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Antigen–immunostimulatory oligonucleotide conjugates: mechanisms and applications

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Summary: Conjugation of protein antigen with immunostimulatory oligonucleotides creates a potent immunogen. Physical linking of oligonucleotides to antigen enhances antigen uptake and targets the adjuvant properties of the oligonucleotides to the antigen-presenting cell. In addition, the conjugated oligonucleotides appear to have improved immunostimulatory abilities compared to free oligonucleotides, presumably due to enhanced activation of Toll-like receptor 9. Immunization with these conjugate preparations elicits antigen-specific antibody responses, a T-helper cell 1-biased cytokine profile from CD4 T cells, and CD8 cytotoxic T-lymphocyte activity that is CD4 independent. The humoral and cellular immune responses induced by these conjugates suggest they can be used to create effective vaccines against infectious pathogens and tumors and to beneficially modulate allergic responses. Indeed, recent clinical trial data show symptom relief and immunomodulation of the allergic response in patients with allergic rhinitis. This review considers the mechanisms of action of antigen–oligonucleotide conjugates and discusses available data regarding their use for the prevention and treatment of infectious, oncologic, and allergic diseases.

Introduction

The discovery that mycobacterial DNA is largely responsible for the anti-tumor activities of *Mycobacterium bovis* bacille Calmette–Guérin (BCG) (1, 2) has stimulated significant research on the immunostimulatory properties of bacterial DNA. This research has led to the identification of immunostimulatory sequences (ISSs) in bacterial DNA that are structurally defined by their content of unmethylated CpG motifs (5'-purine-purine/T-CpG-pyrimidine-pyrimidine-3') (3) and are underrepresented in mammalian DNA (4). ISS-containing oligodeoxyribonucleotides (ISS-ODNs) induce natural killer (NK) cells to secrete interferon- γ (IFN- γ) (5), stimulate B-cell proliferation and immunoglobulin M (IgM) secretion (3), and induce costimulatory molecule expression and cytokine secretion from antigen-presenting cells (APCs) that shape a T-helper cell 1 (Th1)-biased adaptive immune response (6–8).

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The ability of ISS-ODNs to prime Th1-biased CD4 T-cell responses (8) and cross-prime CD8 cytotoxic T-lymphocyte (CTL) responses against codelivered soluble antigen (9–13) holds important therapeutic implications for modulating immune responses in infectious, oncologic, and allergic diseases. However, ISS-ODNs do not act directly on T cells. Instead, ISS-ODNs directly activate cells of the innate immune system, such as dendritic cells (DCs) and other APCs, through Toll-like receptor 9 (TLR9) (14). The activated APCs then express a unique cytokine and costimulatory molecule profile that shape the subsequent adaptive immune responses described above.

Initial studies of ISS-ODNs as a therapeutic agent in animals and humans suggest a favorable safety profile (15). However, the potential for ISS-ODNs to induce local inflammation, systemic septic shock-like symptoms, and autoimmune disease remains a concern (15). To avoid these potential adverse reactions, it is desirable to minimize the dose of ISS-ODNs needed to achieve therapeutic effects. Multiple studies have now shown that covalently conjugating ISS-ODNs to antigen creates a more potent immunogen than mixtures of antigen with an amount of ISS-ODNs equivalent to that in the conjugates (11, 12, 16, 17), thereby achieving therapeutic effects at smaller doses. In this review, data from our laboratory regarding the use of antigen–ISS-ODN conjugates (AICs) are summarized, and their therapeutic implications for the treatment of oncologic, infectious, and allergic diseases are discussed.

Chemical synthesis of AICs

For most studies involving AICs, the protein antigen was covalently linked to ISS-ODNs by mixing maleimide-activated proteins with thiolated ISS-ODNs (18). Maleimide groups are incorporated onto the protein by incubation of the protein with excess sulfosuccinimidyl-4-[N-maleimidomethyl]-cyclohexane-1-carboxylate. A 5'-disulfide group on the ODN is reduced by incubation with tricarboxyethylphosphine.

Incubation of the maleimide-modified protein with the reduced disulfide-modified ODN results in conjugation of protein to the ODN through covalent maleimide-sulfur thioether linkages (Fig. 1A). Fast protein liquid chromatography can then be used to isolate protein with various numbers of conjugated ODN molecules. Conjugation has also been done with thiolated proteins and maleimide-activated ISS-ODNs (19), or by physically linking biotinylated protein and biotinylated ISS-ODNs with polyvalent avidin (20). The conjugation process in these various studies yielded average protein to

ISS-ODN ratios ranging between 1:1 and 1:8, and similar immunomodulatory effects were obtained regardless of the ratio. When compared with the native protein by sodium dodecyl sulfate polyacrylamide gel electrophoresis, the conjugates are visualized as higher molecular weight bands that also stain for DNA (Fig. 1B).

AICs: mechanisms of action

Initial reports documenting the enhanced potency of AICs compared to mixtures of antigen with ISS-ODNs hypothesized that this potency was attributable to the forced colocalization of antigen and ISS-ODN to the same APC (11). Administration of a mixture of unconjugated protein antigen and ISS-ODN results in the following inefficiency: a portion of the ISS-ODN is taken up by cells that do not come in contact with antigen, and a portion of the antigen is taken up by cells that do not come in contact with ISS-ODN. The colocalization forced by chemical conjugation should result in any APC that processes the antigen to also incur the adjuvant effects of ISS-ODN, resulting in enhanced T-cell-stimulatory abilities. Indeed, *in vitro* studies have shown that DCs that ingest AICs are the ones that upregulate costimulatory molecules and cytokine expression (21, S.K. Datta and E. Raz, unpublished data).

In addition to targeting antigen and adjuvant to the same cell, the presence of ODN enhances uptake of the conjugated antigen. Experiments comparing uptake of conjugated and unconjugated ovalbumin (OVA) showed that OVA conjugated to ODN was taken up by DCs more efficiently than free OVA or OVA mixed with ODN (Fig. 2A). The enhanced uptake of AICs did not depend on the presence of CpG motifs within the ODNs (data not shown). AICs not only improved the uptake of antigen but also enhanced the immunostimulatory activity of the ISS-ODNs. DCs incubated with AICs secreted higher levels of interleukin-12 (IL-12) than that of DCs incubated with similar concentrations of free ISS-ODNs (Fig. 2B). Presumably, the protein acts as a scaffold for the ODN molecules that are conjugated to it, allowing for better interaction and cross-linking of TLR9 with the ISS-ODN.

In the above experiments, the enhanced uptake of AICs by DCs led to presentation of antigen on major histocompatibility complex (MHC) class II (Fig. 2C), as measured by proliferation of OVA-specific CD4 T cells from OT-II mice (13). Presentation of native OVA on MHC class II occurred if higher concentrations of OVA were used (data not shown). Cross-presentation, the mechanism by which exogenous antigens are presented on MHC class I, also occurred (Fig. 2C), as measured by proliferation of OVA-specific CD8 T cells from OT-I mice (13). Like

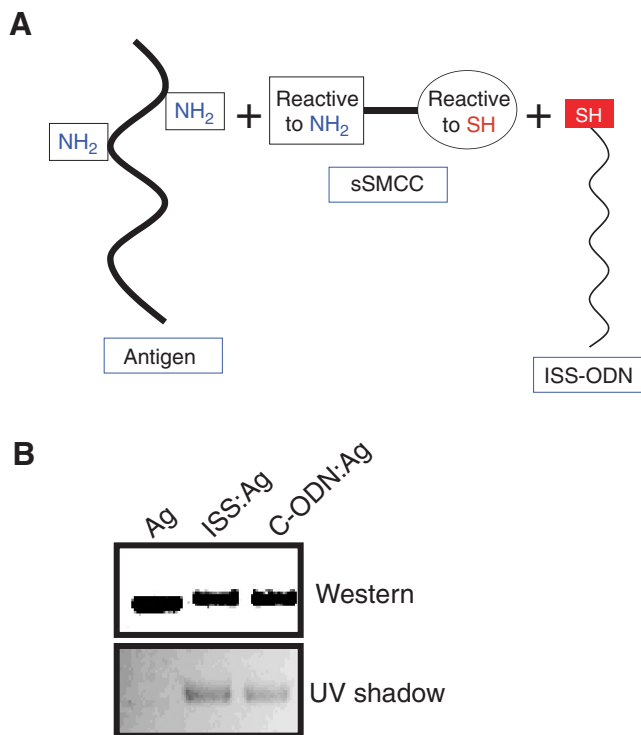


Fig. 1. Synthesis of antigen-ISS-ODN conjugates (AICs). (A) AICs are synthesized by using sulfosuccinimidyl-4-[N-maleimidomethyl]-cyclohexane-1-carboxylate (sSMCC) to chemically cross-link NH_2 groups on protein antigen with sulfhydryl (SH) groups on ODNs. (B) Visualization of antigen (Ag), ISS-ODN:Ag conjugate (ISS:Ag), and control ODN:Ag conjugate (C-ODN:Ag) by Western blot after sodium dodecyl sulfate polyacrylamide gel electrophoresis shows higher molecular weight bands for the conjugates compared to native protein. The ODNs contained in the conjugates are also visible by ultraviolet (UV) shadowing of the gel.

antigen uptake, antigen presentation was enhanced by conjugated ODN in a largely sequence nonspecific manner. However, the *in vivo* development of T-cell immunity after AIC immunization required the presence of CpG motifs (11, 12). This reflects the necessity for TLR9-mediated adjuvant effects of CpG motifs *in vivo*, whereas the *in vitro* proliferation of T cells primarily reflects antigen presentation and is largely independent of adjuvant effects such as costimulatory molecule induction. Studies from other groups have reported similar findings (21–23).

AICs: applications for cancer

Considering that the discovery of immunostimulatory DNA arose from investigation of the anti-tumor properties of *M. bovis* BCG, it seems appropriate to review the role of AICs in cancer immunotherapy. The concept of mobilizing immune responses against tumors through the use of vaccines or other immune stimulants dates back to the late nineteenth

and early twentieth centuries when it was observed that streptococcal infections appeared to induce regression and in some cases complete remission of tumors. Several physicians and scientists, notably William Coley, spent decades attempting to harness the so-called ‘bacterial toxins’ as immune stimulants for cancer (24, 25). These efforts continued through the twentieth century; however, with the exception of BCG preventing second primary tumors in superficial bladder cancer (26), most of these efforts failed to demonstrate reproducible results against tumors. In retrospect, it is likely that the initial anti-tumor effects seen with infectious agents were actually due to the effects of cytokine production in response to the bacterial toxins. Recently, key discoveries in the regulation of adaptive immunity, particularly the pivotal role of DCs in priming antigen-specific T-cell activity (27, 28), have revitalized the drive to develop antigen-specific vaccines for cancer immunotherapy.

AIC vaccines act upon DCs, largely by type I IFN-dependent mechanisms, to promote transporter associated with antigen presentation-mediated cross-presentation of exogenous antigens on MHC class I and to upregulate expression of surface costimulatory molecules (29). As a result, these vaccines can efficiently prime CTL activity independent of CD4 and/or MHC class II-restricted T-cell help (Fig. 3) (11). By regulating both signal 1 (antigen in the context of MHC class I) and signal 2 (costimulatory molecules), AIC vaccines effectively replace the CD40/CD40 ligand-dependent ‘licensing’ interaction between DCs and T-helper cells that is usually needed for CTL activation (30–32).

The efficient priming of CTL activity by AIC vaccines, even under conditions where T-cell help is deficient or absent, makes them a promising strategy for cancer immunotherapy. It was our hypothesis that AIC vaccines could stimulate protective anti-tumor CTL activity. We investigated this hypothesis in both a preventive and therapeutic experimental model of cancer (11).

In the preventive model, wildtype mice received two vaccinations with OVA AICs or other OVA-based vaccines at 2-week intervals and then received a lethal tumor challenge with EG7-OVA, a syngeneic murine thymoma that stably expressed OVA as an artificial tumor-specific antigen. As shown in Fig. 4(A), mice vaccinated with AIC were able to restrict the growth of EG7-OVA, whereas other vaccine preparations conferred moderate or no resistance to tumor growth.

In the therapeutic model, test animals received lethal tumor challenge and were then immunized with OVA AIC vaccines on days 0, 3, and 7 (early treatment) or days 3, 7, and 10 (late treatment) (Fig. 4B). In both treatment protocols, mice were

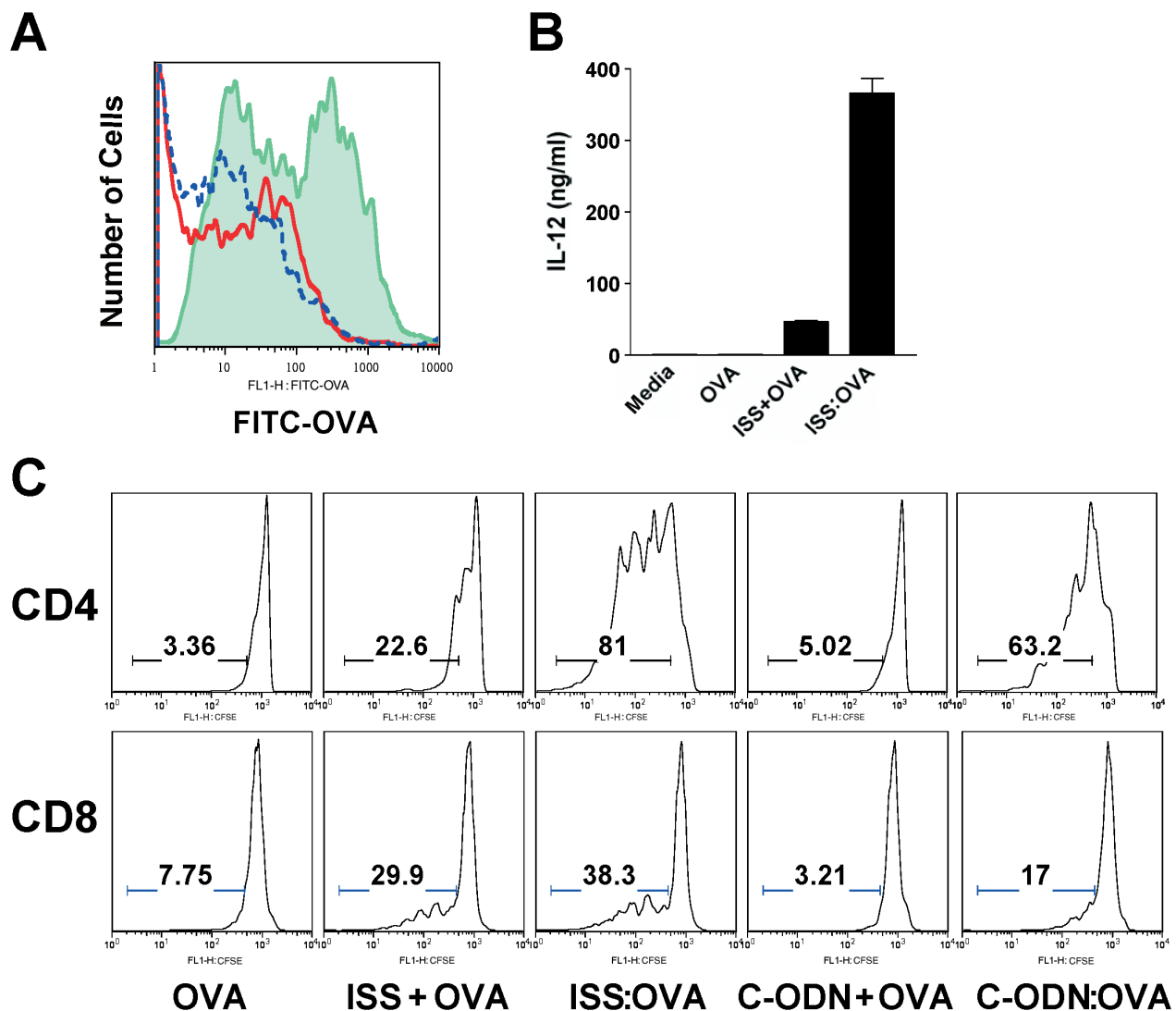


Fig. 2. Conjugation leads to enhanced antigen uptake and immunostimulatory activity. (A) Bone marrow-derived dendritic cells (DCs) were incubated for 2 h with 10 μ g/ml of fluorescein isothiocyanate (FITC)-labeled ovalbumin (OVA) (Molecular Probes) alone (dashed histogram), a mixture of 5 μ g/ml of immunostimulatory sequence oligodeoxyribonucleotide (ISS-ODN) and 10 μ g/ml of FITC-OVA (open histogram), or ISS-ODN:FITC-OVA conjugate (filled histogram; 10 μ g/ml based on OVA content, corresponding to approximately 5 μ g/ml of ISS-ODN). The cells were then washed and analyzed by flow cytometry for OVA uptake. (B) DCs were incubated overnight with media, OVA, a mixture of ISS-ODN and OVA (ISS + OVA), or ISS:OVA conjugate at the concentrations described above, and the supernatant was then assessed for interleukin-12 content by routine enzyme-linked immunosorbent assay (BD PharMingen,

San Diego, CA, USA). (C) DCs were incubated overnight with OVA, ISS + OVA, ISS:OVA conjugate, C-ODN + OVA, or C-ODN:OVA conjugate. For all groups, OVA was used at 10 μ g/ml and 0.1 μ g/ml for the CD4 and CD8 assays, respectively. In the conjugate preparations, the concentration of ODN was approximately half the OVA concentration, and the corresponding concentration of free ODN was used in these assays. The DC were then washed and incubated for 3 days with carboxyfluorescein diacetate succinimidyl ester (CFSE)-labeled OVA-specific CD4 cells from OT-II mice (CD4) or OVA-specific CD8 cells from OT-I mice (CD8) as described in Datta et al. (13). T-cell proliferation, indicated by decreased CFSE fluorescence, was then assessed by flow cytometry. The percentage of cells undergoing at least one round of proliferation is noted within each histogram. The data shown is representative of at least three independent experiments.

able to restrict growth of tumor. In the late-treatment group, initial tumor growth was followed by regression, suggesting that even an established tumor could be eradicated by the developing immune response. Similar experiments with gene-deficient mice demonstrated that CD8 T cells were required to restrict tumor growth, whereas CD4 T cells were not (11). Thus, CD8 CTL activity, which was efficiently

primed in a CD4-independent fashion by AIC vaccines, was responsible for anti-tumor immunity in this model.

Humans and mice differ in their DC subsets. Murine plasmacytoid and myeloid DCs express both TLR3 (double-stranded RNA receptor) and TLR9 (ISS-DNA receptor) and mature in response to either ligand (33). In contrast, human plasmacytoid DCs express TLR9 but not TLR3, whereas the converse is

true in human myeloid DCs. Human plasmacytoid dendritic cells upregulate surface costimulatory molecules and secrete IFN- α when stimulated with ISS-ODN (34, H.J. Cho, unpublished data). Human plasmacytoid DCs also upregulate CCR7, a chemokine receptor that is important for homing to secondary lymphoid tissue, when stimulated with ISS-ODNs (H.J. Cho, unpublished data). Interestingly, unlike their murine counterparts, human plasmacytoid DCs do not upregulate CD40 when stimulated with ISS-ODNs (H.J. Cho, unpublished data). The T-helper-independent priming of CTLs by AIC vaccines in mice predicts that this will not affect CTL priming in the human system.

Translation of AIC vaccines to human cancers is dependent upon the identification of target antigens. Ideally, these target antigens should be exclusively expressed on tumor cells. Monoclonal idiotypes expressed by B-cell lymphomas (35, 36) and the cancer-testis family of tumor-associated antigens (37) are examples of tumor antigens that can be targeted by cancer vaccines. Formulation of AIC vaccines with such antigens should elicit a polyclonal CTL response against the target protein, even in the setting of relative immunosuppression often seen in cancer patients, evoking hope as a promising new strategy for cancer immunotherapy.

AICs: applications for infectious diseases

The majority of currently licensed vaccines against infectious agents are protein-based vaccines that use alum as an adjuvant. These vaccine formulations induce an antibody response but are unable to induce cellular immunity, a crucial requirement for control of intracellular pathogens such as *Mycobacterium tuberculosis* and human immunodeficiency virus (HIV).

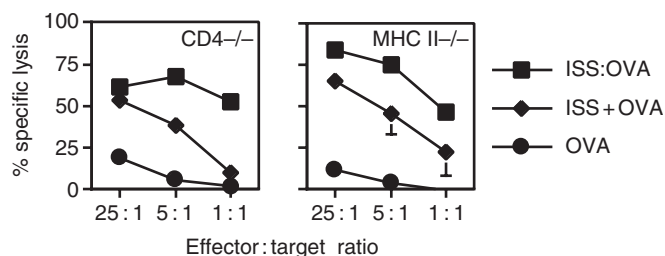


Fig. 3. Antigen-immunostimulatory sequence oligodeoxyribonucleotide conjugates (AICs) primes antigen-specific cytotoxic T lymphocytes (CTLs) independent of major histocompatibility complex (MHC) class II-restricted T-cell help. Mice were immunized twice with ISS:OVA, ISS + OVA, or OVA alone, and OVA-specific CTL activity from total splenocytes was assayed at 6 weeks as described in Cho et al. (11). Results are shown for CD4^{-/-} mice and MHC class II^{-/-} mice. Error bars indicate standard error of the mean.

Currently, the only vaccines that effectively induce cellular immunity are live attenuated vaccines. Although live vaccines are effective, development of live vaccines against most pathogens is limited by their potential for causing disease.

The ability of ISS-ODNs to elicit antigen-specific cellular and humoral immune responses when administered with a protein antigen suggests it can function as a potent vaccine adjuvant. Preliminary trials in humans using ISS-ODNs as an adjuvant with currently approved hepatitis B vaccine preparations show improved antibody responses (38). The improved potency of AICs when compared with antigen-ISS-ODNs mixtures, as discussed above, suggests AIC vaccines will have further improved efficacy with less potential for toxicity.

The potent and wide-ranging immune responses that can be elicited with an AIC vaccine have been demonstrated in a murine study using the HIV gp120 protein as an antigen (12). In this study, the AIC vaccine induced high titers of gp120-specific antibody (IgG and IgG2a) (Table 1). Previous studies have shown the ability of ISS-based vaccines to elicit neutralizing antibodies against gp120 (16), although the clinical efficacy of these antibodies against natural HIV infection remains to be seen, and targeting of other HIV antigens to effectively neutralize this elusive virus will likely be needed. The gp120 AIC vaccine also elicited high levels of antigen-specific IFN- γ (Table 1), a hallmark of Th1-biased immunity that contributes to protection against many viral infections (39, 40). Furthermore, secretion of CCR5-specific β -chemokines, which may inhibit the intercellular spread of HIV and the natural progression of the infection to acquired immunodeficiency disease by competitively inhibiting use of CCR5 as a coreceptor by the virus (41), was induced (Table 1).

In addition to development of the above CD4 T-cell responses, CD8 T cells also secreted these cytokines and developed CTL activity after immunization (Table 2). Because HIV is transmitted across mucosal barriers and depletes CD4 T cells, it is important to note that the antibody, CD4, and CD8 responses occurred at systemic and mucosal sites after mucosal vaccination, and CD8 responses were induced even in the absence of CD4 T cells (Table 2). These findings suggest a role for AIC vaccines in both preventive and therapeutic interventions against HIV.

In summary, a number of desirable immune responses were elicited with the gp120 AIC vaccine: (i) antigen-specific antibody responses, (ii) Th1-biased T-helper cell response, (iii) secretion of β -chemokines, (iv) CTL responses, (v) mucosal immunity, and (vi) efficacy in the absence of CD4 T cells. The AIC vaccine showed improved responses compared to immunization with similar or higher doses of ISS-ODN given as a

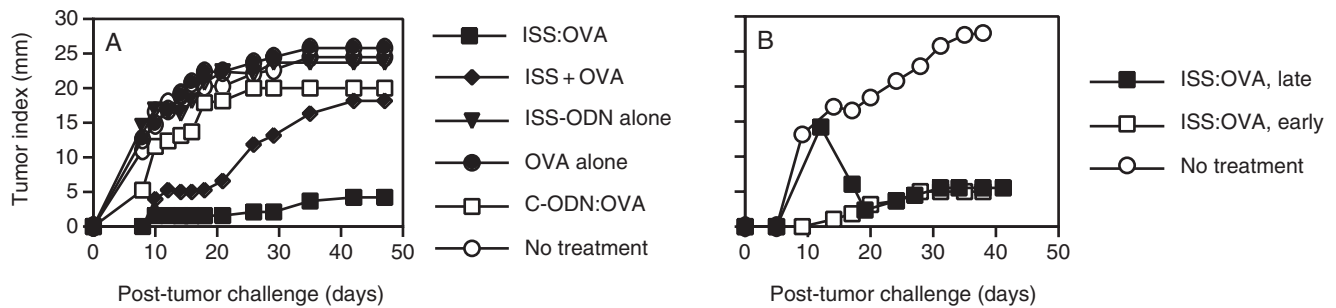


Fig. 4. Antigen-immunostimulatory sequence oligodeoxyribonucleotide conjugates (AICs) stimulate protective, antigen-specific immunity in an animal model of cancer. (A) Preventive model. Wildtype mice were immunized twice with the indicated vaccine preparations. The mice then received a lethal tumor challenge with EG7-OVA, a syngeneic thymoma cell line stably transfected to express OVA.

Tumor growth was then followed for 6 weeks. Mice immunized with OVA AIC were able to restrict tumor growth. (B) Therapeutic model. Wildtype mice were immunized with OVA AIC as described in the text and subject to lethal tumor challenge with EG7-OVA. Tumor growth was followed for 6 weeks. Results are expressed as tumor index = square root (length $\times$ width of tumor). Adapted from Cho et al. (11).

mixture with gp120, and the improved efficacy of the conjugate was especially apparent when difficult-to-elicited responses, such as mucosal immunity or responses in the absence of CD4 T cells, were examined. Conjugation of gp120 to an ODN not containing the CpG motif did not show this enhanced immunogenicity (12). The wide range of humoral and cellular immune responses elicited by AIC vaccines holds promise for development of efficacious vaccines against a wide range of intracellular and extracellular pathogens that current protein-based vaccines are unable to control because of the lack of induction of effective cellular immunity.

AICs: applications for allergic diseases

Allergen immunotherapy has been used for the treatment of allergic diseases since the early 1900s. However, low efficacy

and the risk of allergic and sometimes life-threatening anaphylactic reactions have limited the utility of immunotherapy. Attempts to improve efficacy by increasing allergen doses result in more severe allergic side effects, and attempts to reduce allergenicity by modification of allergens generally results in decreased immunogenicity and efficacy.

The ability of ISS-ODNs to bias immune responses toward a Th1 phenotype suggests it may be useful for treatment of Th2-biased allergic diseases. Tighe et al. (16) studied the immune response in mice to the major short ragweed allergen *Amb a 1* when it was either conjugated to or mixed with ISS-ODN. Immunization of mice with the *Amb a 1* AICs induced IFN- γ -secreting CD4 T cells and high titers of IgG2a, characteristic of a Th1 response. In contrast, immunization with *Amb a 1* alone resulted in IgG1, IgE, and IL-5 responses, characteristic of Th2 responses. Furthermore, AIC treatment of mice with pre-existing anti-*Amb a 1* Th2 responses resulted in increasing amounts

Table 1. gp120 antigen-immunostimulatory sequence oligodeoxyribonucleotide conjugates (AICs) vaccines elicit antigen-specific immunoglobulin and cytokine responses

	Intradermal immunization			Intranasal immunization		
	gp120	gp120 + ISS	gp120:ISS conjugate	gp120	gp120 + ISS	gp120:ISS conjugate
IgG ($U \times 10^3/ml$)	34 $\pm$ 13	202 $\pm$ 44*	221 $\pm$ 20*	0.3 $\pm$ 0.2	402 $\pm$ 47*	488 $\pm$ 4.1*†
IgG2a ($U \times 10^3/ml$)	0.9 $\pm$ 0.9	255 $\pm$ 74*	280 $\pm$ 15*	Not detectable	45 $\pm$ 22	289 $\pm$ 78*†
Vaginal IgA ($U \times 10^3/ml$)			1.2 $\pm$ 0.2	1.8 $\pm$ 0.5	20 $\pm$ 5.8*	19 $\pm$ 1.6*
Fecal IgA ($U \times 10^3/ml$)			1.2 $\pm$ 3.3	1.2 $\pm$ 0.3	37 $\pm$ 8.5*	37 $\pm$ 9.7*
IFN- γ (pg/ml)	55 $\pm$ 32	2934 $\pm$ 528*	13216 $\pm$ 996*†	1.0 $\pm$ 1.0	2291 $\pm$ 432*	3004 $\pm$ 357*
MIP1 α (pg/ml)	Not detectable	203 $\pm$ 16	908 $\pm$ 142*†	10 $\pm$ 5.2	280 $\pm$ 46	1343 $\pm$ 498*†
MIP1 β (pg/ml)	Not detectable	3273 $\pm$ 755*	4958 $\pm$ 51*†	47 $\pm$ 28	2550 $\pm$ 957*	2476 $\pm$ 587*

Female BALB/c mice were immunized intradermally or intranasally on days 0, 14, and 28 with gp120 (10 μ g) alone, gp120 + ISS (10 μ g), or gp120:ISS conjugate (10 μ g based on gp120 content, corresponding to about 1 μ g of ISS-ODN). gp120-specific serum immunoglobulin G (IgG), serum IgG2a, and vaginal and fecal IgA, represented in relative units based on comparison with respective high titer standards, were determined at week 12 by enzyme-linked immunosorbent assay. gp120-specific interferon- γ (IFN- γ), macrophage inflammatory protein 1 α (MIP1 α), and MIP1 β production from splenocytes restimulated *in vitro* with gp120 protein was determined by enzyme-linked immunosorbent assay. Results reflect averages of four mice per group and are representative of three independent experiments. Results are reported as mean $\pm$ SEM. Groups whose means are significantly different from gp120-immunized mice are denoted with a * ($P < 0.05$). Groups whose means are significantly higher than gp120 + ISS-immunized mice are denoted with a † ($P < 0.05$).

Table 2. gp120 antigen-immunostimulatory sequence oligodeoxyribonucleotide conjugates (AICs) elicit major histocompatibility complex (MHC) class I-restricted cytokine responses that are independent of CD4 T cells

	gp120	gp120 + ISS	gp120:ISS conjugate	gp120:ISS conjugate (CD4 depleted)
IFN- γ (pg/ml)	Not detectable	1010 $\pm$ 134*	1472 $\pm$ 344*	1584 $\pm$ 534*
MIP1 α (pg/ml)	Not detectable	119 $\pm$ 53*	122 $\pm$ 19*	259 $\pm$ 18*
MIP1 β (pg/ml)	Not detectable	543 $\pm$ 142*	1228 $\pm$ 200*†	1446 $\pm$ 17*†
Splenic CTL (% specific lysis)	5 $\pm$ 2	63 $\pm$ 8*	84 $\pm$ 5*	65 $\pm$ 6*
Mucosal CTL (% specific lysis)	7	17	66	84

IFN- γ , interferon- γ ; MIP1 α , macrophage inflammatory protein 1 α . Splenocytes from non-depleted or CD4 T-cell-depleted mice immunized intradermally as described in Table 1 were restimulated *in vitro* with MHC class I-restricted gp120 peptide, and cytokine levels in the supernatant were measured by enzyme-linked immunosorbent assay. Splenic cytotoxic T lymphocytes (CTLs) after intradermal immunization and mucosal (*lamina propria*) after intranasal immunization were determined as previously described. Mucosal CTLs reflect pooled samples from four mice per group. All other results reflect averages of four mice per group and are representative of three independent experiments. Results are reported as mean $\pm$ SEM. Groups whose means are significantly different from gp120-immunized mice are denoted with a * ($P < 0.05$). Groups whose means are significantly higher than gp120 + ISS-immunized mice are denoted with a † ($P < 0.05$).

of IgG2a antibodies without the rise in IgE titers seen in saline-treated mice. Challenge of these treated mice with Amb a 1 after 14 weeks showed increased IgG2a and splenic IFN- γ production and decreased IgE and IL-5 production in the AIC-treated group compared to that in the saline-treated group. These results show the ability of AIC to upregulate Th1 responses and downregulate pre-existing Th2 responses as a potential therapy for allergic patients. Similar results have also been obtained in later studies (42) using an OVA AIC preparation (Table 3).

Studies with Amb a 1 and OVA have shown that the AIC preparations were not only more immunogenic for inducing Th1 responses than the protein mixed with ISS-ODNs, but the AIC preparations were also less allergenic than native protein. The bound ODNs prevented recognition of epitopes by antigen-specific IgG and IgE (Fig. 5A), resulting in decreased mast cell degranulation and histamine release (Fig. 5B) and decreased anaphylaxis-induced death upon challenge (Table 4) (16, 42, 43). Furthermore, in a mouse asthma model where OVA-sensitized mice were treated with ISS-ODNs alone, a mixture of ISS-ODNs and OVA, or OVA AIC, the AIC-treated group had the least airway hypersensitivity 8 weeks after treatment (44). Rechallenge of the untreated OVA-sensitized mice with AICs also resulted in less airway reactivity and airway eosinophilia than rechallenge with OVA or OVA + ISS-ODNs (Fig. 5C) (44).

AICs are the first immunotherapy reagent to enhance immunogenicity while decreasing allergenicity, accomplishing this feat by using an immunomodulatory compound (i.e. ISS-ODNs) to shield allergenic/anaphylactogenic epitopes on the allergen. AICs should thus be an attractive form of immunotherapy that can be used at adequate doses to induce Th1 responses without triggering allergic reactions in allergic patients.

Initial clinical trials demonstrated that AICs are less allergenic and more clinically effective than allergen extracts for immunotherapy in patients with allergic rhinitis (45, 46). In a recently published

study, ragweed-sensitive patients with allergic rhinoconjunctivitis were followed after six weekly subcutaneous treatments with escalating doses of ragweed-derived Amb a 1-containing AIC ($n = 28$) or placebo ($n = 29$) prior to the 2001 ragweed season (47). AIC treatment was safe and well tolerated with no serious adverse events reported and no difference from the placebo group in reported mild to moderate adverse events such as injection site reactions, headaches, rhinitis, and upper respiratory tract infections. Clinical chemistry, hematology, and urinalysis results, including anti-nuclear and anti-DNA antibodies, remained normal after treatment. Nasal biopsy evaluation 24 h after intranasal ragweed challenge upon completion of the injection protocol and before the ragweed season had begun showed no difference between AIC- and placebo-treated groups. However, AIC treatment resulted in decreased eosinophilia, decreased IL-4-producing cells, and increased IFN- γ -producing cells in nasal biopsy specimens upon rechallenge after the 2001 ragweed season, approximately 4–5 months after the initial injections. Although symptom and medication-use scores were not significantly different between groups in the 2001 season, the change in balance from Th2 to Th1 responses suggested by the nasal biopsies appeared to translate into improved clinical outcome during the following ragweed season, with AIC treatment conferring significant

Table 3. Antigen-immunostimulatory sequence oligodeoxyribonucleotide conjugates (AICs) induce T-helper 1 immune responses

Immunization	IgG2a (U/ml)	IgG1 (U/ml)	IFN- γ (pg/ml)	IL-10 (pg/ml)
OVA	193 $\pm$ 79	1020 $\pm$ 608	194 $\pm$ 75	Not detectable
ISS + OVA	596 $\pm$ 113	2330 $\pm$ 743	409 $\pm$ 98	Not detectable
ISS:OVA	4840 $\pm$ 2075*	1157 $\pm$ 379	989 $\pm$ 151*	378 $\pm$ 143*

IFN- γ , interferon- γ ; IgG, immunoglobulin G; IL-10, interleukin-10. Four mice per group received a single intradermal immunization with ovalbumin (OVA), ISS + OVA, or ISS:OVA conjugate. Antigen-specific serum antibody and splenic cytokine responses were determined 6 weeks later by enzyme-linked immunosorbent assay. Results are presented as mean $\pm$ SEM.

* $P < 0.01$ compared to OVA vaccination.

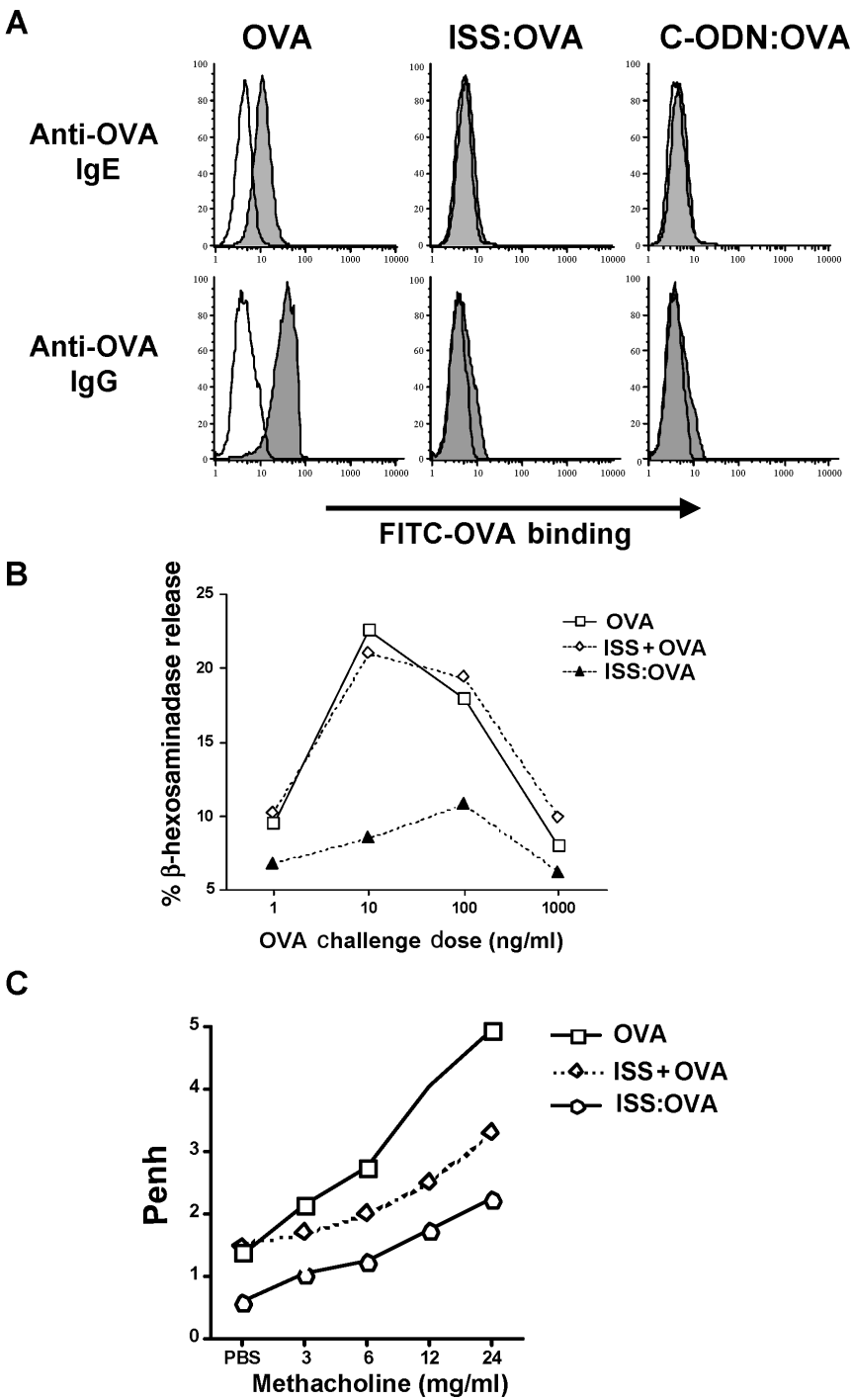


Fig. 5. Antigen-immunostimulatory sequence oligodeoxynucleotide conjugates (AICs) decrease allergenicity of immunotherapy. (A) Cells were left unloaded (open histograms) or loaded (filled histograms) with ovalbumin (OVA)-specific immunoglobulin E (IgE) or IgG as described in Horner et al. (42). The cells were then incubated with fluorescently labeled OVA, ISS:OVA, or C-ODN:OVA, and binding was assessed by flow cytometry. (B) Bone marrow-derived mast cells were loaded with IgE-enriched OVA-specific sera, incubated with varying concentrations of OVA, ISS + OVA, or ISS:OVA, and assessed for mast cell degranulation by β -hexosaminidase release as previously described (42). (C) Mice with an asthmatic phenotype after sensitization with OVA and alum received two intranasal challenges with OVA, OVA + ISS, or OVA:ISS conjugate. Bronchial reactivity (Penh) to methacholine was then determined as previously described (44). PBS, phosphate-buffered saline.

decrease in chest symptoms ($P = 0.047$) and a trend toward decreased nasal symptoms ($P = 0.065$) during the 2002 ragweed season.

Conclusion

Conjugation of protein antigen to ISS-ODN creates a potent immunogen with therapeutic implications for a wide range of

human diseases. The potency of AIC is at least partially explained by the efficient colocalization of antigen and adjuvant within the same APC that leads to robust innate and adaptive immune responses. Each of the two components of the AIC compound, namely protein antigen and ISS-ODN, seems to facilitate the immunological activity of the other. The ODN enhances uptake of the conjugated antigen by APCs. Conversely, the protein platform to which the ODNs

Table 4. Antigen-immunostimulatory sequence oligodeoxy-ribonucleotide conjugates (AICs) decrease anaphylactic reactions

Challenge	Histamine (μM)	Survival
Saline	none detected	6/6*
OVA	28 ± 2	0/14
ISS + OVA	27 ± 1	0/10
ISS:OVA	2 ± 1	10/10*

Mice sensitized to ovalbumin (OVA) were intravenously challenged with equivalent doses of OVA, ISS + OVA, or ISS:OVA conjugate. Plasma histamine levels (mean $\pm$ SEM) and mortality from anaphylaxis was determined.

* $P < 0.01$ compared to OVA challenge.

are linked seems to potentiate their immunostimulatory activities, possibly by creating a scaffold for the ODN molecules that results in improved activation of TLR9.

References

- Tokunaga T, et al. Antitumor activity of deoxyribonucleic acid fraction from *Mycobacterium bovis* BCG. I. Isolation, physicochemical characterization, and antitumor activity. *J Natl Cancer Inst* 1984;**72**:955–962.
- Yamamoto S, Kuramoto E, Shimada S, Tokunaga T. In vitro augmentation of natural killer cell activity and production of interferon- α /beta and -gamma with deoxyribonucleic acid fraction from *Mycobacterium bovis* BCG. *Jpn J Cancer Res* 1988;**79**:866–873.
- Krieg AM, et al. CpG motifs in bacterial DNA trigger direct B-cell activation. *Nature* 1995;**374**:546–549.
- Bird AP. Functions for DNA methylation in vertebrates. *Cold Spring Harb Symp Quant Biol* 1993;**58**:281–285.
- Yamamoto S, Yamamoto T, Kataoka T, Kuramoto E, Yano O, Tokunaga T. Unique palindromic sequences in synthetic oligonucleotides are required to induce IFN [correction of INF] and augment IFN-mediated [correction of INF] natural killer activity. *J Immunol* 1992;**148**:4072–4076.
- Martin-Orozco E, Kobayashi H, Van Uden J, Nguyen MD, Kornbluth RS, Raz E. Enhancement of antigen-presenting cell surface molecules involved in cognate interactions by immunostimulatory DNA sequences. *Int Immunol* 1999;**11**:1111–1118.
- Klinman DM, Yi AK, Beaucage SL, Conover J, Krieg AM. CpG motifs present in bacteria DNA rapidly induce lymphocytes to secrete interleukin 6, interleukin 12, and interferon gamma. *Proc Natl Acad Sci USA* 1996;**93**:2879–2883.
- Roman M, et al. Immunostimulatory DNA sequences function as T helper-1-promoting adjuvants. *Nat Med* 1997;**3**:849–854.
- Kobayashi H, et al. Immunostimulatory DNA pre-priming: a novel approach for prolonged Th1-biased immunity. *Cell Immunol* 1999;**198**:69–75.
- Wild J, Grusby MJ, Schirmbeck R, Reimann J. Priming MHC-I-restricted cytotoxic T lymphocyte responses to exogenous hepatitis B surface antigen is CD4⁺ T cell dependent. *J Immunol* 1999;**163**:1880–1887.
- Cho HJ, et al. Immunostimulatory DNA-based vaccines induce cytotoxic lymphocyte activity by a T-helper cell-independent mechanism. *Nat Biotechnol* 2000;**18**:509–514.
- Horner AA, et al. Immunostimulatory dna-based vaccines elicit multifaceted immune responses against hiv at systemic and mucosal sites. *J Immunol* 2001;**167**:1584–1591.
- Datta SK, et al. A subset of Toll-like receptor ligands induces cross-presentation by bone marrow-derived dendritic cells. *J Immunol* 2003;**170**:4102–4110.
- Hemmi H, et al. A Toll-like receptor recognizes bacterial DNA. *Nature* 2000;**408**:740–745.
- Verthelyi D, Klinman DM. CpG ODN – Safety Considerations. In: Raz E, ed. *Microbial DNA and Host Immunity*. Totowa, New Jersey: Humana Press Inc., 2002:385–396.
- Tighe H, 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* 2000;**106**:124–134.
- 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* 2000;**164**:5575–5582.
- Takabayashi K, Tighe H, Beck L, Spiegelberg HL. Protein-immunostimulatory DNA-conjugate: a novel immuogen. In: Raz E, ed. *Microbial DNA and Host Immunity*. Totowa, New Jersey: Humana Press Inc., 2002:175–188.
- Tighe H, 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* 2000;**30**:1939–1947.
- Klinman DM, Barnhart KM, Conover J. CpG motifs as immune adjuvants. *Vaccine* 1999;**17**:19–25.
- Shirota H, Sano K, Hirasawa N, Hattori T, Shirato K, Tamura G. Nover roles of CpG oligodeoxynucleotides as a leader for the sampling and presentation of CpG-tagged antigen by dendritic cells. *J Immunol* 2001;**167**:66–74.
- Maurer T, et al. CpG-DNA aided cross-presentation of soluble antigens by dendritic cells. *Eur J Immunol* 2002;**32**:2356–2364.
- Heit A, et al. Toll-like receptor 9 expression is not required for CpG DNA-aided cross-presentation of DNA-conjugated antigens but essential for cross-priming of CD8 T cells. *J Immunol* 2003;**170**:2802–2805.
- Starnes CO. Coley's toxins in perspective. *Nature* 1992;**357**:11–12.
- Nauts HC, McLaren JR. Coley toxins – the first century. *Adv Exp Med Biol* 1990;**267**:483–500.
- Bohle A, Brandau S. Immune mechanisms in bacillus Calmette–Guerin immunotherapy for superficial bladder cancer. *J Urol* 2003;**170**:964–969.
- Banchereau J, et al. Immunobiology of dendritic cells. *Annu Rev Immunol* 2000;**18**:767–811.
- Steinman RM, Dhodapkar M. Active immunization against cancer with dendritic cells: the near future. *Int J Cancer* 2001;**94**:459–473.

The immune responses triggered by AICs are characterized by antigen-specific antibody production, induction of Th1 cytokine responses and suppression of existing Th2 cytokine responses, and cross-priming of CD8 CTL responses against soluble antigen in a CD4-independent manner. The robust humoral and cellular responses elicited by AIC vaccines make them ideal candidates for use against a variety of infectious pathogens and tumors. AICs also hold promise as immunomodulatory agents for the attenuation of allergic and asthmatic disease by deviating antigen-specific T-cell responses to a non-allergic phenotype, while masking allergenic epitopes that have limited the utility of traditional immunotherapy. Ongoing and future clinical trials will be needed to further confirm the safety and efficacy of AICs in this wide spectrum of human diseases.

29. Cho HJ, et al. IFN- α beta promote priming of antigen-specific CD8⁺ and CD4⁺ T lymphocytes by immunostimulatory DNA-based vaccines. *J Immunol* 2002;**168**:4907–4913.
30. Bennett SR, Carbone FR, Karamalis F, Flavell RA, Miller JF, Heath WR. Help for cytotoxic-T-cell responses is mediated by CD40 signalling. *Nature* 1998;**393**:478–480.
31. Schoenberger SP, Toes RE, van der Voort EI, Offringa R, Melief CJ. T-cell help for cytotoxic T lymphocytes is mediated by CD40-CD40L interactions. *Nature* 1998;**393**:480–483.
32. Ridge JP, Di Rosa F, Matzinger P. A conditioned dendritic cell can be a temporal bridge between a CD4⁺ T-helper and a T-killer cell. *Nature* 1998;**393**:474–478.
33. Mellman I, Steinman RM. Dendritic cells: specialized and regulated antigen processing machines. *Cell* 2001;**106**:255–258.
34. Kadowaki N, et al. Subsets of human dendritic cell precursors express different toll-like receptors and respond to different microbial antigens. *J Exp Med* 2001;**194**:863–869.
35. Hsu FJ, et al. Tumor-specific idiotype vaccines in the treatment of patients with B-cell lymphoma – long-term results of a clinical trial. *Blood* 1997;**89**:3129–3135.
36. Kwak LW. Induction of immune responses in patients with B-cell lymphoma against the surface-immunoglobulin idiotype expressed by their tumors. *N Engl J Med* 1992;**327**:1209–1215.
37. Knuth A, Wolfel T, Klehmann E, Boon T, Meyerzum Buschenfelde KH. Cytolytic T-cell clones against an autologous human melanoma: specificity study and definition of three antigens by immunoselection. *Proc Natl Acad Sci USA* 1989;**86**:2804–2808.
38. McCluskie MJ, Weeranta R, Davis HL. Comparison of CpG DNA with other adjuvants for vaccination against hepatitis B. In: Raz E, ed. *Microbial DNA and Host Immunity*. Totowa, New Jersey: Humana Press Inc., 2002:229–238.
39. Guidotti LG, Chisari FV. To kill or to cure: options in host defense against viral infection. *Curr Opin Immunol* 1996;**8**:478–483.
40. van den Broek MF, Müller U, Huang S, Zinkernagel RM, Aguet M. Immune defence in mice lacking type I and/or type II interferon receptors. *Immunol Rev* 1995;**148**:5–18.
41. Cocchi F, DeVico AL, Garzino-Demo A, Arya SK, Gallo RC, Lusso P. Identification of RANTES, MIP-1 α , and MIP-1 β as the major HIV-suppressive factors produced by CD8⁺ T cells. *Science* 1995;**270**:1811–1815.
42. Horner AA, et al. Optimized conjugation ratios lead to allergen immunostimulatory oligodeoxynucleotide conjugates with retained immunogenicity and minimal anaphylactogenicity. *J Allergy Clin Immunol* 2002;**110**:413–420.
43. Horner AA, Nguyen MD, Ronaghy A, Cinman N, Verbeek S, Raz E. DNA-based vaccination reduces the risk of lethal anaphylactic hypersensitivity in mice. *J Allergy Clin Immunol* 2000;**106**:349–356.
44. Takabayashi K, Libet L, Chisolm D, Zubeldia J, Horner AA. Intranasal immunotherapy is more effective than intradermal immunotherapy for the induction of airway allergen tolerance in Th2-sensitized mice. *J Immunol* 2003;**170**:3898–3905.
45. Creticos PS, et al. Initial immunotherapy trial to explore the safety, tolerability and immunogenicity of subcutaneous injections of an Amb a 1 immunostimulatory oligonucleotide conjugate (AIC) in ragweed allergic adults. *J Allergy Clin Immunol* 2001;**107**:S216.
46. Creticos PS, et al. Immunostimulatory oligodeoxynucleotides conjugated to Amb a 1: safety, skin test reactivity, and basophil histamine release. *J Allergy Clin Immunol* 2000;**105**:S70 [abstract].
47. Tulic MK, et al. Amb a 1-immunostimulatory oligodeoxynucleotide conjugate immunotherapy decreases the nasal inflammatory response. *J Allergy Clin Immunol* 2004;**113**:235–241.