical RW challenge, and late-phase cellular inflammation of the conjunctiva was evaluated by histology. Due to differences in mouse-strain reactivities, neutrophils and not eosinophils are the predominant inflammatory cells in this model. Nonetheless, 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 control mice (Fig. 10B). 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 anti-allergic activity of ISS-ODN on late-phase conjunctival inflammation.

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 injected with M-ODN or DXM (Fig. 11A). This ISS-ODN-induced antigen-specific IFN- γ response was completely inhibited by co-delivery with IL-12 mAb. In a separate series of experiments RW-sensitized and ISS-ODN- or M-ODN-treated mice were chal-

lenged on day 10 and rechallenged on day 17 with RW. ISS-ODN treatment significantly inhibited the post-challenge RW specific IgE response when compared to M-ODN-treated mice (Fig. 11B).

In summary, our data indicate that ISS-ODN has anti-inflammatory effects on both the immediate and late-phase hypersensitivity responses phases in this model of allergic conjunctivitis. In contrast, dexamethasone primarily inhibited the immediate hypersensitivity response. Consistent with the anti-allergic immunomodulatory activity of ISS-ODN in asthma models, IL-12 is shown to play an important role in mediating the immunomodulatory activity of ISS-ODN in this conjunctivitis model (72).

Discussion

DNA-based therapeutics have shown great promise in rodent models of allergic disease. Presently, four basic DNA-based strategies are being utilized in the modulation of allergic hypersensitivities. 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 prevent-

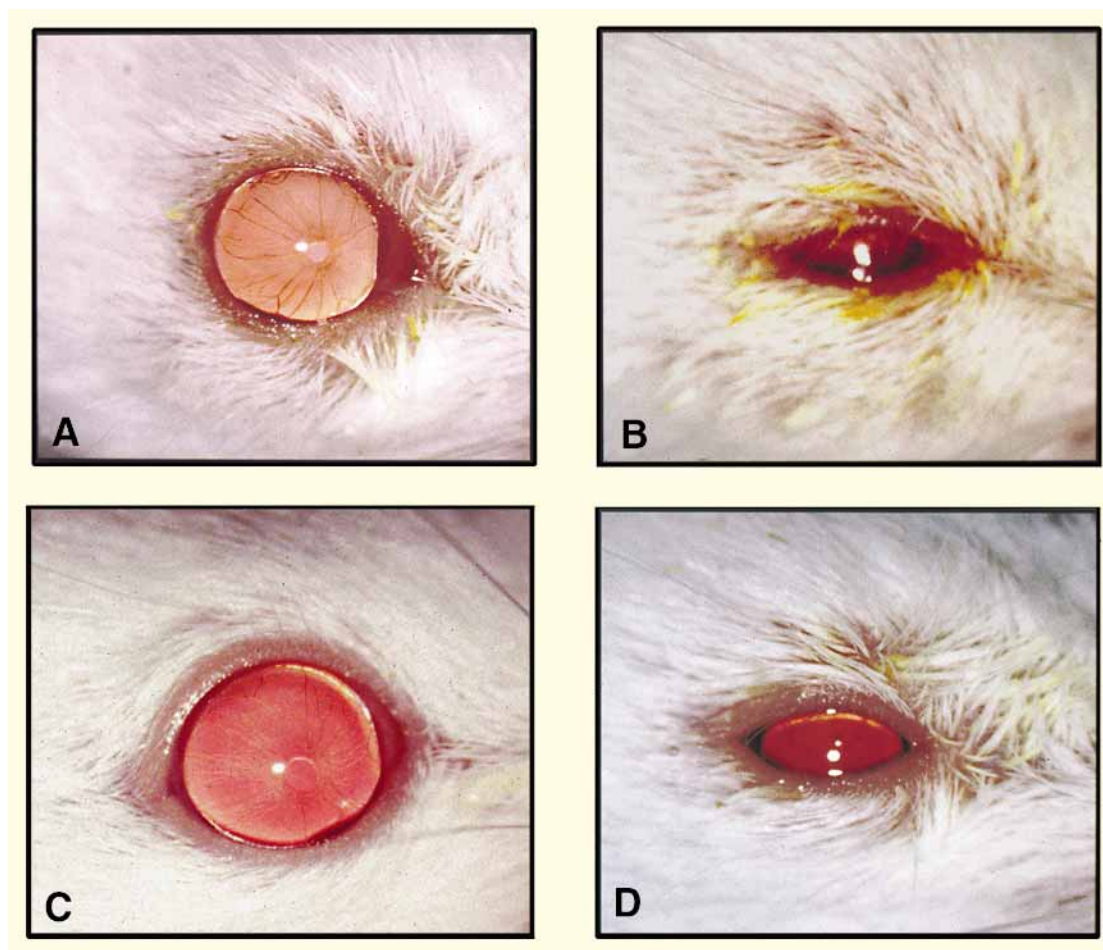


Fig. 9. External appearance of mice 20 min after topical RW challenge. SWR/J mice were sensitized with RW (50 μ g) mixed with alum (5 ng) injected into a footpad on day 0. On day 10, conjunctivitis was induced by topical application of 2 mg RW into each eye. Three days prior to challenge, selected mice received i.p. injections of ISS-ODN

(100 μ g), M-ODN (100 μ g) or DXM (5 mg/kg). An additional group received ISS-ODN on day 7 followed by IL-12 mAb (200 μ g) 15 h before challenge. The observed yellow pigment results from the mixture of ocular discharge and RW pollen. **A)** ISS-ODN, **B)** M-ODN, **C)** DXM, **D)** ISS-ODN plus IL-12 mAb.

ing and reversing underlying allergic sensitivities, these DNA-based vaccination strategies have been further shown to attenuate the immediate hypersensitivity response of anaphylaxis, and the late-phase allergic response of asthma (12–14, 18, 28, 55). Recently, physical conjugates of allergen and ISS-ODN (AIC) have been developed. AIC have proven even more immunogenic than allergen gene expression plasmids and 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 (58–62). 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 anti-allergic bioactivity of DNA-based reagents has been well char-

acterized 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 1 year or more after immunization (11, 58, 59) (and unpublished observations).

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 (68, 69, 72). In fact, ISS-ODN has proven to be as effective an immunomodulator as corticosteroids in the prevention of the immediate-phase hypersensitivity response of allergic conjunctivitis, and more effective than corticosteroids in the prevention of the late-phase inflammatory responses seen in murine allergic

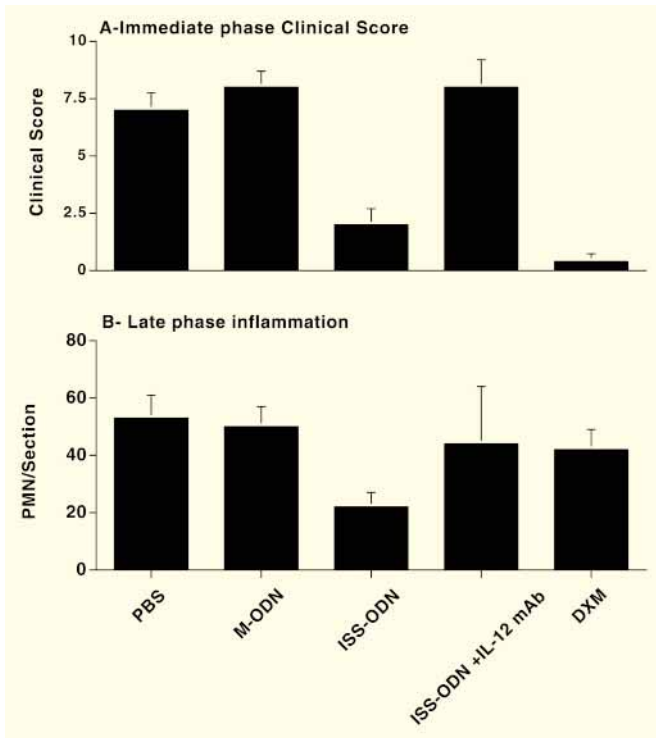


Fig. 10. Immediate hypersensitivity scores, and late-phase PMN inflammation. Mice were RW sensitized. They then received ISS-ODN, M-ODN, DXM, or ISS-ODN + IL-12 mAb prior to RW challenge, as outlined in Fig. 9. **A) Immediate hypersensitivity scores.** Clinical scores were determined 20 min post-challenge (range 0–12). Bar graph results represent mean scores $\pm$ S.E. from 5 or more mice per group. A p -value of ≤ 0.0006 was determined for ISS-ODN- and DXM- vs. M-ODN- and PBS-treated mice by studies of central tendency in a non-Gaussian distribution. Interleukin-12 mAb neutralized the effect of ISS-ODN on the immediate hypersensitivity response. **B) Late-phase PMN infiltration.** Eyes were enucleated 24 h after RW challenge, fixed, and then stained with Giemsa, hematoxylin, eosin, and periodic acid Schiff. Results were determined by a “blinded” ocular pathologist. ISS-ODN-treated mice demonstrated significantly lower PMN inflammation of the conjunctiva than M-ODN- and PBS-treated mice ($p \leq 0.0003$ by ANOVA analysis). DXM treatment did not reduce late-phase conjunctival inflammation, while IL-12 mAb neutralized the effect of ISS-ODN on the PMN infiltrate. Bar graph results represent mean PMNs/section $\pm$ S.E. from 5 or more mice per group.

conjunctivitis and asthma models. 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.

In further consideration of the dichotomy between DNA-based vaccination and ISS-ODN-mediated immunomodulation, we have found that in contrast to the long-standing and antigen-specific immune responses elicited with DNA-

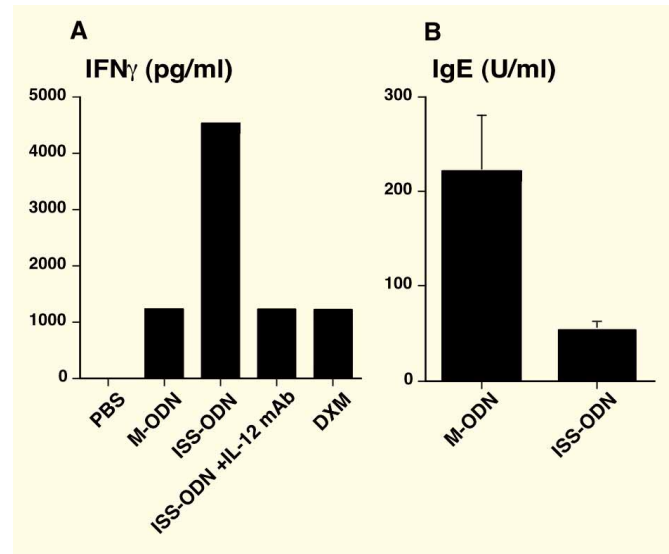


Fig. 11. IFN- γ and IgE production from ISS-ODN-treated mice. A) IFN- γ response. Mice were RW sensitized. They then received ISS-ODN, M-ODN, DXM, or ISS-ODN + IL-12 mAb prior to RW challenge, as outlined in Fig. 9. Lymphocytes were harvested from popliteal lymph nodes draining the site of RW sensitization on day 11, pooled and cultured with RW extract or in media alone. **B) IgE.** Mice were treated as in 11A but instead of sacrifice on day 11, the mice were rechallenged with RW on day 17, and serum was collected on day 18. RW specific IgE was quantitated by standard ELISA protocols. Results represent mean RW specific IgE levels $\pm$ S.E. from 10 mice per group. ISS-ODN inhibited IgE production significantly compared to M-ODN treatment ($p < 0.05$ by Student's t -test).

based vaccination, allergen non-specific ISS-ODN-induced immunomodulation is relatively short lived. In a series of unpublished studies we have found that a single delivery of ISS-ODN shortly before a primary allergen challenge leads 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 are 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 are similar to those of mice that did not receive ISS-ODN.

The principles of vaccination and immunomodulation, as discussed in this review, represent two distinct yet interrelated strategies for the utilization of DNA-based reagents in the treatment of allergic diseases (Fig. 12). With DNA-based vaccination, a long-lasting Th1-biased adaptive immune response develops. 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

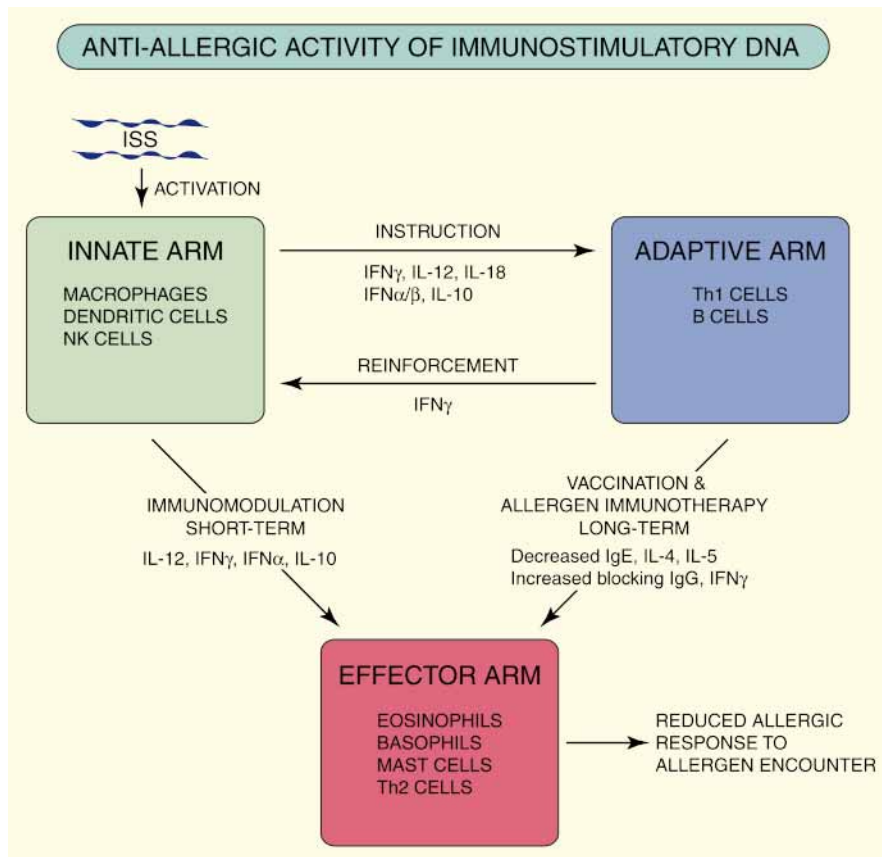


Fig. 12. Simplified schema for the anti-allergic activities of immunostimulatory DNA sequences (ISS). Cryptic immunostimulatory DNA contained in allergen-gene vaccination plasmids and synthetic ISS-ODN directly activate cells of the innate arm of the immune system to produce a number of anti-allergic and Th1-promoting cytokines. This acute burst of cytokines directly inhibits the effector arm of the allergic hypersensitivity response, including the response of precommitted Th2 cells. In addition, ISS-induced innate immune activation promotes the development of Th1- and inhibits Th2-biased adaptive responses to allergens. This Th1-biased adaptive response reinforces the activities of innate immunocytes, while mediating long-lasting inhibition of the effector arm of the allergic hypersensitivity response. Thus, ISS activation of two separate but complementary arms of the immune response (innate and adaptive) has the potential to provide both rapid and long-lasting inhibition of the allergic response.

for an extended period of time. In contrast, ISS-ODN-induced immunomodulation appears to depend on the anti-allergic innate cytokine response which is generated by immunocytes upon exposure to ISS-ODN. Our unpublished observations suggest that ISS-ODN-induced cytokine production attenuates hypersensitivity responses in previously sensitized mice upon challenge for a limited period of time. 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. This schema for the interrelationship between the ISS-ODN effect on innate and adaptive immunity in the context of allergic disease is presented in Fig. 12.

Conclusions

Over the past decade, a growing body of experimental evidence suggests that DNA-based immunotherapeutics may be effective in the treatment of allergic diseases and the reversal

of the allergen-specific hypersensitivities which mediate them. DNA-based reagents have proven effective in the prevention and treatment of allergic diseases when used according to the principles of immunization. ISS-ODN has further proven to have utility as an immunomodulator when used independently of allergen, in much the same way that corticosteroids are presently used to treat allergic diseases. While their effects on immediate and late-phase allergic hypersensitivity responses appear similar, DNA-based vaccination appears to have a much longer effect on adaptive immunity than ISS-ODN-induced immunomodulation. However, if utilized in clinical practice, ISS-ODN-induced immunomodulation has a practical advantage over DNA-based vaccination, as it does not require that a patient's specific allergic hypersensitivities be identified. In the years to come, a number of clinical trials evaluating the safety and efficacy of DNA-based immunotherapeutics for the treatment of allergic diseases are anticipated, and some are already under way. If these reagents prove as effective in humans as they have proved to be in rodents and non-human primates, then DNA-based immunotherapeutics are likely to revolutionize the standard of care for the treatment of allergic diseases.

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