

Molecular mechanisms in allergy and clinical immunology

(Supported by a grant from Merck & Co, Inc, West Point, Pa)

Series editor: Lanny J. Rosenwasser, MD

Immunostimulatory DNA and applications to allergic disease

John Van Uden and Eyal Raz, MD *La Jolla, Calif*

The vertebrate immune system reacts to certain sequences of DNA with a strong T_H1-inducing innate response. These sequences, termed immunostimulatory DNA sequences, are not fully defined but generally consist of a central nonmethylated CG dinucleotide, flanked by less highly conserved sequences (hence the alternate name CpG motifs). These sequences seem to be rare in vertebrates but relatively common in many lower organisms, including bacteria and viruses. It is likely that these sequences represent a danger signal to the immune system; a powerful T_H1 response is induced against colocalized foreign antigen. This can be used to modify an allergic response away from a pathogenic T_H2-dominated immune profile toward a nonpathogenic and even protective T_H1 profile. (*J Allergy Clin Immunol* 1999;104:902-10.)

Key words: Immunostimulatory DNA sequences, CpG motifs, DNA immunotherapy, DNA immunotherapeutics, allergic disease

It has recently been appreciated that DNA carries information about its originating organism in addition to its genetic blueprints. Rodent and primate immune systems react strongly to certain sequences (immunostimulatory DNA sequences [ISS]) that are very rare in vertebrates but are present in many lower, potentially pathogenic organisms. DNA containing these sequences is apparently perceived as likely to be associated with a dangerous invader and is thus a powerful adjuvant for activation of the innate arm of the immune response, detailed below. Briefly, professional antigen-presenting cells (APCs) such as macrophages and dendritic cells produce IL-12, IL-18, TNF- α , and IFN- α/β , while altering their surface molecule profile, and, in the case of macrophages, becoming cytotoxic. B cells also alter their surface markers for enhanced antigen presentation while

Abbreviations used

APC:	Antigen-presenting cell
BCG:	Bacillus Calmette Guerin mycobacterium
CTL:	Cytotoxic T lymphocyte
dsRNA:	Double-stranded RNA
ISS:	Immunostimulatory DNA sequences
JNK:	c-Jun NH2-terminal kinase
MAPK:	Mitogen-activated protein kinase
NF- κ B:	Nuclear factor- κ B
NK:	Natural killer
ODN:	Oligodeoxynucleotide
ps:	Phosphorothioate
ROS:	Reactive oxygen species

proliferating and producing IL-6 and IL-12. Natural killer (NK) cells become cytotoxic and produce early IFN- γ . This concerted response then biases the adaptive arm of the immune system toward a T_H1 response against any locally encountered foreign proteins. Many researchers are attempting to harness the potency of T_H1 immune activation, the favorable safety profile, and the ease of use of ISS-containing DNA (oligodeoxynucleotides [ODNs] and plasmid) to treat or prevent disease.

IMMUNOSTIMULATORY NUCLEOTIDES: HISTORICAL PERSPECTIVES

Freund's famous adjuvant consisting of mycobacterial extract in oil immersion was initially characterized 60 years ago¹ yet remains one of the most potent modalities for generation of a T_H1 response. CFA contains many immunologically active substances, but it is likely that some of its activity is the result of mycobacterial genomic DNA.²

In the 1960s it was shown that natural double-stranded RNA (dsRNA) and its more stable analog polyinosinic, poly-cytidilic ribonucleic acid could stimulate the antiviral response by production of IFN- α and IFN- β .^{3,4} The IFN-induced, dsRNA-activated protein kinase is responsible for this phenomenon, which probably evolved as a mechanism for sensing the dsRNA produced during the life cycle of some viruses.^{5,6}

Yet it wasn't until the 1980s that the first reports of the immunostimulatory properties of DNA appeared. Tokunaga and his group in Japan found that it was the mycobacterial genomic DNA activation of NK cells that

From the Department of Medicine, University of California, San Diego, La Jolla, Calif.

Supported in part by grant No. AI 40682 from the National Institutes of Health and by Dynavax Technologies. J. V. U. is a Medical Scientist Training Program Trainee (National Institute of General Medical Sciences grant No. GM 07198) and a Lucille P. Markey Charitable Trust Fellow.

Received for publication Aug 4, 1999; revised Aug 17, 1999; accepted for publication Aug 18, 1999.

Reprint requests: John Van Uden, University of California, San Diego, Department of Medicine, 128 Stein Clinical Research Bldg, 9500 Gilman Dr, La Jolla, CA 92093-0663.

Copyright © 1999 by Mosby, Inc.
0091-6749/99 \$8.00 + 0 1/1/102307

TABLE I. Potent immunostimulatory DNA sequences

Sequence	Notes	References
Stimulatory activity shown reproducibly by several groups:		
AACGTT	One of the first sequences discovered; we and others use this sequence frequently; stimulates human cells	13-18
AACGTTCCG	More active than AACGTT in our hands; this is sequence we use most often	2, 15
AGCGCT	One of first sequences discovered	13-15, 19
GACGTC	One of first sequences discovered; active as a 6-mer ODN; stimulates human cells	13, 15, 16, 20
GACGTT	Active as 6-mer ODN; stimulates human cells; modified flanking sequences can give good response with low TNF- α ; this sequence has been used in many studies	20-23
Limited reports of stimulatory activity:		
AACGAT	Moderate B-cell activation	19
AACGCT		18, 19
ACGCGT	Active as a 6-mer ODN	20
AGCGTT		24
ATCGAT	Active as a 6-mer ODN	14, 20
CACGTG	Active as a 6-mer ODN	17, 20
CACGTT		25
CTCGAC		19
CTCGCA	From acute retrovirus genome	26
CTCGTA	From acute retrovirus genome	26
GACGAT		25
GACGCT		25
GACGTA		25
GACGTG		25
GGCGTT		25
GTCGAC	Active as a 6-mer ODN	20
GTCGAT		17, 27
GTCGCT	May stimulate human cells	21, 24, 27
GTCGTC	May stimulate human cells	21
GTCGTT		17, 24
TACGTA	Active as a 6-mer ODN	20
TACGTT		25
TCGCGA	Active as a 6-mer ODN	14, 20
TGACGTT	Version of GACGTT that is claimed to be optimal mouse B-cell stimulatory sequence	24
Conflicting reports of stimulatory ability:		
CGCGCG	Has been shown to be stimulatory, ¹⁴ has been shown to be inhibitory ²⁸	14, 28
GACGGT	Demonstrated activity, ^{16,24} demonstrated lack of activity ²⁵	16, 24, 25
GCGCGC	Has been shown to be stimulatory, ¹⁴ has been shown to be inhibitory ²⁸	14, 28
GGCGCC	Report likely activity, ²⁹ but not as 6-mer ODNs ²⁰	20, 29
GGCGGT	Demonstrated moderate activity, ²⁴ demonstrated lack of activity ²⁵	24, 25
GTCGGT	Demonstrated low stimulatory ability, ¹⁷ demonstrated high stimulatory ability ^{19,24}	17, 19, 24
TGCGCA	Report likely activity ²⁹ but not as 6-mer ODNs ²⁰	20, 29

This table is a compilation of the central core sequences of ODNs that have been shown to be immunostimulatory. However, except where noted that the 6-mer ODN is stimulatory, the actual ODN used was longer than this core sequence. There is some indirect evidence, but no direct evidence, that these core sequences constitute the fundamental stimulatory units (see text for details). It is also important to note that most of these studies referenced used different assays for immune stimulation under different treatment conditions. The most common assays include induction of proliferation (B cells), cytokines (APCs and NK cells), or cytolytic activity (mostly NK cells, some macrophages) on incubation of murine splenocytes with short (12-30-mer) phosphorothioate (ps)-modified ODNs. The work to define the exact motifs required for immune stimulation is still in progress; this table is not meant to be a final and authoritative listing, only a general guide to what has been shown to date. There are other sequences that have been demonstrated to have lesser but significant immunostimulatory ability; only the most potent sequences are shown.

was responsible for the potent antitumor activity of an extract of *Mycobacterium bovis* Bacillus Calmette-Guerin mycobacterium (BCG).⁷⁻¹²

DEFINING IMMUNOSTIMULATORY SEQUENCES

Even after intensive attempts to precisely define the DNA sequence structure required for immune stimulation, this most fundamental aspect of ISS is only partially

understood (Table I). After their initial characterization of the immune stimulatory properties of mycobacterial DNA, Tokunaga and colleagues found that 6 of 13 synthetic 45-mer ODNs taken from the known sequence of the mycobacterial genome had some activity. Further dissection with overlapping and shorter ODNs led these researchers to the conclusion that a few palindromic hexamers containing CpG dinucleotides were the active units.^{13,14,20,29,30}

Later studies by Krieg et al¹⁹ revealed a tentative con-

sensus sequence of single-stranded 5'-purine-purine-C-G-pyrimidine-pyrimidine-3' to have optimal B cell stimulatory ability. This consensus also seems to hold for production of IL-6, IL-12, and IFN- γ by mouse splenocytes¹⁸ and for NK activation in mouse and human cells.¹⁷ However, this consensus is only a loose rule: there are many sequences that are stimulatory that do not follow the motif and many sequences that would be included within the consensus that are not particularly stimulatory. One aspect of ISS that does appear to be generally required is the inclusion of CpG, preferably near the center of an ODN.^{14,19} Nevertheless, some sequences without CpG have some stimulatory ability,²¹ which is why we prefer the functionally defined name ISS to the structurally defined name CpG motif.

There is a further layer of complexity in the structural requirements for ISS activity—epigenetics. It has been shown that ISS lose their stimulatory ability with C-5 methylation of the cytosine at the CpG core.^{17,19,31,32} This is particularly interesting because vertebrates methylate about 70% of their CpG DNA (which is already reduced about 5-fold in frequency) and nearly all of their CpG sequences that are not in active promoter or enhancer regions.^{33,34} Methylation within promoters and enhancers shuts down transcriptional activity and is used in long-term gene regulation, X-chromosome inactivation, and suppression of invading and endogenous viruses and transposons.^{35,36} Additionally, methylation is believed to be responsible for the drastic reduction in frequency of CpG in the vertebrate genome. ^{me}CpG is a hotspot for mutation to TpG during replication, and in fact organisms with low CpG tend to have more TpG and its complement CpA than expected.³³ Thus it makes sense that there are clusters of unmethylated CpGs (CpG islands) found near the promoters of genes (where CpGs are not methylated) because the rest of the genome has undergone methylation and mutation over evolutionary time. This is in contrast to lower organisms, where the frequency of CpG dinucleotides varies widely but in general is near the expected occurrence of 1:16, and the C-5 positions on the cytosines are not as heavily methylated.³⁷ Not all CpGs are ISS, and we have found that the frequencies of active ISS are even further reduced in mammalian genomes and are altered in pathogen genomes (Van Uden and Raz, manuscript in preparation) beyond dinucleotide CpG frequencies.

Although ISS are generally considered by researchers in this field to be modular 6-mer units, it has been difficult to determine the minimum stimulatory motif length. One study showed that a minimum length of 18 bases was required but that a length of 22 bases gave greater activity.³⁸ Another study demonstrated good activity with a 15-mer ODN.¹⁷ Still another study used cationic lipid transfection to show a stimulatory effect with a 6-mer ODN.²⁰ Phosphorothioate (ps) ODNs are nuclease resistant and were shown to have activity when as short as 8 bases.¹⁹ It is possible that the minimum motif is around 6 bases but that exonucleases within target cells destroy short ODNs before they can have significant activity.

Another possibility consistent with some of our new work is that the CpG core is the primary active unit and that a variety of flanking sequences are permissive for activity. This would cause each ISS to appear to have a different minimum length because crucial flanking bases would be variably distant from the core (Van Uden and Raz, manuscript in preparation).

Nuclease resistant ps ODNs can be as much as 200-fold more potent than natural phosphodiester ODNs while apparently maintaining the original sequence specificity and spectrum of stimulation.¹⁹ The ps modification replaces 1 of the 2 nonbridging oxygens of the internucleotide phosphate with a sulfur and thus prevents nuclease activity and greatly extends the half-life.^{39,40} The ps ODNs have been used extensively for antisense, are commercially available, have been approved for use in humans, and are therefore gaining in popularity.

Almost all this work has been done in mice, but it is clear that human cells and primates also respond to ISS, sometimes with different sequence specificities. More work in this area is required and is underway, but one study has addressed some differences between sequences that stimulate mouse splenocytes and those that stimulate human peripheral B cells.²¹

Recently an entirely new functionality of ISS has been discovered. Repeating sequences containing Gs and Cs have been found that specifically neutralize ISS activity, which have been named CpG-N motifs.²⁸ We have found another class of sequences that not only inhibit the T_H1-biasing activity of ISS but also promote a T_H2 response (Van Uden and Raz, submitted for publication). Therefore there seems to be an important balance between the number and types of sequences in any given DNA that determines its ability to stimulate the immune system. This concept is likely to be important in understanding the net stimulatory properties of plasmids and genomes.

DNA IMMUNOSTIMULATION: NK CELL ACTIVATION

Activation of NK tumoricidal activity was the first effect of ISS that was described,⁹ and it has subsequently been shown that ISS-activated NK cells are more than 90% of the early IFN- γ -producing cells (later CD4⁺ cells contribute the majority of IFN- γ).⁴¹ Purified NK cells are not directly activated by ISS; the IL-12, TNF- α , and IFN- α/β produced by APCs are required (however, lack of B and T cells in mice with severe combined immunodeficiency syndrome does not reduce NK activation) (Fig 1).^{13,17,42} Although most of the NK characterization has been done with mouse cells, human blood NK cells are also responsive to ISS.¹⁷

DNA IMMUNOSTIMULATION: MACROPHAGE AND DENDRITIC CELL ACTIVATION

ISS directly activate macrophages and macrophage-like cell lines^{17,31,42,43} but indirect activation by IFN- γ

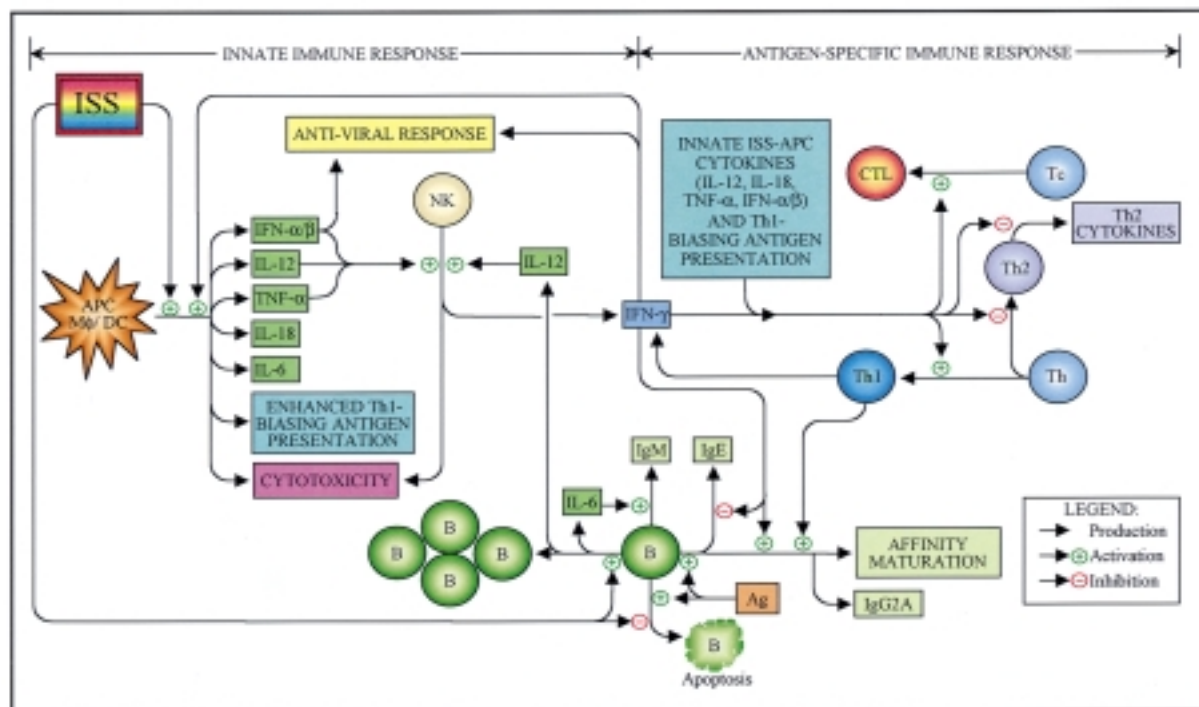


FIG 1. Intercellular mechanisms of ISS activation. This diagram summarizes known intercellular mechanisms of ISS activation of innate and antigen-specific arms of immune response, as detailed in text. Note that direct effects of ISS are apparently limited to stimulation of professional APCs and B cells within the innate arm and that it is the products of these stimulations that then drive the characteristic T_H1 development in the antigen-specific arm. *Mφ*, monocyte/macrophage; *DC*, dendritic cell; *Ag*, antigen; *Tc*, naïve $CD8^+$ CTL precursor; *Th*, naïve $CD4^+$ T_H1 or T_H2 precursor.

appears to account for the majority of quantitative activation of macrophages in mixed cell cultures and in vivo.^{11,13} ISS-activated macrophages become cytotoxic and produce the T_H1 -biasing cytokines $TNF-\alpha$, IL-12, IL-18, and $IFN-\alpha/\beta$.^{2,15,31,42,43} These cytokines stimulate $IFN-\gamma$ production by NK cells in the innate phase of the response to ISS and by T_H1 cells in the adaptive phase. $IFN-\gamma$, in turn, potently activates macrophages further, forming a positive feedback loop (Fig 1). The reactivity of macrophages was defined in mouse systems, but human macrophages are similarly activated by ISS.¹⁵ More recently it has been appreciated that the antigen processing and presentation apparatus of macrophages is also amplified on ISS stimulation. Bone marrow-derived macrophages treated with ISS-ODN up-regulate their expression of class I MHC, CD40, intercellular adhesion molecule-1, and CD16/32 (IgG receptor for bound antigen internalization and antibody-dependent cellular cytotoxicity) and consequently function more effectively as APCs.⁴⁴

Dendritic cells are similarly activated by ISS. They produce IL-12, IL-6, and $TNF-\alpha$ in response to ISS, and they increase their class II MHC and B7-2 levels.^{45,46} ISS-treated dendritic cells also function better as APCs in allogeneic mixed lymphocyte reactions and with superantigen stimulation⁴⁵ and may be particularly important in ISS-stimulated responses in vivo because of their pro-

posed role in priming of immune responses.⁴⁷ Thus the 2 most important classes of professional APCs are potentially stimulated by ISS.

DNA IMMUNOSTIMULATION: B-CELL ACTIVATION

B cells also are directly stimulated by ISS. The profound mitogenic effect of ISS on B cells was first described in 1991⁴⁸ and more than 95% of purified primary B cells in vitro will enter the cell cycle.¹⁹ In addition, purified B cells stimulated with ISS will produce polyclonal IgM antibodies independent of T-cell help or antigen exposure.^{19,21,49} ISS also rescues B cells from surface IgM cross-linking-induced apoptosis.^{25,27,50,51} B cells produce high levels of IL-6 and IL-12 in direct response to ISS (Fig 1).^{18,24,49} B-cell proliferation is independent of IL-6 production (as determined with blocking antibodies and with knockout mice) (Martin-Orozco and Raz, unpublished observation), but neutralization of IL-6 reduces IgM production by 90%,^{18,24} suggesting autocrine stimulation (Fig 1). The relative importance of B cell versus macrophage and dendritic cell production of IL-12 in establishment of the T_H1 response is unclear.^{15,18,42}

The surface molecule expression profile of B cells is also drastically altered by ISS activation. Class I MHC,

class II MHC, B7-1, B7-2, CD40, CD16/32, intercellular adhesion molecule-1, IFN- γ R, and IL-2R are all up-regulated in vivo or in vitro, whereas CD23 (the low-affinity IgE receptor) is down-regulated.⁴⁴

Although ISS can effectively activate B cells as part of the innate response (in an antigen-independent fashion), ISS also acts as a powerful costimulatory signal for B cells, which receive signals through their B-cell reactivity. Proliferation, IL-6 production, and IgM secretion are all synergistically increased with low-level surface B-cell reactivity cross-linking combined with ISS-ODN treatment (Fig 1).^{19,24} In addition, ISS appears to allow cooperation between the cellular and humoral responses. Normally IFN- γ inhibits the IgM produced by LPS activation of B cells, but with ISS IFN- γ increases the IgM and IL-6 produced.⁴⁹ Human B cells also respond to ISS; 95% of human peripheral B cells stimulated with ISS-ODN increase their expression of B7-2 and IL-2R.²¹

DNA IMMUNOSTIMULATION: T-CELL ACTIVATION AND T_H1 BIASING

T cells are also activated by ISS, although not in a direct way. Simple stimulation of purified T cells with ISS-ODN does not result in activation. Treatment of splenocytes with α -CD3-activating antibody combined with ISS-ODN leads to slightly increased levels of CD69 and B7-2 on T cells, but this effect is mediated by IFN- α/β .⁵² Similar treatments with ISS-ODN and α -CD3 led to increased levels of IFN- γ and to reduced levels of the T_H2 cytokines IL-3, IL-4, IL-5, and GM-CSF⁵³ (Van Uden and Raz, submitted for publication). Blocking studies have revealed that IFN- α/β , IL-12, and IFN- γ from APCs and NK cells contribute to the above alteration in the cytokine profile of T cells.⁵³

T cells are not required for the innate response to ISS,^{17,21,31,42,43,48} but both CD4⁺ and CD8⁺ T cells are vital for the characteristic T_H1 response to genetic vaccination.^{54,55} The T_H1 response to genetic vaccination and to ISS-ODN/antigen codelivery includes generation of CD8⁺ cytotoxic T lymphocyte (CTL), T_H1 cells, IgG2a > IgG1, and IFN- γ > IL-4, IL-5, and IL-10 (Fig 1).^{2,15,23,54,56-60} Generation of this strong T_H1 response can prevent the generation of, or even suppress a continuing, T_H2 response to the same antigen (see below). We have shown that this crucial profile of adaptive immune response activation in gene vaccination is at least largely the result of the presence of ISS in the backbone of all plasmids, which are bacterially derived.¹⁵ Therefore the plasmid can be thought of as 2 distinct immunologic components: the antigen expression cassette and the various ISS located throughout that act as T_H1-promoting adjuvants.⁶¹

One prominent exception to the T_H1 rule is the T_H0/T_H2 bias usually seen with intradermal particle-mediated transfer of plasmid DNA (the gene gun).^{62,63} This system is significantly different in that only about 1% of the amount of adjuvant DNA is administered relative to syringe injection and because a significant quantity of potentially immunologically active gold particles are also present.^{2,64}

Recent reports of the first generation of gene vaccination trials in monkeys and humans have met with only partial success.⁶⁵⁻⁶⁸ One of the major limitations to successful gene vaccination is the very low level of antigen expression, but we and others have reported that coadministration of ISS-ODN with protein results in strong CTL, antibody, and T_H1 cytokine production.^{2,23,69} An even more potent way to increase the response to the antigen is to chemically conjugate it to the ISS-ODN adjuvant to facilitate entry into the same APCs (Raz, manuscript in preparation).

MOLECULAR MECHANISMS

The molecular pathway from sensation of ISS-containing DNA to production of the characteristic cytokines and cell surface molecules is still largely undiscovered territory and an area of active investigation. Probably the most important of these areas is identification of the mechanism directly responsible for sensing ISS in or on a cell. There is likely a sequence-specific DNA binding protein(s) that is an ISS receptor, but there are still no published reports of such a protein. There is some evidence that ISS need to be internalized for activity. ISS are generally more potent and can be shorter when they are directly transfected into cells.^{20,70} Also, ISS-ODNs covalently bound to a solid support do not stimulate mouse B cells.¹⁹

The intracellular signaling events after ISS activation have been partially investigated. Several common pathways have been shown to not be involved: tyrosine phosphorylation, inositol triphosphate generation, calcium ion (Ca⁺⁺) flux, protein kinase C activation, protein kinase A activation, and nitric oxide production.^{19,24} The first positive finding was that reactive oxygen species (ROS) are generated within 20 minutes of stimulation with ISS, before IL-6 messenger RNA, which appears at 30 minutes.²⁴ Blockade of ROS activation with antioxidants completely blocks B-cell activation by ISS.²⁴ Nuclear factor- κ B (NF- κ B) has also been shown to be activated, and it is apparently necessary for ISS stimulation, although the NF- κ B inhibitors used are not entirely specific.^{31,50} The time course of NF- κ B activation is similar to that of cytokine messenger RNA induction, which is inconsistent with its proposed central role in ISS signal transduction. ROS activate NF- κ B, and both are involved in a great variety of immunologic signaling cascades and are thus not specific to ISS.

The mitogen-activated protein kinase (MAPK) and stress kinase pathways are also activated by ISS. Specifically, c-Jun NH2-terminal kinase (JNK), JNK kinase 1, p38, activating transcription factor-2 (ATF-2), c-Jun, MAPK-activated protein kinase-2, and activator protein-1 (AP-1) have been implicated in ISS signaling.^{71,72} The p38 pathway appears to be crucial because its blockade results in lack of cytokine production and of downstream signaling component activation.^{71,72}

Curiously, blockade of endosome acidification/maturation by chloroquine, monensin, or bafilomycin A inhibits the earliest aspects of the ISS response, includ-

ing the known signaling pathways.^{71,72} This could represent the ISS escaping the endosome, or perhaps some kind of potentiation or sensing of ISS.

A host of apoptosis-related genes are differentially regulated when ISS is used to block apoptosis in B cells. ISS appears to up-regulate *myn*, *bcl-2*, *bcl-x_L*, and *egr-1*, whereas *c-myc* was up-regulated in one study and down-regulated in another.^{27,73}

There is not yet a clear picture of how these disparate signaling clues form the greater picture of ISS activation of immune genes, but it is apparent that there are multiple interacting pathways.

CLINICAL APPLICATION: ALLERGIC DISEASE

There are many different potential areas where the ISS T_H1-inducing profile of adjuvanticity would be useful, such as in infectious disease vaccines, in cancer therapeutic vaccines, and in infectious disease immunotherapy. However, this review will focus on the implications for treatment of allergic disease.

Asthma and other allergic diseases are largely mediated by profound T_H2 responses against environmental antigens. It has been proposed that strong T_H1 responses can suppress and even down-regulate T_H2 reactions in an antigen-specific manner, thus ameliorating the disease.⁷⁴ The only currently available option for antigen-specific treatment is standard allergen immunotherapy, which involves injecting sensitized individuals with extremely small but increasing doses of allergen over the course of years, thus diverting the immune response. This immunotherapy only works for a select set of allergic diseases and carries a small but significant risk of side effects, including anaphylaxis.⁷⁵ We and others have shown that the T_H1-inducing properties of ISS can be used to prevent or treat allergic disease in rodent models.^{15,53-56,76-79}

It has been shown that genetic vaccination encoding a model antigen, β -gal, leads to a T_H1 response. This response dominates over subsequent or previous T_H2 sensitizations with β -gal in alum. IgE in particular is almost completely inhibited when plasmid is given before β -gal/alum, and IgE is drastically reduced when plasmid is given after β -gal/alum.⁵⁶ In a prophylaxis model, another group has shown that vaccination with a plasmid encoding the dust mite allergen Der p 5 inhibits the induction of allergy at a later time as measured with IgE production, airway hyperreactivity, and histamine release. Interestingly, this protection could be adoptively transferred by CD8⁺ T cells.^{55,80} It has been shown that this T_H1-promoting and T_H2-inhibiting effect of genetic vaccination can be largely accounted for by the presence of ISS in the plasmid backbone of genetic vaccines.^{15,32}

In addition to these antigen-specific effects of ISS as a T_H1-promoting (and therefore T_H2-inhibiting) adjuvant, ISS also have direct T_H2-inhibiting effects. We have demonstrated that only one injection (or mucosal administration) of ISS-ODN given before allergen challenge to allergically sensitized mice inhibits lung and blood

eosinophilia better than does 7 daily injections of dexamethasone. Levels of antigen-specific IL-3 and IL-5 and airway hyperreactivity were also reduced, whereas IFN- γ levels were increased.⁵³ This phenomenon of immediate inhibition of the allergic response by ISS-ODN has recently been confirmed with a different allergen, ragweed. This investigation further showed that the longer-term T_H1 diversion of the allergen-specific response is stable for at least 6 weeks.⁷⁸ This observation has recently been extended to other allergic diseases. ISS-ODN administration has been shown to block the development of both the early and late phases of murine allergic conjunctivitis, even when it is given after allergen sensitization (Magone and Raz, submitted for publication). Although the above studies use ISS primarily as a pharmacologic inhibitor of established T_H2 immunity, the ability of ISS-ODN to act as a T_H2-inhibiting adjuvant in a mouse model of asthma with use of shistosoma eggs has also been shown. When the T_H2-inducing eggs were given with ISS-ODN, lung eosinophilia, airway hyperreactivity, and lavage IL-4 were reduced and lavage IFN- γ and IL-12 were increased.⁷⁷

Thus the use of ISS both as prophylaxis and treatment of allergic disease is promising. ISS have shown utility both as short-term antigen-nonspecific inhibitors of allergic inflammation and as adjuvants for long-term immune deviation. Studies in monkey models of allergic disease have also been very promising (Raz, unpublished data) and human clinical trials are not far away.

POTENTIAL SIDE EFFECTS

Advantages of ISS for use as adjuvants include their powerful activation of multiple immune cell types and of T_H1 responses. However, the immune system is delicately balanced between immunity and tolerance, between T_H1 and T_H2, and between inflammation and unresponsiveness. There is always the possibility of unwanted effects of the powerful immune stimulation that ISS delivers.

LPS is similar to ISS in that it is a conserved bacterial product that is recognized by the immune system as a danger signal and it stimulates the production of many of the same cytokines. A systemic overload of LPS, however, can lead to lethal septic shock by production of excessive IL-1 and TNF- α . Additionally, high levels of IL-6 are predictive of a poor outcome in septic shock.⁸¹ ISS also induces the production of TNF- α and IL-6, so it is possible that it could lead to shock in environmentally or genetically sensitized individuals. It has been shown that mice given ISS followed by sublethal doses of LPS die of shock at an increased frequency (control at 0% to ISS-treated at 75%) in an IFN- γ -dependent manner.⁴¹ In a separate study it was found that mice presensitized with D-galactosamine then given ISS also undergo lethal shock; this model demonstrated that TNF- α but not T, B, or NK cells are required.⁴³ Interestingly, these researchers found that some immunostimulatory sequences retain their ability to stimulate IL-12 production without

producing TNF- α and are safe in their shock model.²² Although these reports demonstrate the possibility of shock in extreme cases of sensitization or concurrent LPS exposure, there has never been a reported case of ISS alone causing shock in any kind of healthy animal at any dose.

Another possibility is that ISS could cause excessive local inflammation, as seen with other powerful T_H1 adjuvants such as CFA. We and others have never observed gross inflammation in response to ISS in ODN or plasmid form in any experimental animals or humans.⁵⁷ There can be immune-mediated destruction of antigen-expressing tissue in gene vaccination, but this effect is antigen-specific, not a generalized inflammatory process.⁸² In contrast, it has been reported that delivery of ISS-containing bacterial DNA or ODNs to the lung can cause increases in the levels of neutrophils and other immune cells while increasing lavage fluid levels of TNF- α , IL-6, and macrophage inflammatory protein-2.⁸³ Fluid from the lungs of patients with cystic fibrosis was able to reproduce this effect in mouse lungs, implying that the copious amounts of bacterial DNA found in these patients' lungs could be responsible for some of the immune-mediated damage seen clinically.⁸³ However, this study never shows any clinically significant inflammation in their mouse model, only correlates of inflammation that could also be interpreted as important for proper adjuvanticity. In addition, this same group also found that systemically administered ISS can actually reduce LPS-induced airway inflammation in an IL-12-dependent manner.⁸⁴

When ISS-containing ODNs (ISS-ODNs) are injected into mice, the most obvious effect is a massive but transient splenomegaly within the first 2 weeks, primarily caused by extramedullary hematopoiesis in the spleen.⁸⁵ This is not likely to be relevant to humans because extramedullary hematopoiesis is not usually observed past the neonatal period and because this phenomenon has not been observed in humans and nonhuman primates treated with ISS-containing antisense ODNs.⁸⁶

Many autoimmune diseases are T_H1 in nature, and the danger exists of precipitating disease in genetically or environmentally predisposed individuals. In one model of experimental allergic encephalomyelitis, T cells primed in vivo with myelin basic protein in CFA (which contains bacterial DNA), and then restimulated with antigen and ISS in vitro will exit quiescence and transfer disease to recipient mice in an IL-12-dependent fashion.⁸⁷ Another group of researchers has shown that bacterial DNA can generate anti-DNA antibodies in a model of systemic lupus erythematosus. To make the DNA antigenic, bacterial DNA is complexed with methylated bovine serum albumin in CFA and injected into mice. Normal healthy mice make anti-DNA antibodies and even develop low levels of proliferative glomerulonephritis and proteinuria.^{88,89} When the mixture is given to preautoimmune NZB/NZW F1 mice, they develop antibodies that cross-react with mammalian DNA, but surprisingly they are actually protected from their spontaneous autoimmune disease.^{90,91} There still are no examples of ISS directly

causing any type of autoimmune disease in animal models.

SUMMARY AND PERSPECTIVES

Immunostimulatory DNA sequences in plasmid DNA or in ODNs potently and rapidly activate the innate immune system, triggering NK cells, macrophages, dendritic cells, and B cells. These cells produce antigen-nonspecific cytotoxicity, polyclonal IgM antibodies, and an environment that is both antiviral and conducive to generation of an antigen-specific T_H1 response. The resulting adaptive immune response consists of CD4⁺ T_H1 cells, CD8⁺ CTL cells, and high-affinity T_H1-type antibody (IgG2a in mice). This profile is especially well suited to attack intracellular pathogens and also to inhibit T_H2-mediated diseases such as allergy.

There are also cases in which the stimulatory properties of DNA should be minimized, such as in gene replacement therapy. The recent discovery of sequences that can inhibit the function of ISS, or even act as T_H2 adjuvants, opens up an exciting new arena for the modulation of the immunologic activity of DNA.

Most of the work on ISS has been in mice, but it is clear that human cells also respond. It may be important for those who wish to take ISS to the clinic to optimize sequences for potency and consistency in humans; it is already clear that the best sequences in mice are not necessarily the best in people.

ISS are also exciting from the basic science point of view. This system is assumed to have evolved to sense dangerous pathogens, but there are to date no reports of any experiments to determine the actual importance of ISS in host-pathogen interactions or what countermeasures successful pathogens may have evolved to avoid or take advantage of this system.

REFERENCES

- Freund J, Casals J, Hosmer EP. Sensitization and antibody formation after injection of tubercule bacilli and paraffin oil. *Proc Soc Exp Biol Med* 1937;37:509-13.
- Roman M, Martin-Orozco E, Goodman JS, et al. Immunostimulatory DNA sequences function as T helper-1-promoting adjuvants. *Nat Med* 1997;3:849-54.
- Lampson GP, Tytell AA, Field AK, Nemes MM, Hilleman MR. Inducers of interferon and host resistance, I: double-stranded RNA from extracts of *Penicillium funiculosum*. *Proc Natl Acad Sci U S A* 1967;58:782-9.
- Field AK, Tytell AA, Lampson GP, Hilleman MR. Inducers of interferon and host resistance, II: multistranded synthetic polynucleotide complexes. *Proc Natl Acad Sci U S A* 1967;58:1004-10.
- Zilberstein A, Kimchi A, Schmidt A, Revel M. Isolation of two interferon-induced translational inhibitors: a protein kinase and an oligo-isoadenylate synthetase. *Proc Natl Acad Sci U S A* 1978;75:4734-8.
- Meurs E, Chong K, Galabru J, et al. Molecular cloning and characterization of the human double-stranded RNA-activated protein kinase induced by interferon. *Cell* 1990;62:379-90.
- Tokunaga T, Yamamoto H, Shimada S, et al. Antitumor activity of deoxyribonucleic acid fraction from *Mycobacterium bovis* BCG, I: isolation, physicochemical characterization, and antitumor activity. *J Natl Cancer Inst* 1984;72:955-62.
- Shimada S, Yano O, Inoue H, et al. Antitumor activity of the DNA fraction from *Mycobacterium bovis* BCG, II: effects on various syngeneic mouse tumors. *J Natl Cancer Inst* 1985;74:681-8.

9. Shimada S, Yano O, Tokunaga T. In vivo augmentation of natural killer cell activity with a deoxyribonucleic acid fraction of BCG. *Jpn J Cancer Res* 1986;77:808-16.
10. Mashiba H, Matsunaga K, Tomoda H, Furusawa M, Jimi S, Tokunaga T. In vitro augmentation of natural killer activity of peripheral blood cells from cancer patients by a DNA fraction from *Mycobacterium bovis* BCG. *Jpn J Med Sci Biol* 1988;41:197-202.
11. 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;79:866-73.
12. Kuramoto E, Toizumi S, Shimada S, Tokunaga T. In situ infiltration of natural killer-like cells induced by intradermal injection of the nucleic acid fraction from BCG. *Microbiol Immunol* 1989;33:929-40.
13. Yamamoto S, Yamamoto T, Kataoka T, Kuramoto E, Yano O, Tokunaga T. Unique palindromic sequences in synthetic oligonucleotides are required to induce IFN and augment IFN-mediated natural killer activity. *J Immunol* 1992;148:4072-6.
14. Kuramoto E, Yano O, Kimura Y, et al. Oligonucleotide sequences required for natural killer cell activation. *Jpn J Cancer Res* 1992;83:1128-31.
15. Sato Y, Roman M, Tighe H, et al. Immunostimulatory DNA sequences necessary for effective intradermal gene immunization. *Science* 1996;273:352-4.
16. Yamamoto T, Yamamoto S, Kataoka T, Komuro K, Kohase M, Tokunaga T. Synthetic oligonucleotides with certain palindromes stimulate interferon production of human peripheral blood lymphocytes in vitro. *Jpn J Cancer Res* 1994;85:775-9.
17. Ballas ZK, Rasmussen WL, Krieg AM. Induction of NK activity in murine and human cells by CpG motifs in oligodeoxynucleotides and bacterial DNA. *J Immunol* 1996;157:1840-5.
18. 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 U S A* 1996;93:2879-83.
19. Krieg AM, Yi AK, Matson S, et al. CpG motifs in bacterial DNA trigger direct B-cell activation. *Nature* 1995;374:546-9.
20. Sonehara K, Saito H, Kuramoto E, Yamamoto S, Yamamoto T, Tokunaga T. Hexamer palindromic oligonucleotides with 5'-CG-3' motif(s) induce production of interferon. *J Interferon Cytokine Res* 1996;16:799-803.
21. Liang H, Nishioka Y, Reich CF, Pisetsky DS, Lipsky PE. Activation of human B cells by phosphorothioate oligodeoxynucleotides. *J Clin Invest* 1996;98:1119-29.
22. Lipford GB, Sparwasser T, Bauer M, et al. Immunostimulatory DNA: sequence-dependent production of potentially harmful or useful cytokines. *Eur J Immunol* 1997;27:3420-6.
23. Chu RS, Targoni OS, Krieg AM, Lehmann PV, Harding CV. CpG oligodeoxynucleotides act as adjuvants that switch on T helper 1 (Th1) immunity. *J Exp Med* 1997;186:1623-31.
24. Yi AK, Klinman DM, Martin TL, Matson S, Krieg AM. Rapid immune activation by CpG motifs in bacterial DNA: systemic induction of IL-6 transcription through an antioxidant-sensitive pathway. *J Immunol* 1996;157:5394-402.
25. Yi AK, Chang M, Peckham DW, Krieg AM, Ashman RF. CpG oligodeoxynucleotides rescue mature spleen B cells from spontaneous apoptosis and promote cell cycle entry. *J Immunol* 1998;160:5898-906.
26. Lapatschek MS, Gilbert RL, Wagner H, Miethke T. Activation of macrophages and B lymphocytes by an oligodeoxynucleotide derived from an acutely pathogenic simian immunodeficiency virus. *Antisense Nucleic Acid Drug Dev* 1998;8:357-70.
27. Yi AK, Hornbeck P, Lafrenz DE, Krieg AM. CpG DNA rescue of murine B lymphoma cells from anti-IgM-induced growth arrest and programmed cell death is associated with increased expression of c-myc and bcl-xL. *J Immunol* 1996;157:4918-25.
28. Krieg AM, Wu T, Weeratna R, et al. Sequence motifs in adenoviral DNA block immune activation by stimulatory CpG motifs. *Proc Natl Acad Sci U S A* 1998;95:12631-6.
29. Tokunaga T, Yano O, Kuramoto E, et al. Synthetic oligonucleotides with particular base sequences from the cDNA encoding proteins of *Mycobacterium bovis* BCG induce interferons and activate natural killer cells. *Microbiol Immunol* 1992;36:55-66.
30. Kataoka T, Yamamoto S, Yamamoto T, et al. Antitumor activity of synthetic oligonucleotides with sequences from cDNA encoding proteins of *Mycobacterium bovis* BCG. *Jpn J Cancer Res* 1992;83:244-7.
31. Stacey KJ, Sweet MJ, Hume DA. Macrophages ingest and are activated by bacterial DNA. *J Immunol* 1996;157:2116-22.
32. Klinman DM, Yamshchikov G, Ishigatsubo Y. Contribution of CpG motifs to the immunogenicity of DNA vaccines. *J Immunol* 1997;158:3635-9.
33. Bird AP. DNA methylation and the frequency of CpG in animal DNA. *Nucleic Acids Res* 1980;8:1499-504.
34. Bird AP. CpG islands as gene markers in the vertebrate nucleus. *Trends Genet* 1987;3:342-7.
35. Bird AP. Functions for DNA methylation in vertebrates. *Cold Springs Harb Symp Quant Biol* 1993;58:281-5.
36. Bestor TH. The host defence function of genomic methylation patterns. *Novartis Found Symp* 1998;214:187-9, 95-9.
37. Bird AP, Taggart MH. Variable patterns of total DNA and rDNA methylation in animals. *Nucleic Acids Res* 1980;8:1485-97.
38. Yamamoto T, Yamamoto S, Kataoka T, Tokunaga T. Ability of oligonucleotides with certain palindromes to induce interferon production and augment natural killer cell activity is associated with their base length. *Antisense Res Dev* 1994;4:119-22.
39. Crooke RM, Graham MJ, Cooke ME, Crooke ST. In vitro pharmacokinetics of phosphorothioate antisense oligonucleotides. *J Pharmacol Exp Ther* 1995;275:462-73.
40. Cossum PA, Truong L, Owens SR, Markham PM, Shea JP, Crooke ST. Pharmacokinetics of a 14C-labeled phosphorothioate oligonucleotide, ISIS 2105, after intradermal administration to rats. *J Pharmacol Exp Ther* 1994;269:89-94.
41. Cowdery JS, Chace JH, Yi AK, Krieg AM. Bacterial DNA induces NK cells to produce IFN-gamma in vivo and increases the toxicity of lipopolysaccharides. *J Immunol* 1996;156:4570-5.
42. Halpern MD, Kurlander RJ, Pisetsky DS. Bacterial DNA induces murine interferon-gamma production by stimulation of interleukin-12 and tumor necrosis factor-alpha. *Cell Immunol* 1996;167:72-8.
43. Sparwasser T, Miethke T, Lipford G, et al. Bacterial DNA causes septic shock. *Nature* 1997;386:336-7.
44. Martin-Orozco E, Kobayashi H, Van Uden J, Nguyen M-D, Kornbluth RS, Raz E. Enhancement of antigen-presenting cell surface molecules involved in cognate interactions by immunostimulatory DNA sequences. *Int Immunol* 1999;11:1111-8.
45. Sparwasser T, Koch ES, Vabulas RM, et al. Bacterial DNA and immunostimulatory CpG oligonucleotides trigger maturation and activation of murine dendritic cells. *Eur J Immunol* 1998;28:2045-54.
46. Häcker H, Mischak H, Miethke T, et al. CpG-DNA-specific activation of antigen-presenting cells requires stress kinase activity and is preceded by non-specific endocytosis and endosomal maturation. *Embo J* 1998;17:6230-40.
47. Banchereau J, Steinman RM. Dendritic cells and the control of immunity. *Nature* 1998;392:245-52.
48. Messina JP, Gilkeson GS, Pisetsky DS. Stimulation of in vitro murine lymphocyte proliferation by bacterial DNA. *J Immunol* 1991;147:1759-64.
49. Yi AK, Chace JH, Cowdery JS, Krieg AM. IFN-gamma promotes IL-6 and IgM secretion in response to CpG motifs in bacterial DNA and oligodeoxynucleotides. *J Immunol* 1996;156:558-64.
50. Yi AK, Krieg AM. CpG DNA rescue from anti-IgM-induced WEHI-231 B lymphoma apoptosis via modulation of I kappa B alpha and I kappa B beta and sustained activation of nuclear factor-kappa B/c-Rel. *J Immunol* 1998;160:1240-5.
51. Macfarlane DE, Manzel L, Krieg AM. Unmethylated CpG-containing oligodeoxynucleotides inhibit apoptosis in WEHI 231 B lymphocytes induced by several agents: evidence for blockade of apoptosis at a distal signalling step. *Immunology* 1997;91:586-93.
52. Sun S, Zhang X, Tough DF, Sprent J. Type I interferon-mediated stimulation of T cells by CpG DNA. *J Exp Med* 1998;188:2335-42.
53. Broide D, Schwarze J, Tighe H, et al. Immunostimulatory DNA sequences inhibit IL-5, eosinophilic inflammation, and airway hyperresponsiveness in mice. *J Immunol* 1998;161:7054-62.
54. Lee DJ, Tighe H, Corr M, et al. Inhibition of IgE antibody formation by plasmid DNA immunization is mediated by both CD4+ and CD8+ T cells. *Int Arch Allergy Immunol* 1997;113:227-30.
55. Hsu CH, Chua KY, Tao MH, et al. Immunoprophylaxis of allergen-induced immunoglobulin E synthesis and airway hyperresponsiveness in vivo by genetic immunization. *Nat Med* 1996;2:540-4.

56. Raz E, Tighe H, Sato Y, et al. Preferential induction of a Th1 immune response and inhibition of specific IgE antibody formation by plasmid DNA immunization. *Proc Natl Acad Sci U S A* 1996;93:5141-5.
57. Raz E, Carson DA, Parker SE, et al. Intradermal gene immunization: the possible role of DNA uptake in the induction of cellular immunity to viruses. *Proc Natl Acad Sci U S A* 1994;91:9519-23.
58. Inchauspe G, Vitvitski L, Major ME, et al. Plasmid DNA expressing a secreted or a nonsecreted form of hepatitis C virus nucleocapsid: comparative studies of antibody and T-helper responses following genetic immunization. *DNA Cell Biol* 1997;16:185-95.
59. Ulmer JB, Donnelly JJ, Parker SE, et al. Heterologous protection against influenza by injection of DNA encoding a viral protein. *Science* 1993;259:1745-9.
60. Shiver JW, Davies ME, Perry HC, Freed DC, Liu MA. Humoral and cellular immunities elicited by HIV-1 vaccination. *J Pharm Sci* 1996;85:1317-24.
61. Tighe H, Corr M, Roman M, Raz E. Gene vaccination: plasmid DNA is more than just a blueprint. *Immunol Today* 1998;19:89-97.
62. Pertmer TM, Roberts TR, Haynes JR. Influenza virus nucleoprotein-specific immunoglobulin G subclass and cytokine responses elicited by DNA vaccination are dependent on the route of vector DNA delivery. *J Virol* 1996;70:6119-25.
63. Feltquate DM, Heaney S, Webster RG, Robinson HL. Different T helper cell types and antibody isotypes generated by saline and gene gun DNA immunization. *J Immunol* 1997;158:2278-84.
64. Fynan EF, Webster RG, Fuller DH, Haynes JR, Santoro JC, Robinson HL. DNA vaccines: protective immunizations by parenteral, mucosal, and gene-gun inoculations. *Proc Natl Acad Sci U S A* 1993;90:11478-82.
65. Bagarazzi ML, Boyer JD, Ugen KE, et al. Safety and immunogenicity of HIV-1 DNA constructs in chimpanzees. *Vaccine* 1998;16:p1836-41.
66. Ugen KE, Nyland SB, Boyer JD, et al. DNA vaccination with HIV-1 expressing constructs elicits immune responses in humans. *Vaccine* 1998;16:p1818-21.
67. Ockenhouse CF, Sun PF, Lanar DE, et al. Phase I/IIa safety, immunogenicity, and efficacy trial of NYVAC-Pf7, a pox-vectored, multiantigen, multistage vaccine candidate for *Plasmodium falciparum* malaria. *J Infect Dis* 1998;177:1664-73.
68. Wang R, Doolan DL, Le TP, et al. Induction of antigen-specific cytotoxic T lymphocytes in humans by a malaria DNA vaccine. *Science* 1998;282:p476-80.
69. Klinman DM, Barnhart KM, Conover J. CpG motifs as immune adjuvants. *Vaccine* 1999;17:19-25.
70. Yamamoto T, Yamamoto S, Kataoka T, Tokunaga T. Lipofection of synthetic oligodeoxyribonucleotide having a palindromic sequence of AACGTT to murine splenocytes enhances interferon production and natural killer activity. *Microbiol Immunol* 1994;38:831-6.
71. Yi AK, Krieg AM. Rapid induction of mitogen-activated protein kinases by immune stimulatory CpG DNA. *J Immunol* 1998;161:4493-7.
72. Yi AK, Tuetken R, Redford T, Waldschmidt M, Kirsch J, Krieg AM. CpG motifs in bacterial DNA activate leukocytes through the pH-dependent generation of reactive oxygen species. *J Immunol* 1998;160:4755-61.
73. Han S-S, Chung S-T, Robertson DA, Chelvarajan RL, Bondada S. CpG oligodeoxynucleotides rescue BKS-2 immature B cell lymphoma from anti-IgM-mediated growth inhibition by up-regulation of egr-1. *Int Immunol* 1999;11:871-9.
74. Romagnani S. Atopic allergy and other hypersensitivities editorial overview: technological advances and new insights into pathogenesis prelude novel therapeutic strategies. *Curr Opin Immunol* 1995;7:745-50.
75. Weber RW. Immunotherapy with allergens. *JAMA* 1997;278:1881-7.
76. Goodman JS, Van Uden JH, Kobayashi H, Broide D, Raz E. DNA immunotherapeutics: new potential treatment modalities for allergic disease. *Int Arch Allergy Immunol* 1998;116:177-87.
77. Kline JN, Waldschmidt TJ, Businga TR, et al. Modulation of airway inflammation by CpG oligodeoxynucleotides in a murine model of asthma. *J Immunol* 1998;160:2555-9.
78. Sur S, Wild JS, Choudhury BK, Sur N, Alam R, Klinman DM. Long term prevention of allergic lung inflammation in a mouse model of asthma by CpG oligodeoxynucleotides. *J Immunol* 1999;162:6284-93.
79. Broide D, Raz E. DNA-based immunization for asthma. *Int Arch Allergy Immunol* 1999;118:453-6.
80. Hsu CH, Chua KY, Tao MH, Huang SK, Hsieh KH. Inhibition of specific IgE response in vivo by allergen-gene transfer. *Int Immunol* 1996;8:1405-11.
81. Dinarello CA. Cytokines as mediators in the pathogenesis of septic shock. *Curr Top Microbiol Immunol* 1996;216:133-65.
82. Davis HL, Millan CL, Watkins SC. Immune-mediated destruction of transfected muscle fibers after direct gene transfer with antigen-expressing plasmid DNA. *Gene Ther* 1997;4:181-8.
83. Schwartz DA, Quinn TJ, Thorne PS, Sayeed S, Yi AK, Krieg AM. CpG motifs in bacterial DNA cause inflammation in the lower respiratory tract. *J Clin Invest* 1997;100:68-73.
84. Schwartz DA, Wohlford-Lenane CL, Quinn TJ, Krieg AM. Bacterial DNA or oligonucleotides containing unmethylated CpG motifs can minimize lipopolysaccharide-induced inflammation in the lower respiratory tract through an IL-12-dependent pathway. *J Immunol* 1999;163:224-31.
85. Sparwasser T, Hültner L, Koch ES, Luz A, Lipford GB, Wagner H. Immunostimulatory CpG-oligodeoxynucleotides cause extramedullary murine hemopoiesis. *J Immunol* 1999;162:2368-74.
86. Crooke ST. Therapeutic applications of oligonucleotides. Austin: RG Landes; 1995.
87. Segal BM, Klinman DM, Shevach EM. Microbial products induce autoimmune disease by an IL-12-dependent pathway. *J Immunol* 1997;158:5087-90.
88. Gilkeson GS, Grudier JP, Karounos DG, Pisetsky DS. Induction of anti-double stranded DNA antibodies in normal mice by immunization with bacterial DNA. *J Immunol* 1989;142:1482-6.
89. Gilkeson GS, Ruiz P, Howell D, Lefkowitz JB, Pisetsky DS. Induction of immune-mediated glomerulonephritis in normal mice immunized with bacterial DNA. *Clin Immunol Immunopathol* 1993;68:283-92.
90. Gilkeson GS, Phippen AM, Pisetsky DS. Induction of cross-reactive anti-dsDNA antibodies in preautoimmune NZB/NZW mice by immunization with bacterial DNA. *J Clin Invest* 1995;95:1398-402.
91. Gilkeson GS, Ruiz P, Phippen AM, Alexander AL, Lefkowitz JB, Pisetsky DS. Modulation of renal disease in autoimmune NZB/NZW mice by immunization with bacterial DNA. *J Exp Med* 1996;183:1389-97.