

# Prevention of Acute Allergic Conjunctivitis and Late-Phase Inflammation with Immunostimulatory DNA Sequences

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**PURPOSE.** To evaluate the therapeutic potential of immunostimulatory sequence oligodeoxynucleotides (ISS-ODN) administration in ocular allergy, using a mouse model of ragweed-specific conjunctivitis.

**METHODS.** A murine model of allergic conjunctivitis involving SWR/J mice sensitized and challenged with short ragweed was used to test immunostimulatory DNA sequences for therapeutic potential. ISS-ODN or control ODN (0.1 mg/mouse) was administered intraperitoneally or topically to the conjunctiva 3 days before final allergen challenge. Multiple parameters of clinical symptoms evident during the acute-phase reaction and the cellular components of the late-phase reaction were evaluated in both groups of mice.

**RESULTS.** All parameters of clinical symptoms were markedly inhibited after intraperitoneal injection of ISS-ODN, whereas topical application to the conjunctiva did not inhibit clinical symptoms significantly. Remarkably, a single topical treatment with ISS-ODN (as well as by intraperitoneal injection) completely inhibited both eosinophilia and neutrophilia in the late-phase reaction.

**CONCLUSIONS.** Systemic or conjunctival administration of ISS-ODN was shown to significantly inhibit allergic responses in this mouse model. This indicates that ISS-ODN may be an effective form of immunotherapy for this class of allergic disease. (*Invest Ophthalmol Vis Sci.* 2000;41:3850–3855)

Seasonal allergic conjunctivitis (SAC) is the most common allergic disease, with over 40 million patients affected in the United States alone.<sup>1</sup> More severe forms of ocular allergy, such as vernal conjunctivitis (VC), also frequently exhibit an IgE-mediated pathogenesis. In both severe SAC and VC, the allergic response can interfere with normal vision, and damage to the cornea can be a consequence.<sup>2</sup> As a result, allergic conjunctivitis constitutes a significant medical problem, absorbing an inordinately high proportion of the ophthalmic health care budget. Despite this, current treatment regimens are grossly inadequate, spurring the intensive search for new and more effective drugs.

Ocular allergies are most commonly initiated in response to airborne allergens,<sup>1,2,3</sup> with short ragweed (SRW) pollen being the major cause of late summer rhinoconjunctivitis in North America.<sup>4</sup> In Japan, allergic responses to cedar pollen constitute a problem of even greater relative magnitude. Antihistamines or steroids are commonly used to treat ocular allergies; however, they only provide temporary relief for a subset of patients. Moreover, use of steroids can increase the inci-

dence of serious complications such as the development of glaucoma or cataracts.

For these reasons, immunotherapy may be preferable for providing long-term relief without medication. Conventional immunotherapy through desensitization by repeated exposure of low-dose allergen has achieved some success, although the majority of patients remain unresponsive. Recently, considerable interest has materialized in the potential use of immunostimulatory sequence oligodeoxynucleotides (ISS-ODN) in the treatment of allergic diseases.<sup>5–8</sup> This field developed from the finding by Tokunaga et al.<sup>9</sup> that bacterial DNA has immunostimulatory effects. After years of searching for the responsible sequences, Krieg et al.<sup>10</sup> reported that the unmethylated Cytidine-phosphate-Guanosine (CpG) dinucleotide motif has immunostimulatory activity. These ISS-containing CpG motifs have now been shown to bias the immune response toward the development of an antigenspecific T helper (Th) cell type 1 response.<sup>11,12</sup> Indeed, ISS-ODNs have been strong therapeutic agents in murine models of airway inflammation.<sup>12–14</sup>

As is the case for allergic reactions in other mucosal tissues, conjunctival allergy results from a prevalent Th2 response to allergen (as determined by the analysis of conjunctiva-derived T-cell clones from patients with SAC).<sup>15,16</sup> Thus, skewing of the Th1–Th2 response to an allergen in patients with SAC and VC could have significant therapeutic value, as has been shown in studies of airway hyperresponsiveness and allergic responses in the skin.

We show in this study that intraperitoneal injection of ISS-ODN prevents clinical effects of SAC in a mouse model, and effectively suppresses the late-phase reaction in the conjunctiva. Moreover, although a single topical administration of ISS-ODN does not significantly affect the acute-phase reaction

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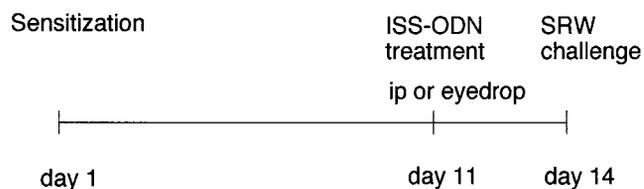
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**FIGURE 1.** Immunization and ISS-ODN treatment protocol. Mice were sensitized by footpad injection of 50  $\mu$ g of SRW with alum or alum only on day 1. ISS-ODN or control ODN was administered once at a dose of 100  $\mu$ g, either by intraperitoneal injection or eyedrop in PBS on day 11. All mice were challenged with SRW suspension (1.5 mg of SRW/eye) on day 14, and clinical scores and late-phase reaction were evaluated.

in this model, it completely inhibits inflammatory cell recruitment in late-phase reaction. Together with a recent report, these data indicate that ISS-ODN treatment of conjunctival allergy may be a highly promising future immunotherapy for this major human allergy.<sup>17</sup>

## MATERIALS AND METHODS

### Animals

Female 6-week-old SWR/J mice were obtained from Jackson Laboratory (Bar Harbor, ME). To prevent any contamination of allergen, all mice were kept in cages with filter-topped lids. The present study conformed to the principles for laboratory animal research outlined by the Animal Welfare Act and the National Institutes of Health guidelines for the experimental use of animals. The work also complied with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

### Allergen Challenge Protocol

Mice were sensitized to SRW pollen according to the protocol originally reported by Magone et al.<sup>18</sup> A suspension of 50  $\mu$ g of SRW (ICN, Aurora OH) and 1 mg of aluminum hydroxide (Sigma, St. Louis, MO) were injected into the left hind footpad under anesthesia. On day 14, conjunctivitis was induced by topical application of 1.5 mg SRW suspended in 10  $\mu$ l of phosphate-buffered saline (PBS; Fig. 1).

### ISS-ODN Administration Protocol

Endotoxin-free phosphorothioate ISS-ODN 5'-TGACTGTGAACGTTTCGAGATGA-3' or control ODN 5'-TGACTGTGAAGGTTAGAGATGA-3' were prepared as previously described<sup>11</sup> and used in the in vivo experiments. ISS-ODN is reported to have a prepriming effect as an immune modulator,<sup>19</sup> exerting an effect when administered 3 days before antigen challenge. To test the therapeutic efficacy on allergic conjunctivitis, we administered ISS-ODN once, 3 days before final SRW challenge, intraperitoneally (0.1 mg/mice) or topically (as an eyedrop, 0.05 mg/eye; Fig. 1).

### Clinical Evaluation

Mice were observed in a double-blind study for clinical symptoms of immediate hypersensitivity response 20 minutes after the topical challenge with SRW. Chemosis, conjunctival redness, lid edema and redness, and tearing and discharge were scored 0 to 4, after evaluation by slit lamp, according to the

modified criteria described by Magone et al.<sup>18</sup> For assessment of severity of the symptoms, representative photographs of the mouse eyes were used. The cumulative clinical score was calculated as the sum of the scores of each of these four parameters.

### Histology

To evaluate the cellular infiltrates in the conjunctiva during the late-phase reaction, eyes were enucleated with the attached lids and conjunctiva intact (24 hours after SRW challenge) and immediately fixed in 4% paraformaldehyde. The tissue was then embedded in resin (Histo-resin; Leica Instruments, Heidelberg, Germany) and serially sectioned (3  $\mu$ m thick). The sagittal sections were stained with Giemsa or hematoxylin and eosin. Five conjunctival tissue sections from each eye were examined and counted by a masked observer under a microscope, using a  $\times 400$  high-powered field.

### Statistical Analysis

Data were analyzed using Mann-Whitney test or ANOVA, as appropriate, and differences were considered significant at  $P < 0.05$ .

## RESULTS

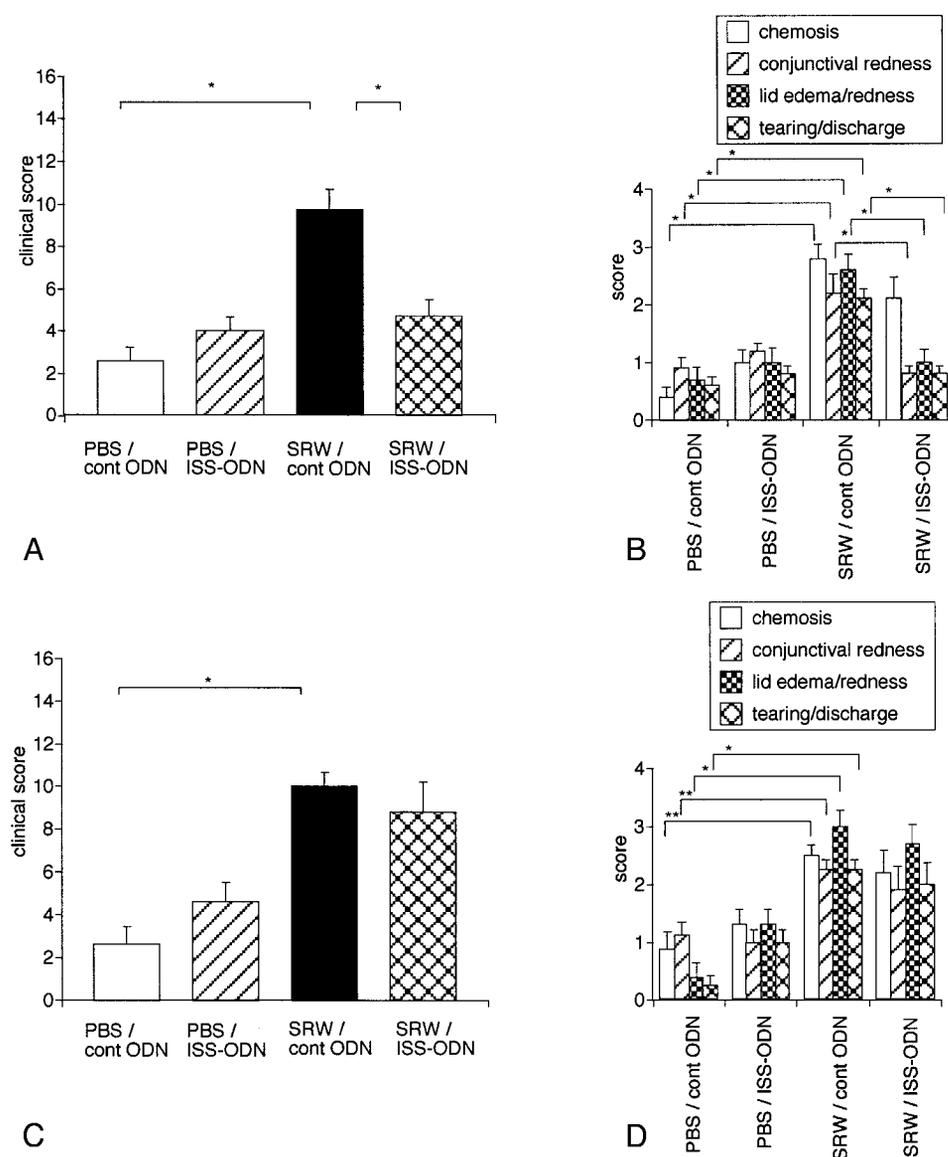
### Prevention of Acute-Phase Reaction by Intraperitoneal, but Not Topical ISS-ODN Treatment

Three days before final allergen challenge, mice were treated by intraperitoneal injection of ISS-ODN or control ODN (0.1 mg/mouse). The mice were then challenged with SRW (14 days after initial sensitization). This provoked clear clinical symptoms, including conjunctival redness, lid edema and redness, and tearing and discharge in the SRW-immunized group. The total clinical response (as well as each symptom measured) was markedly inhibited by treatment with ISS-ODN intraperitoneal injection (Fig. 2A). Each symptom, including conjunctival redness, lid edema and redness, and tearing and discharge, was significantly inhibited, whereas reduction of chemosis scores was not significant ( $P = 0.07$ , Fig. 2B).

To determine whether ISS-ODN could also be effective as a topical treatment of allergic conjunctivitis, we treated SRW-sensitized mice with a single topical instillation of ISS-ODN 3 days before final challenge. In contrast to the systemic administration, clinical symptoms including conjunctival edema, conjunctival redness, lid edema and redness, and tearing and discharge were not inhibited significantly with this dose regimen (Fig. 2C, 2D). Furthermore, to examine whether longer pre-priming may improve therapeutic effect on the clinical symptoms, we also treated the animals from day 0 to day 3 using ISS-ODN eyedrops. However, the inhibitory effect on clinical symptoms corresponded to the effect shown in Figure 2, and no significant inhibition was detected (data not shown).

### Inhibition of Late-Phase Reaction by Both Systemic and Topical ISS-ODN Treatment

The late-phase reaction of antigen-challenged conjunctival tissue is characterized by a profound inflammatory cell recruitment consisting of eosinophils and neutrophils. This chronic inflammation is pronounced in cases of severe SAC and VC. We



**FIGURE 2.** Effect of ISS-ODN administration on clinical symptoms of allergic conjunctivitis. (A, B) Inhibition of clinical symptoms by intraperitoneal ISS-ODN administration. The clinical score is a composite of clinical evaluation of allergic conjunctivitis with chemosis, conjunctival redness, lid edema and redness, and tearing and discharge as symptoms.<sup>18</sup> The maximum possible clinical score is 16. All groups of mice were challenged with SRW 3 days after ISS-ODN (or control ODN) injection. PBS/Cont ODN, nonsensitized SRW-challenged mice treated with control ODN; PBS/ISS-ODN, nonsensitized SRW-challenged mice treated with ISS-ODN; SRW/control ODN, sensitized SRW-challenged mice treated with control ODN; SRW/ISS-ODN, sensitized SRW-challenged mice treated with ISS-ODN.  $n = 10/\text{group}$ ,  $*P < 0.001$ . (C, D) Effect of topical administration of ISS-ODN on clinical symptoms. All groups of mice were challenged with SRW 3 days after a single instillation of ISS-ODN (or control ODN) as an eyedrop ( $n = 10/\text{group}$ ,  $*P < 0.001$ ;  $**P < 0.05$ ).

examined whether systemic or topical ISS-ODN treatment could inhibit the late-phase reaction in this mouse model. Conjunctival eosinophilia was evaluated by counting eosinophils attracted to the fornical region of the conjunctiva. SRW challenge to the sensitized mice (control animals and those treated with control ODN) resulted in a massive eosinophil recruitment into the conjunctival fornix, as expected. Intraperitoneal injection of ISS-ODN abolished the conjunctival eosinophilia, consistent with that observed in lung eosinophilia. Surprisingly, a single topical application of ISS-ODN also abolished eosinophil recruitment, although the effect on clinical symptoms was insufficient (Fig. 3).

Because neutrophilia is a hallmark of the late-phase reaction in SAC, we also evaluated whether ISS-ODN treatment had any effect on neutrophil attraction. As is shown in Figure 3B, SRW-sensitized mice treated with control ODN showed significant neutrophilia in the conjunctival fornix compared with the PBS-treated (allergen-challenged) mice. The conjunctival neutrophilia was completely abolished by the single topical application of ISS-ODN (Fig. 3B). Thus, two key parameters of chronic inflammation in ocular allergy: Eosinophilia and neu-

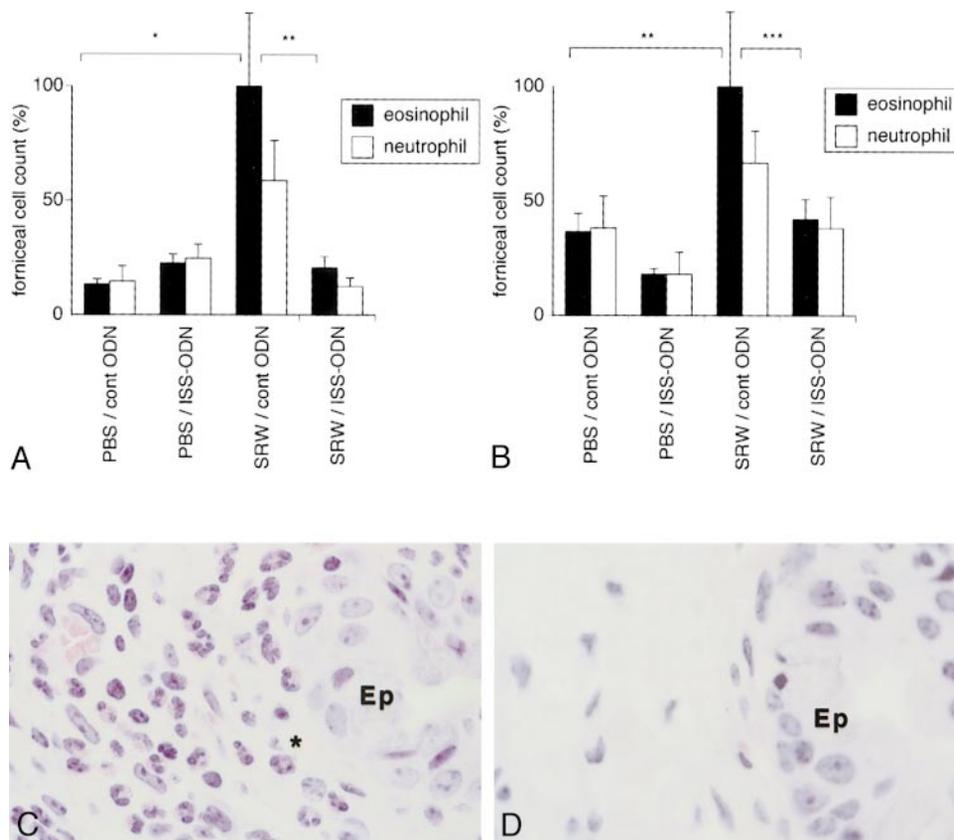
trophilia are shown to be markedly inhibited by a single topical treatment with ISS-ODN.

## DISCUSSION

In this study, the results demonstrate that ISS-ODN was an effective treatment for ocular allergy, as assessed in an SRW-induced mouse model of SAC. Systemic treatment with ISS-ODN markedly inhibited clinical parameters of SAC (Fig. 2), and blocked conjunctival eosinophilia in the late-phase reaction (Fig. 2). The current data also show that ISS-ODN treatment effectively blocked neutrophilia, a hallmark of the late-phase reaction in SAC (Fig. 3). Thus, ISS-ODN may be a versatile antiallergic compound, exhibiting efficacy in various mucosal tissues. We also have shown that a single topical application of ISS-ODN effectively inhibited both eosinophilia and neutrophilia, although it did not affect the acute-phase reaction with this dose regimen.

These experiments also raise interesting questions about the mechanism of action of ISS-ODN. As discussed previously,

**FIGURE 3.** Prevention of the late-phase reaction by systemic or topical ISS-ODN administration. **(A)** Inhibition of eosinophil and neutrophil recruitment into the conjunctiva by systemic administration of ISS-ODN. All groups of mice were challenged with SRW and evaluated for histology 24 hours after challenge. Inflammatory cells present in the conjunctival fornix were counted under a  $\times 400$  field ( $n = 10/\text{group}$ ,  $*P < 0.001$ ,  $**P < 0.01$ ). **(B)** Inhibition of eosinophil and neutrophil recruitment into the conjunctiva by topical administration of ISS-ODN. **(C)** Histology of representative conjunctival section from a sensitized SRW-challenged and control ODN-treated (by topical application) mouse. (\*), Eosinophil; Ep, conjunctival epithelium. **(D)** Histology of representative conjunctival section from sensitized, SRW-challenged, and ISS-ODN treated (by topical application) mouse. Note the dramatic inhibition of the late-phase cellular infiltrate. **(D, E)** Hematoxylin and eosin; magnification,  $\times 1000$ . Abbreviations defined in Figure 2.



bacterial DNA was initially shown to have immune stimulatory properties<sup>9</sup> and the responsible element was found to be the CpG motif now known as ISS-ODN. ISS-ODN are known to affect both the innate immune system (by dendritic cells and macrophages) and the acquired immune system (by activation of B cells). The dendritic cells and macrophages have two major roles in priming the immune responses. First, antigen-presenting cells (APCs) process antigens and present antigenic peptides to T cells through class II major histocompatibility complex (MHC) molecules. Second, APCs secrete proinflammatory cytokines, leading to T- and B-cell activation. Because polarization of activated T-helper cells into Th1 or Th2 phenotypes is observed in ISS-ODN-treated animals, the APCs are thought to be a major target of ISS-ODN with respect to the deviation toward the Th1 phenotype.

It is now known that ISS-ODN induce the expression of various cytokines that in total contribute to the skewing of the T-cell response toward the Th1 phenotype systemically.<sup>14</sup> Using *in vivo* administration of ISS-ODN, splenic antigen-presenting cells (APCs) efficiently activate naïve T cells and bias their differentiation toward a Th1 phenotype.<sup>20</sup> Importantly, ISS-ODN stimulate APCs to upregulate the Th1-like cytokines interleukin (IL)-12 and IL-18.<sup>7</sup> In addition, ISS-ODN induces IL-2 receptor and interferon- $\gamma$  receptor expression on macrophages and B cells.<sup>21</sup> Systemically, ISS-ODN administration is known to promote high IgG2a titers and lower IgG1 and IgE titers and reduce serum IL-4 and IL-5 levels, also dampening the allergic response.<sup>6,22</sup>

Using murine allergic airway models, three research groups have shown that ISS-ODN treatment can effectively inhibit airway hyperresponsiveness and inflammation.<sup>12-14</sup> Al-

though interferon  $\gamma$  and IL-12 usually oppose Th2-mediated inflammation, Kline et al.<sup>22</sup> also showed that neither of these cytokines is absolutely required, by using allergen-sensitized knockout mice, which suggests an IL-12/interferon- $\gamma$ -independent mechanism of ISS-ODN action. While preparing this manuscript, we have identified an abstract by Magone et al.,<sup>17</sup> which concurs with this article's conclusion about the therapeutic potential of ISS-ODN for allergic conjunctivitis. Also using a mouse model, they report that systemic ISS-ODN treatment can block early- and late-phase reactions after antigen challenge, agreeing completely with our findings. Because the protective effect was abolished by systemic anti-IL-12 treatment, they concluded that ISS-ODN action is mediated by IL-12. This is consistent with the data of Kline et al.,<sup>22</sup> who also showed, in the absence of either IL-12 or interferon- $\gamma$ , that smaller amounts of ODN do not provide protection against airway eosinophilia.

The treatment protocol is likely to have an important impact on the effectiveness of this new form of therapy. Kobayashi et al.<sup>19</sup> reported that ISS-ODN have prepriming effects that last for up to 2 weeks with an optimal effect observed when animals are treated between 3 and 7 days before challenge. The ISS-ODN prepriming induces Th1 bias, characterized by interferon- $\gamma$  and IL-12 production by splenocyte and elevated serum IgG2a levels and cytotoxic T lymphocyte (CTL) responses. The activated interferon- $\gamma$  production by splenocytes peaks when prepriming is conducted 3 days before antigen challenge. In addition, other successful treatment protocols for disease models support that ISS-ODN triggers an immunostimulatory cascade that matures over a period of sev-

eral days.<sup>12,23</sup> For all these reasons, we treated the animals with ISS-ODN 3 days before the final challenge.

For clinical use, we believe that ISS-ODN treatment in the form of eyedrops may provide significant benefits. However, as was shown in Figure 2, we could not achieve sufficient inhibition of clinical symptoms, despite a clear inhibition of late-phase reaction. To exclude the possibility that the prepriming period was not optimal, we also treated the animals from day 0 to day 3. However, the outcome of inhibitory effect on clinical symptoms corresponded to Figure 2, and any inhibitory effect was subtle at best (data not shown). These data indicate that there are limitations for ISS-ODN eyedrop treatment of allergic conjunctivitis. However, we cannot exclude the possibility that other drug delivery methods may permit topical ISS-ODN administration to inhibit both phases of the allergic reaction in the eye.

The molecular mechanisms of immune stimulation by ISS-ODN also remain unknown. For the signaling pathway of ISS-ODN, downstream signaling steps including the nuclear factor (NF) $\kappa$ B and mitogen-activated protein kinase (MAPK) pathways.<sup>24-26</sup> To transmit the signals, ISS-ODN must be internalized into the cell by endocytosis. Intracellular CpG receptors have not been found. To understand the signaling pathway, it is essential to identify such intracellular binding proteins. Following the signaling pathway of ISS-ODN, multiple early-response genes, proto-oncogenes, and cytokine genes are induced.<sup>27</sup> For cytokine gene expression, *in vitro* studies have shown that ISS-ODN induces IL-12, tumor necrosis factor (TNF)- $\alpha$ , IL-6, IL-1 $\beta$ , IL-1RA, macrophage inflammatory protein (MIP)-1 $\beta$ , monocyte chemoattractant protein (MCP)-1, interferon- $\alpha/\beta/\gamma$ , and IL-18.<sup>27</sup> However, there is little knowledge concerning the mechanisms by which these genes are activated by ISS-ODN. In the case of conjunctival allergy, virtually nothing is known. Because the pathophysiology of SAC and atopic asthma are considered to be distinct, targeted research on the mechanism of ISS-ODN action in both clinical entities is required.<sup>13,28</sup>

It is difficult to attribute the impressive therapeutic effect of topical ISS-ODN treatment on conjunctival eosinophilia and neutrophilia solely to the known mechanism by which ISS-ODN skews the Th response. Other mechanisms, including induced expression of cell surface MHC, costimulatory and adhesion molecules, and cytokines and Fc $\gamma$  receptors,<sup>20</sup> may play significant roles in the conjunctiva. In the lung models, inhibition of eosinophilia seems to be derived in large part from systemic effects, which is more difficult to envision in the current experiments. Thus, the cytokine milieu of the target tissue must be considered in the ISS-ODN response, because this has a significant impact on the outcome of ISS-ODN treatment.<sup>29,30</sup> In the conjunctiva, the well known abundance of dendritic cells may substantially affect or deviate the outcome of the treatment. Although the relative importance of each target cell *in vivo* is not yet well understood, dendritic cells and macrophages seem to have a crucial role in the release of inflammatory mediators, including IL-12, IL-18, and interferon- $\alpha$ , which then induce interferon- $\gamma$  from natural killer cells.<sup>22</sup> In this Th1-like environment, T cells are skewed toward the Th1 type, whereas no clear evidence has been obtained that ISS-ODN directly skews Th cell subsets.

In conclusion, these studies indicate that ISS-ODN treatment (together with other novel approaches) may be useful in the treatment of SAC and VC, the most common allergic dis-

eases of humans.<sup>31,32</sup> Acting by distinct pathways from existing therapies, ISS-ODN has the potential to significantly enhance our ability to treat these diseases.

### Note Added in Proof

Since the submission of the manuscript by Miyazaki et al., *IOVS* notes the publication of a paper by Eyal Raz in the *European Journal of Ophthalmology* indicating results similar to those reported in this paper.

### Acknowledgments

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