

Immunostimulatory DNA for Asthma Better than Eating Dirt

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Innate Immunity and the Hygiene Hypothesis

The innate immune system provides an early line of defense against infection by recognizing and processing evolutionarily conserved structural motifs, so-called "pathogen-associated molecular patterns (PAMP)," on bacteria, viruses, and other invading pathogens and responding to these "danger signals" by priming the adaptive immune system for appropriate action. Macrophages, dendritic cells, and other cells of the innate immune system lack the highly-specific antigen receptors of T cells and B cells of the adaptive immune system, but can recognize these foreign motifs by a limited number of germline-encoded "pattern recognition receptors (PRRs)." The binding of PAMP to PRR can enhance phagocytosis, cell proliferation, and cytokine expression, and may have profound and long-lasting immunostimulatory effects. These effects are essential for the elimination of infectious organisms, but may also be important for immune system development and homeostasis (1).

In the absence of early life PAMP stimulation, immune balance may be upset and individuals may be susceptible to the development of disease reflecting a skewing of the immune system toward the T-helper type 2 (T_H2) phenotype. This concept, known as the "hygiene hypothesis," is thought to be a major reason for the dramatic increase in asthma prevalence in Western countries over the past 40 years (2–4). The developing immune system of humans has a T_H2 bias *in utero* and in early life that gradually diminishes over time in individuals without asthma. Exposure to PAMP during childhood infections may provide a T_H1 stimulus that diminishes T_H2 responses and the likelihood of developing asthma. Furthermore, exposure to PAMP may dampen both T_H1 and T_H2 immune responses by increasing the expression of anti-inflammatory cytokines such as interleukin-10 and trans-

forming growth factor- $\beta 1$ (TGF- $\beta 1$) from T regulatory (Tr) cells and antigen-presenting cells (4). The "hygiene hypothesis" predicts that asthma is increasing in highly developed regions of the world because of decreased exposure to PAMP in affluent settings and disturbing the homeostasis of a human immune system that has evolved to fight infection. Is there a way, other than exposure to childhood disease, to keep the immune system in balance?

One way of modifying immune responses is to use immunostimulatory DNA; these are specific DNA sequences that have been shown by several groups to be effective in the prevention and treatment of allergen-induced airway inflammation in animals, and may have a role in the prevention and treatment of asthma. In this issue of the *AJRCMB*, Ikeda and colleagues compared the ability of immunostimulatory DNA and glucocorticosteroids to treat allergen-induced airway inflammation in mice (5). Does the use of immunostimulatory DNA make sense for asthma treatment?

Immunostimulatory Effects of CpG DNA

For more than a century it has been known that bacterial extracts have immunostimulatory effects that can be used to fight disease. Important work over the past 10 years by a number of investigators has proven that many of these effects are mediated by unmethylated CpG dinucleotides in the DNA of bacteria (6–9). CpG dinucleotides are common in bacterial genomes, occurring at the expected frequency of ~ 1 in 16 dinucleotides, and less than 5% of the cytosines in these dinucleotides are methylated. In contrast, CpG dinucleotides are greatly underrepresented in vertebrate genomes, occurring at a frequency of ~ 1 in 125 dinucleotides, and 70–90% of the cytosines in these dinucleotides are methylated, which greatly diminishes their immunostimulatory effects (7, 10). Moreover, the sequences flanking CpG dinucleotides are not random in vertebrates or bacteria, and can dramatically impact the immunostimulatory activity of the DNA sequence. In general, strong immunostimulatory effects are elicited by unmethylated CpG sequences within a palindromic hexamer that follows the formula 5'- $R_1R_2CGY_1Y_2$ -3', where R_1 is a purine (preference for G), R_2 is a purine or T, and Y_1 and Y_2 are pyrimidines (e.g., 5'-GACGTC-3', 5'-AGCGCT-3', and 5'-AACGTT-3') (8, 11). In vertebrates, the most common 5' flanking base is C and the most common 3' flanking base is a G, which produces a sequence that does not evoke a strong

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Abbreviations: interleukin, IL; immunostimulatory sequence-ODN, ISS-ODN; natural killer, NK; oligodeoxynucleotides, ODN; pathogen-associated molecular patterns, PAMP; pattern recognition receptors, PRRs; transforming-growth factor- $\beta 1$, TGF- $\beta 1$; T helper, T_H ; toll-like receptor 9, TLR9; T regulatory, Tr.

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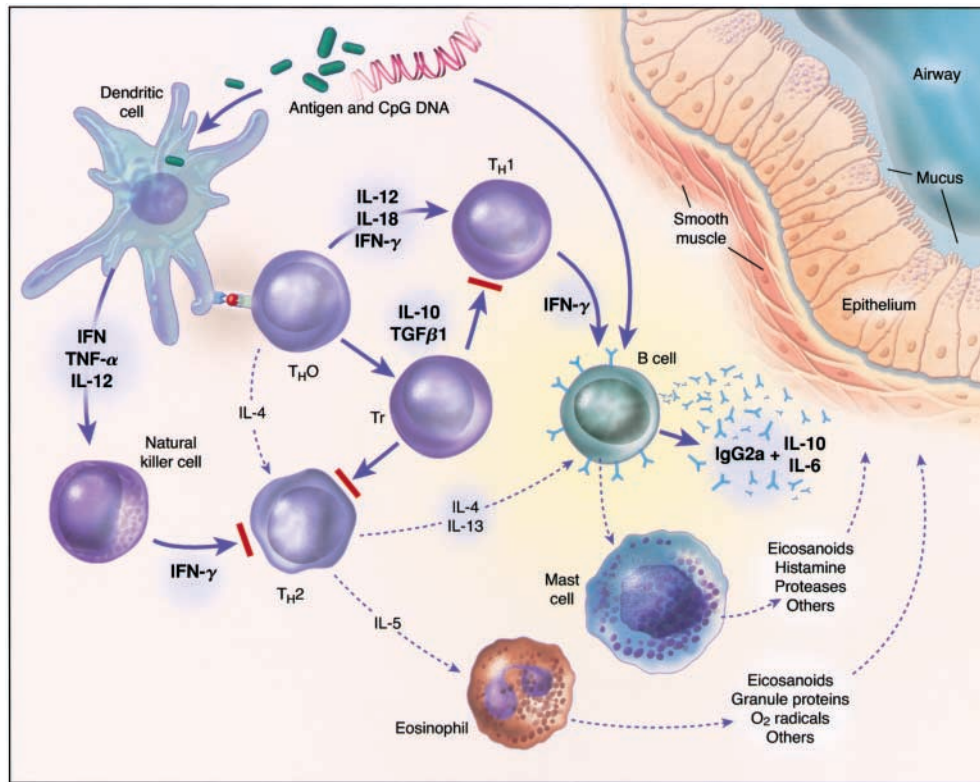


Figure 1. Immunomodulatory effects of CpG DNA. Allergen is presented by dendritic cells to CD4⁺ T_H0 lymphocytes and may result in their differentiation into T_H2 cytokine-producing cells. Interleukin (IL)-4, IL-13, and IL-5 regulate the asthmatic response. IL-4 directs the growth and differentiation of T cells toward a T_H2 cytokine profile. IL-4 and IL-13 regulate the synthesis of IgE by B cells. IL-5 regulates the differentiation and egress of eosinophils from the bone marrow into the blood. Tissue eosinophils and activated mast cells release a variety of inflammatory mediators that increase mucus production, airway edema, and smooth muscle contraction. In general, CpG DNA elicits T_H1-biased immune responses that prevent or reverse T_H2-mediated inflammation. CpG DNA activated pathways and critical mediator are shown in **bold**. CpG DNA-inhibited pathways are shown with *dashed lines*. ISS-ODN increase production of T_H1

cytokines interleukin (IL)-12, IL-18, interferon- γ , and IL-6, and decreased production of T_H2 cytokines IL-4 and IL-5. T cells are inactivated by increased production of anti-inflammatory cytokines IL-10 and TGF- β 1 by dendritic and T regulatory (Tr) cells. B-cells proliferate and produce IgG2 antibody. Natural killer (NK) cells are activated by IL-12 and IL-18. Modified from Ref. 4 with permission.

immunostimulatory effect and may actually antagonize the effects of more potent immunostimulatory sequences (7, 12). It has been hypothesized that evolutionary forces have suppressed immunostimulatory motifs in the genomic DNA of vertebrates so that PRR can more effectively distinguish endogenous DNA from the DNA of foreign invaders. It is also likely that evolutionary forces have suppressed immunostimulatory motifs in certain pathogens to avoid their detection and elimination (13).

The immunostimulatory effects of bacterial DNA can be reproduced *in vivo* by synthetic oligodeoxynucleotides (ODN) containing unmethylated CpG dinucleotides, so-called immunostimulatory sequence-ODN (ISS-ODN). The effects of ISS-ODN on the immune system depend on the specific sequence, animal species, dose, time course, and route of delivery, so generalizations must be made with care. For example, the sequence GACGTT activates innate immune cells of the mouse much more efficiently than similar cells from humans (9). The sequence, GTCGTT confers optimal immunostimulatory effects in human cells (14). Some of the differences in response to ISS-ODN variants between species appear to be caused by genetically determined toll-like receptor 9 (TLR9) sequence-binding selectivity (15). Chemical modifications are being systematically incorporated into ISS-ODN to design second generation reagents with more potent anti-inflammatory effects and less potential for adverse reactions (16).

In general, ISS-ODN elicit T_H1-biased immune responses

in mammals that may prevent and reverse T_H2-mediated inflammation associated with asthma and other allergic disease. CpG DNA directly activates dendritic cells and B-cells that express TLR9, a transmembrane receptor that binds CpG DNA and transduces its immunostimulatory effects (17–19), and indirectly activates other cells of the immune system such as T cells, NK cells, and neutrophils, by altering the expression of specific cytokines, receptors, costimulatory molecules, and adhesion molecules. Figure 1 is a simplified scheme of how these immunostimulatory events may block or reverse the T_H2-mediated inflammation associated with asthma and other allergic disease. A detailed discussion of these complex immunomodulatory effects is beyond the scope of this Perspective. Comprehensive reviews of the effects of CpG DNA on the immune system are available (7, 19).

ISS-ODN Therapy for Airway Inflammation: Animal Models

Ikeda and colleagues, as well as others, have shown that ISS-ODN treatment decreases eosinophilic airway inflammation, blood and bone marrow eosinophilia, IgE levels, T_H2 cytokine expression (interleukin [IL]-5), and airway hyperresponsiveness, and increases the expression of T_H1 cytokines (interferon- γ , IL-12) and anti-inflammatory cytokines (TGF- β 1) (5, 20–25). It has also been shown that ISS-ODN attenuate allergen-induced airway remodeling in mice as manifested by decreased subepithelial collagen deposition

and goblet cell metaplasia (26). ISS-ODN may be effective in the context of airway inflammation caused by infectious agents. Administration of ISS-ODN before respiratory syncytial virus infection inhibited viral replication, airway inflammation, and mucus secretion, but not airway hyper-responsiveness (27). The minor inconsistencies in some of the many studies examining the role of ISS-ODN in airway inflammation may be due to differences in the mouse strain and allergen studied, specific ISS-ODN sequence, and timing, frequency, and route of administration.

ISS-ODN have an effect in animal models similar to those observed with asthma treatments in current use (28, 29). For example, one treatment of ISS-ODN was as effective as seven daily injections of dexamethasone in protecting sensitized mice from inflammation with nebulized allergen challenge (23). Ikeda and colleagues have now shown that the combination of ISS-ODN and corticosteroid is more effective at treating established inflammation than either agent alone and discuss some of the distinct and shared anti-inflammatory pathways by which these agents mediate their effects (5). On the downside, the effects of ISS-ODN therapy appear to be transient, suggesting that optimal ISS-ODN therapy may require repeat administrations (20). The combination of ISS-ODN with allergen in the form of allergen-ISS conjugates may offer longer-lasting and allergen-specific protection (30).

ISS-ODN Therapy for Asthma: Will It Work?

ISS-ODN are just beginning to be tested in human asthma. If the murine models are a true beacon, we may find that this form of immunotherapy can decrease many of the inflammatory effects characteristic of human asthma. Although ISS-ODN treatment may be better than eating dirt as a way to keep our immune system in balance (2), it makes sense to be concerned about the effects of too much immune suppression or too marked a skew toward the T_H1 phenotype. Phase 1 clinical trials suggest that ISS-ODN are well tolerated in humans, but long-term effects are unknown (7, 31). In a choice between autoimmune hepatitis or diabetes and asthma, most people would choose asthma.

References

- Janeway, C. A., Jr., and R. Medzhitov. 2002. Innate immune recognition. *Annu. Rev. Immunol.* 20:197–216.
- Weiss, S. T. 2002. Eat dirt—the hygiene hypothesis and allergic diseases. *N. Engl. J. Med.* 347:930–931.
- Yazdanbakhsh, M., P. G. Kremsner, and R. van Ree. 2002. Allergy, parasites, and the hygiene hypothesis. *Science* 296:490–494.
- Wills-Karp, M., J. Santeliz, and C. L. Karp. 2001. The germless theory of allergic disease: revisiting the hygiene hypothesis. *Nat. Rev. Immunol.* 1: 69–75.
- Ikeda, R. K., J. Nayar, J. Y. Cho, M. Miller, M. Rodriguez, E. Raz, and D. H. Broide. 2003. Resolution of airway inflammation following ovalbumin inhalation: comparison of ISS DNA and corticosteroids. *Am. J. Respir. Cell Mol. Biol.* 28:655–663.
- Tokunaga, T., T. Yamamoto, and S. Yamamoto. 1999. How BCG led to the discovery of immunostimulatory DNA. *Jpn. J. Infect. Dis.* 52:1–11.
- Krieg, A. M. 2002. CpG motifs in bacterial DNA and their immune effects. *Annu. Rev. Immunol.* 20:709–760.
- Horner, A. A., J. H. Van Uden, J. M. Zubeldia, D. Broide, and E. Raz. 2001. DNA-based immunotherapeutics for the treatment of allergic disease. *Immunol. Rev.* 179:102–118.
- Krieg, A. M., A. K. Yi, S. Matson, T. J. Waldschmidt, G. A. Bishop, R. Teasdale, G. A. Koretzky, and D. M. Klinman. 1995. CpG motifs in bacterial DNA trigger direct B-cell activation. *Nature* 374:546–549.
- Lander, E. S., L. M. Linton, B. Birren, C. Nusbaum, M. C. Zody, J. Baldwin, K. Devon, K. Dewar, M. Doyle, W. FitzHugh, R. Funke, D. Gage, K. Harris, A. Heaford, J. Howland, et al. 2001. Initial sequencing and analysis of the human genome. *Nature* 409:860–921.
- Yamamoto, S., T. Yamamoto, T. Kataoka, E. Kuramoto, O. Yano, and T. Tokunaga. 1992. Unique palindromic sequences in synthetic oligonucleotides are required to induce IFN and augment IFN-mediated natural killer activity. *J. Immunol.* 148:4072–4076.
- Krieg, A. M., T. Wu, R. Weeratna, S. M. Efler, L. Love-Homan, L. Yang, A. K. Yi, D. Short, and H. L. Davis. 1998. Sequence motifs in adenoviral DNA block immune activation by stimulatory CpG motifs. *Proc. Natl. Acad. Sci. USA* 95:12631–12636.
- Karlin, S., W. Doerfler, and L. R. Cardon. 1994. Why is CpG suppressed in the genomes of virtually all small eukaryotic viruses but not in those of large eukaryotic viruses? *J. Virol.* 68:2889–2897.
- Hartmann, G., and A. M. Krieg. 2000. Mechanism and function of a newly identified CpG DNA motif in human primary B cells. *J. Immunol.* 164: 944–953.
- Bauer, S., C. J. Kirschning, H. Hacker, V. Redecke, S. Hausmann, S. Akira, H. Wagner, and G. B. Lipford. 2001. Human TLR9 confers responsiveness to bacterial DNA via species-specific CpG motif recognition. *Proc. Natl. Acad. Sci. USA* 98:9237–9242.
- Agrawal, S., and E. R. Kandimalla. 2002. Medicinal chemistry and therapeutic potential of CpG DNA. *Trends Mol. Med.* 8:114–121.
- Hemmi, H., O. Takeuchi, T. Kawai, T. Kaisho, S. Sato, H. Sanjo, M. Matsumoto, K. Hoshino, H. Wagner, K. Takeda, and S. Akira. 2000. A Toll-like receptor recognizes bacterial DNA. *Nature* 408:740–745.
- Wagner, H. 2002. Interactions between bacterial CpG-DNA and TLR9 bridge innate and adaptive immunity. *Curr. Opin. Microbiol.* 5:62–69.
- Ashkar, A. A., and K. L. Rosenthal. 2002. Toll-like receptor 9, CpG DNA and innate immunity. *Curr. Mol. Med.* 2:545–556.
- Broide, D. H., G. Stachnick, D. Castaneda, J. Nayar, M. Miller, J. Y. Cho, M. Roman, J. Zubeldia, T. Hayashi, E. Raz, and T. Hyashi. 2001. Systemic administration of immunostimulatory DNA sequences mediates reversible inhibition of Th2 responses in a mouse model of asthma. *J. Clin. Immunol.* 21:175–182.
- Choudhury, B. K., J. S. Wild, R. Alam, D. M. Klinman, I. Boldogh, N. Dharajiya, W. J. Mileski, and S. Sur. 2002. *In vivo* role of p38 mitogen-activated protein kinase in mediating the anti-inflammatory effects of CpG oligodeoxynucleotide in murine asthma. *J. Immunol.* 169:5955–5961.
- Sur, S., J. S. Wild, B. K. Choudhury, N. Sur, R. Alam, and D. M. Klinman. 1999. Long term prevention of allergic lung inflammation in a mouse model of asthma by CpG oligodeoxynucleotides. *J. Immunol.* 162:6284–6293.
- Broide, D., J. Schwarze, H. Tighe, T. Gifford, M. D. Nguyen, S. Malek, J. Van Uden, E. Martin-Orozco, E. W. Gelfand, and E. Raz. 1998. Immunostimulatory DNA sequences inhibit IL-5, eosinophilic inflammation, and airway hyperresponsiveness in mice. *J. Immunol.* 161:7054–7062.
- Kline, J. N., T. J. Waldschmidt, T. R. Businga, J. E. Lemish, J. V. Weinstock, P. S. Thorne, and A. M. Krieg. 1998. Modulation of airway inflammation by CpG oligodeoxynucleotides in a murine model of asthma. *J. Immunol.* 160:2555–2559.
- Santeliz, J. V., G. Van Nest, P. Traquina, E. Larsen, and M. Wills-Karp. 2002. A 1-linked CpG oligodeoxynucleotides reverse established airway hyperresponsiveness in a murine model of asthma. *J. Allergy Clin. Immunol.* 109:455–462.
- Jain, V. V., K. Kitagaki, T. Businga, I. Hussain, C. George, P. O'Shaughnessy, and J. N. Kline. 2002. CpG-oligodeoxynucleotides inhibit airway remodeling in a murine model of chronic asthma. *J. Allergy Clin. Immunol.* 110: 867–872.
- Cho, J. Y., M. Miller, K. J. Baek, D. Castaneda, J. Nayar, M. Roman, E. Raz, and D. H. Broide. 2001. Immunostimulatory DNA sequences inhibit respiratory syncytial viral load, airway inflammation, and mucus secretion. *J. Allergy Clin. Immunol.* 108:697–702.
- Broide, D., J. Y. Cho, M. Miller, J. Nayar, G. Stachnick, D. Castaneda, M. Roman, and E. Raz. 2000. Modulation of asthmatic response by immunostimulatory DNA sequences. *Springer Semin. Immunopathol.* 22:117–124.
- Hussain, I., and J. N. Kline. 2001. CpG oligodeoxynucleotides in asthma. *Curr. Opin. Investig. Drugs* 2:914–918.
- Horner, A. A., and E. Raz. 2002. Immunostimulatory sequence oligodeoxynucleotide-based vaccination and immunomodulation: two unique but complementary strategies for the treatment of allergic diseases. *J. Allergy Clin. Immunol.* 110:706–712.
- Krieg, A. M. 2002. From A to Z on CpG. *Trends Immunol.* 23:64–65.