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Immunostimulatory DNA Sequences Inhibit IL-5, Eosinophilic Inflammation, and Airway Hyperresponsiveness in Mice¹

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We have used a mouse model of allergen-induced airway hyperresponsiveness to demonstrate that immunostimulatory DNA sequences (ISS) containing a CpG DNA motif significantly inhibit airway eosinophilia and reduce responsiveness to inhaled methacholine. ISS not only inhibited eosinophilia of the airway (by 93%) and lung parenchyma (91%), but also significantly inhibited blood eosinophilia (86%), suggesting that ISS was exerting a significant effect on the bone marrow production of eosinophils. The inhibition of the bone marrow production of eosinophils by 58% was associated with a significant inhibition of T cell-derived cytokine generation (IL-5, granulocyte-macrophage CSF, and IL-3). ISS exerted this inhibitory effect on T cell cytokine production indirectly by stimulating monocytes/macrophages and NK cells to generate IL-12 and IFNs. The onset of the ISS effect on reducing the number of tissue eosinophils was both immediate (within 1 day of administration) and sustained (lasted 6 days), and was not due to ISS directly inducing eosinophil apoptosis. ISS was effective in inhibiting eosinophilic airway inflammation when administered either systemically (i.p.), or mucosally (i.e., intranasally or intratracheally). Interestingly, a single dose of ISS inhibited airway eosinophilia as effectively as daily injections of corticosteroids for 7 days. Moreover, while both ISS and corticosteroids inhibited IL-5 generation, only ISS was able to induce allergen-specific IFN- γ production and redirect the immune system toward a Th1 response. Thus, systemic or mucosal administration of ISS before allergen exposure could provide a novel form of active immunotherapy in allergic diseases. *The Journal of Immunology*, 1998, 161: 7054–7062.

Allergic asthma is characterized by cellular infiltration of the airways with eosinophils and T lymphocytes expressing a Th2 profile of cytokines (1, 2). This characteristic inflammatory response is evident both in bronchial biopsies obtained from asthmatic patients as well as in mouse models of altered airway responsiveness. Following allergen inhalation in sensitized subjects or animals, Th2 cells release a particular set of cytokines (i.e., IL-5, GM-CSF,³ and IL-3) that promote airway eosinophilia by several different mechanisms, including induction of eosinophil proliferation in the bone marrow, promotion of the release of eosinophils from the bone marrow, and inhibition of eosinophil apoptosis (3, 4). In addition to promoting airway eosinophilia, these Th2 cytokines prime and activate eosinophils to release proinflammatory cytoplasmic granule products, lipid mediators, and cytokines that are thought to contribute to the tissue damage, remodeling, and hyperresponsiveness of the asthmatic airways (1). Antiinflammatory medications such as corticosteroids are standard therapy for asthma, but have limitations in that they

may not be disease modifying (asthma recurs when the corticosteroids are discontinued). In addition, corticosteroids, even when delivered by the inhalation route, are associated with the potential for significant side effects, including cataracts, growth retardation, and osteoporosis. Therefore, there is a need to develop safe and effective alternative therapies to corticosteroids to inhibit the critical events (e.g., Th2 cell activation) that initiate and perpetuate eosinophilic inflammation in the airways.

More than a decade ago, Tokunaga and coworkers discovered that DNA purified from mycobacteria induced the release of IFNs from splenocytes (5). Fractionation of the mycobacterial DNA led to the isolation of several short DNA sequences (containing CpG dinucleotide cores) that mediated the immunostimulatory activity (6). Subsequent experiments have shown that oligodeoxynucleotides (ODN) containing ISS (ISS-ODN) induce the release of several additional cytokines, including IFN- α,β (5, 6) IL-6 (7), IL-12 (8, 9), and IL-18 (10) primarily from monocytes (6, 10, 11), and IFN- γ from NK cells (6). The immune response triggered by ISS is similar to the innate immune response evoked by intracellular pathogens (10), triggering the release of cytokines that bias the immune response toward development of an Ag-specific Th1 effector and memory response (10). Cellular activation by ISS DNA such as CpG is not mediated through binding to a cell surface receptor, but requires cellular uptake by adsorptive endocytosis (12). Studies suggest that CpG DNA, taken up by B cells and monocytes by adsorptive endocytosis, is acidified in an intracellular endosomal compartment (12). Endosomal acidification of DNA is coupled to the rapid generation of reactive oxygen species that leads to nuclear factor- κ B activation and subsequent cytokine expression (12).

In this study, we have used a mouse model of eosinophilic airway inflammation to investigate whether ISS could inhibit both the generation of Th2 cytokines important to eosinophil proliferation

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³ Abbreviations used in this paper: GM-CSF, granulocyte-macrophage CSF; BAL, bronchoalveolar lavage; BALF, bronchoalveolar lavage fluid; DAB, diaminobenzidine; i.n., intranasal; i.t., intratracheal; MCh, methacholine; ISS-ODN, immunostimulatory sequence-oligodeoxynucleotide; M-ODN, mutated-oligodeoxynucleotide.

and survival (IL-5, GM-CSF, IL-3), as well as the subsequent airway hyperreactivity in response to methacholine (MCh) challenge. The data presented in this study indicate that administration of ISS-ODN inhibits both airway hyperresponsiveness and airway eosinophilia by exerting a significant inhibitory effect on the generation of eosinophil-active cytokines (IL-5, GM-CSF, and IL-3) as well as the subsequent bone marrow production of eosinophils. Moreover, while both ISS and corticosteroids inhibited IL-5 generation, only ISS was able to induce IFN- γ , a cytokine that importantly biases the immune system to generate a Th1 (and not Th2) response to subsequently encountered allergens.

Materials and Methods

Oligonucleotides

Endotoxin-free (<1 ng/mg DNA) phosphorothioate ISS-ODN (5'-TGACTGTGAACGTTCCGAGATGA-3') or phosphorothioate M-ODN (5'-TGACTGTGAAGGTTAGAGATGA-3') (Trilink, San Diego, CA), as previously described (10), were used in the *in vivo* and *in vitro* experiments described below.

Animals

Female BALB/c mice (The Jackson Laboratory, Bar Harbor, ME) were used when they reached 8–10 wk of age. All animal experimental protocols were approved by the University of California, San Diego, and the National Jewish Medical Research Center Animal Subjects Committees.

Determination of airway responsiveness to MCh *in vivo*

In experiments performed at the National Jewish Medical and Research Center, BALB/c mice were sensitized by the *i.p.* injection of OVA/alum on days 1 and 10, and subsequently received nebulized 1% OVA on days 22, 23, and 24. Airway responsiveness was assessed on day 26, 48 h after the final OVA inhalation, using a single chamber whole body plethysmograph obtained from Buxco (Troy, NY), as previously described (13). In this system, an unrestrained, spontaneously breathing mouse is placed into the main chamber of the plethysmograph, and pressure differences between this chamber and a reference chamber are recorded. The resulting box pressure signal is caused by volume and resultant pressure changes during the respiratory cycle of the mouse. A low pass filter in the wall of the main chamber allows thermal compensation. From these box pressure signals, the phases of the respiratory cycle, tidal volumes, and the enhanced pause (Penh) can be calculated. Penh is a dimensionless value that represents a function of the proportion of maximal expiratory to maximal inspiratory box pressure signals and of the timing of expiration. It correlates closely with pulmonary resistance measured by conventional two-chamber plethysmography in ventilated mice (13). Penh was used to monitor airway responsiveness in this study. In the plethysmograph, mice were exposed for 3 min to nebulized PBS and subsequently to increasing concentrations of nebulized MCh (Sigma, St. Louis, MO) in PBS using an Aerosonic ultrasonic nebulizer (DeVilbiss). After each nebulization, recordings were taken for 3 min. The Penh values measured during each 3-min sequence were averaged and are expressed for each MCh concentration as the percentage of baseline Penh values following PBS exposure (13). To determine the effect of ISS on airway responsiveness in OVA-sensitized mice, 50 μ g of either ISS-ODN or M-ODN was injected *i.p.* 24 h before each of three OVA inhalation challenges, and airway responsiveness was determined (5 days after the first dose of ISS). The number of bronchoalveolar lavage fluid (BALF) eosinophils was assessed in parallel.

Induction of pulmonary allergic eosinophilic inflammation

To investigate whether ISS inhibits airway eosinophilia, the OVA sensitization and challenge protocol of Corry et al. (14) were used. This protocol (14) differs in the number and route of OVA injections and inhalations compared with the protocol used to assess airway hyperresponsiveness. In these studies, mice were immunized *s.c.* on days 0, 7, 14, and 21 with 25 μ g of OVA (OVA, grade V; Sigma) adsorbed to 1 mg of alum (Aldrich) in 200 μ l normal saline. The OVA inhalation challenge (days 26 and 31) consisted of three 30-min inhalations (separated by 30-min rest intervals) of OVA at a concentration of 10 mg/ml in an inhalation chamber. The nebulizer (DeVILBISS UltraNeb-99; Sunrise Medical, Somers, PA) was set up to aerosolize 80–100 μ l of protein solution in the 30-min inhalation time period. The outflow of the inhalation chamber was attached to a vacuum line and adjusted to a minimal suction rate (enough to prevent excess condensation from occurring in the chamber). Mice were sacrificed,

and BALF, lungs, peripheral blood, and bone marrow were analyzed 24 h after the second OVA inhalation.

Therapeutic intervention with ISS-ODN or corticosteroids

The therapeutic intervention protocol is summarized in Fig. 2. ISS-ODN or M-ODN was injected *i.p.* (100 μ g in 100 μ l of sterile, endotoxin-free PBS) or instilled intranasally (*i.n.*) (50 μ g of ODN/naris, in 20 μ l PBS, under metofane anesthesia). The ODNs were administered either twice (on days 25 and 30, 1 day before each OVA inhalation challenge) or once (on day 25, 1 day before the first OVA inhalation challenge; or on day 30, 1 day before the second OVA inhalation; or on day 31, 30 min before OVA inhalation). Intratracheal (*i.t.*) administration of ISS-ODN or M-ODN (100 μ g of ODN in 50 μ l PBS, under metofane anesthesia) was performed twice on days 25 and 30, 1 day before each OVA inhalation challenge.

Dexamethasone (Sigma), 5 mg/kg in 100 μ l of PBS, was injected *s.c.* either twice (days 25 and 30, 18 h before each OVA inhalation) or daily for 7 days from day 25 through day 31.

Eosinophil counts

Twenty-four hours after the second OVA inhalation challenge (day 32), mice were sacrificed by cervical dislocation, and eosinophil counts of various tissues were performed.

Lung. Lung tissues embedded in OCT in 10 \times 50 \times 50-mm tissue wells were cryosectioned at 10 μ and acetone fixed onto poly(L-lysine)-coated slides. Total eosinophil numbers were enumerated by detection of eosinophil peroxidase using DAB staining and microscopic examination, as described in this laboratory (15). A key code was established for mice groups, and color-coded slides were labeled to designate mouse numbers within groups (*i.e.*, 1–4). Slides were incubated at room temperature for 1 min in the presence of cyanide buffer (10 mM potassium cyanide, pH 6), rinsed in PBS, and incubated for 10 min with the peroxidase substrate DAB (Vector Lab, Burlingame, CA). Slides were subsequently washed in PBS, counterstained with hematoxylin, air dried, and examined by light microscopy (\times 40 magnification) by a “blinded” examiner. Five random fields were selected and eosinophils were counted (cells staining brown) to determine total eosinophil number per microscope field.

BALF. The sacrificed mice had their tracheas surgically exposed and cannulated with 27-gauge silicon tubing attached to a 23-gauge needle on a 1-ml tuberculin syringe. Following instillation of 600 μ l of sterile saline through the trachea into the lung, BALF was withdrawn and cytospun (3 min at 500 rpm) onto microscope slides. Eosinophil counts were performed as described above.

Peripheral blood. Blood was collected from the carotid artery. RBC were lysed using a 1:10 solution of 100 mM potassium carbonate, 1.5 M ammonium chloride. The remaining cells were cytospun (3 min at 500 rpm) onto microscope slides and air dried. Eosinophil counts were performed as described above.

Bone marrow. Bone marrow cells were flushed from femurs with 1 ml PBS and cytospun onto microscope slides, and separate slides were stained with Wright-Giemsa and DAB for cell differential counts.

Stimulation of splenocytes *in vitro* by ISS-ODN and anti-CD3

Abs

Ninety-six-well flat microtiter plates were coated with rat anti-mouse anti-CD3 Abs (1 μ g/ml; PharMingen, San Diego, CA) at room temperature for 2 h and then washed with PBS. Splenocytes (5×10^6 /ml) from each mouse (female BALB/c, $n = 4$) were incubated with ISS-ODN or M-ODN (10 μ g/ml) at 37°C for 2 h and then added to anti-CD3-coated plates (5×10^5 cells/well), in triplicate, with or without neutralizing Abs to mouse IL-12, IFN- γ , and IFN- α (Biosource, Camarillo, CA). The optimal concentration of each neutralizing Ab used was determined previously in pilot experiments performed to neutralize the levels of IL-12, IFN- γ , and IFN- α produced by ISS-ODN-stimulated splenocytes (data not shown). Splenocyte supernatants (24, 48, and 72 h poststimulation) were assayed in duplicate to determine the level of each cytokine. All cytokines were analyzed by ELISA.

Administration of ISS to OVA-sensitized, but not OVA inhalation-challenged mice *in vivo*

Female BALB/c mice were sensitized to develop a Th2 response to OVA, as detailed above and in Fig. 2, but without subsequent aerosol OVA inhalation challenge. Eight weeks after the last OVA/alum injection, mice were injected *i.p.* with 100 μ g of ODN, 1 day (–1d) or 3 days (–3d) before their sacrifice. The cytokine profiles of the supernatants derived from splenocytes incubated for 72 h with 100 μ g/ μ l of OVA were assayed by ELISA.

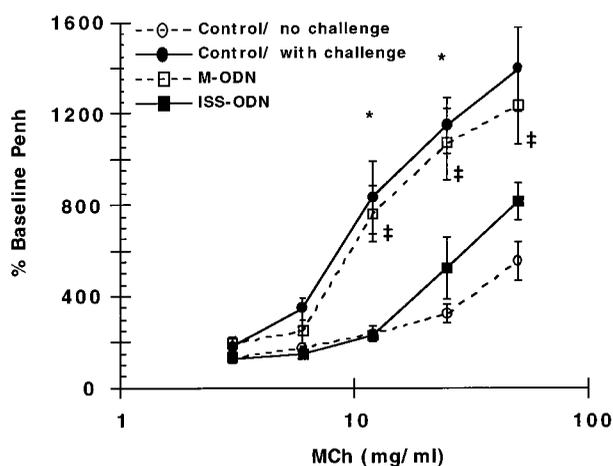


FIGURE 1. Administration of ISS inhibits MCh airway responsiveness. To determine the effect of ISS on airway responsiveness in OVA-sensitized mice, 50 μg of either ISS-ODN ($n = 12$ mice) or M-ODN ($n = 12$ mice) was injected i.p. 24 h before each of three OVA inhalation challenges, and airway responsiveness was determined 48 h after the last OVA challenge (5 days after the first dose of ISS). Airway responsiveness to increasing concentrations of nebulized MCh (0–50 mg/ml) was assessed by barometric whole body plethysmography, and Penh values were calculated. Means \pm SEM of Penh values from three independent experiments are expressed as the percentage of baseline Penh values observed after PBS exposure. Administration of ISS-ODN significantly reduced the increase in MCh airway responsiveness by $69 \pm 9.9\%$ compared with M-ODN-treated mice ($p < 0.05$).

Cytokine assays

The levels of various cytokines (IL-3, IL-5, GM-CSF, and IFN- γ) were measured in cell supernatants following either anti-CD3 Ab or OVA Ag stimulation by ELISA (PharMingen), as was previously described (10, 11, 16).

Eosinophil apoptosis assay

Eosinophils of $>90\%$ purity and $>95\%$ viability were purified from the blood of IL-5 transgenic mice using a Percoll gradient, as previously described in this laboratory (17). The eosinophils were then treated with ISS-ODN (1 $\mu\text{g}/\text{ml}$), M-ODN (1 $\mu\text{g}/\text{ml}$), or controls, including mouse rGM-CSF (1 ng/ml) (PharMingen) and anti-Fas Ab (1 $\mu\text{g}/\text{ml}$) (clone Jo2 from PharMingen), and analyzed at 2, 8, 18, and 32 h after treatment. Eosinophil apoptosis was measured by quantitating the number of apoptotic nuclei relative to healthy nuclei by cell permeabilization, propidium iodide staining, and FACS analysis, as described (18).

Statistical analysis

Statistical analysis was performed with ANOVA and Student's t test, as previously described (10–11). In studies of airway responsiveness, groups were compared by Tukey-Kramer HSD test. A p value of <0.05 was considered statistically significant.

Results

ISS-ODN inhibits allergen-induced airway hyperresponsiveness

Airway responsiveness to MCh was increased significantly in mice following OVA sensitization and OVA inhalation challenge, as opposed to mice sensitized to OVA alone with PBS challenge (14 ± 1.8 -fold increase in Penh values OVA versus PBS following inhalation of 50 mg/ml MCh). Mice sensitized to OVA without inhalation challenge, or mice OVA challenged without OVA sensitization showed minimal change in Penh in response to MCh (data not shown).

Administration of ISS-ODN i.p. before each inhalation challenge significantly reduced the increase in MCh airway responsiveness in OVA-sensitized and OVA-challenged mice by $69 \pm 9.9\%$ (Fig. 1). These changes in airway responsiveness induced by ISS-ODN were accompanied by a significant reduction in BALF eosinophilia (M-ODN-treated mice, $253 \pm 207 \times 10^3$ BALF eosinophils versus ISS-ODN-treated mice, $14 \pm 12 \times 10^3$ BALF eosinophils) ($p < 0.05$).

Kinetics of ISS-ODN-mediated inhibition of airway and lung eosinophilia

To investigate the mechanism by which ISS inhibits airway eosinophilia, the number and route of OVA injections and inhalations in the mouse protocol were modified (depicted in Fig. 2). Both OVA protocols induce significant BAL eosinophilia ($47 \pm 16\%$ BAL eosinophils with protocol used to assess airway responsiveness versus $42 \pm 4\%$ BAL eosinophils with protocol depicted in Fig. 2). However, the OVA protocol depicted in Fig. 2 induces an approximate eightfold greater absolute number of BAL eosinophils/ml compared with the protocol used to assess airway hyperresponsiveness (2075×10^3 BAL eosinophils versus 253×10^3 BAL eosinophils). Using this protocol (Fig. 2), OVA-sensitized and OVA inhalation-challenged mice developed significant airway eosinophilia (Fig. 3) compared with control PBS-challenged mice in the BALF ($42 \pm 4\%$ versus 0% BALF eosinophils) and lungs (67 ± 5 eosinophils/hpf versus 2 ± 1 eosinophils/hpf, $p < 0.05$, Fig. 4). Even with the stronger stimulus for eosinophil recruitment in this protocol, ISS-ODN significantly inhibited eosinophil recruitment to BALF (91% inhibition compared with M-ODN) (Fig. 4A) and lung tissue (90% inhibition compared with M-ODN) (Fig.

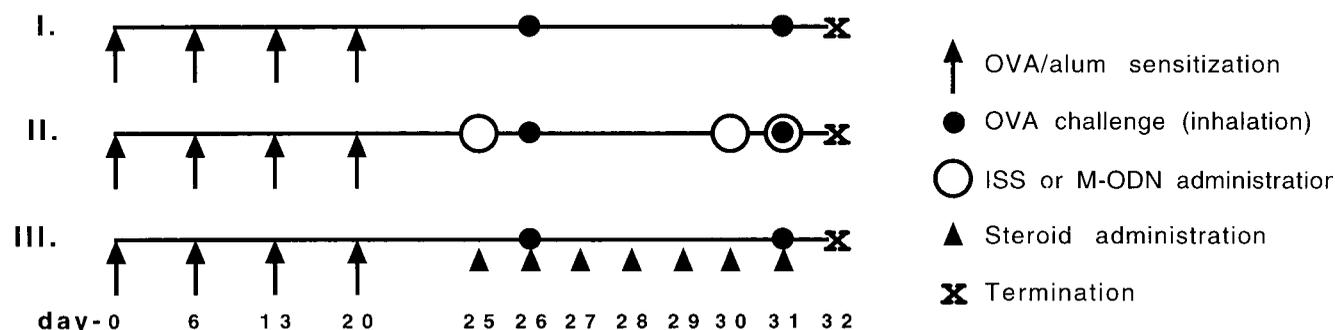


FIGURE 2. Protocol for induction of pulmonary allergic inflammation. *I*, Basic protocol. *II*, Therapeutic intervention with ISS-ODN or M-ODN. *III*, Intervention with corticosteroids. For details, see *Materials and Methods*.

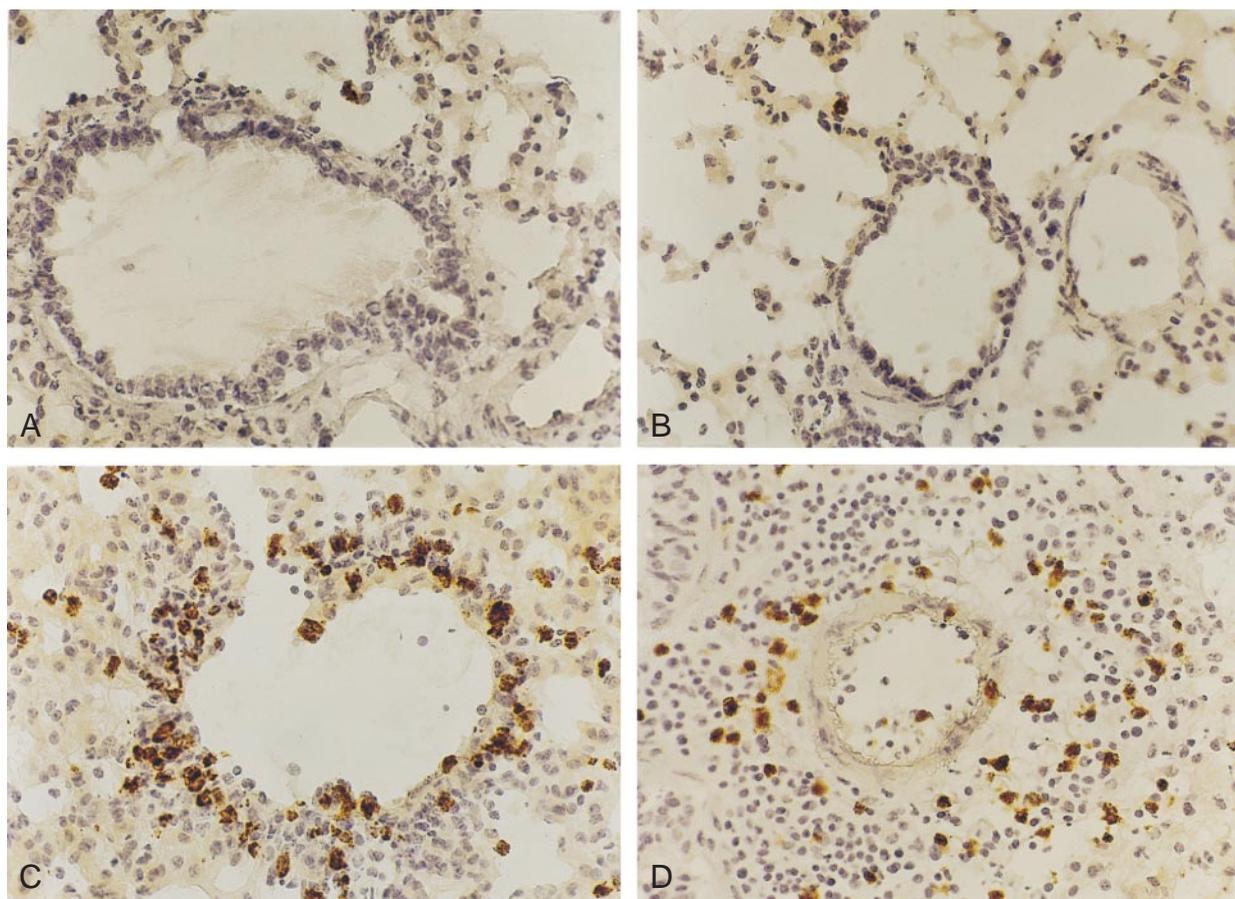


FIGURE 3. Eosinophilic infiltration in the lungs of mice treated with ISS-ODN or M-ODN OVA/alum-sensitized and OVA inhalation-challenged mice were treated i.p. with either ISS-ODN or M-ODN 6 days before the end of the experiment. Lungs were cryosectioned and stained to detect eosinophil peroxidase using the peroxidase substrate DAB. Cells expressing eosinophil peroxidase develop a brown color reaction. Peribronchial pulmonary eosinophilia was inhibited significantly in OVA-sensitized and OVA-challenged ISS-ODN-treated mice (A) compared with M-ODN-treated mice (C) (magnification $\times 40$). Similarly, pulmonary lung eosinophilia was inhibited significantly in ISS-ODN-treated mice (C) compared with M-ODN-treated mice (D). C and D (M-ODN treated) also display the inflammatory mononuclear cell infiltration pattern that is decreased in A and B (ISS-ODN treated). The pattern of inflammation observed in the lungs of the M-ODN-treated mice was similar to those obtained in control mice (see Fig. 4). The same method was applied for eosinophil detection in BALF, peripheral blood, and bone marrow (Figs. 4 and 6).

4B). ISS-ODN administered once 3 to 6 days before the termination of the experiment was the optimal time point (of those examined) for ISS administration in inhibiting eosinophil recruitment into BAL and lung (Fig. 4, and data not shown). These studies demonstrate the sustained 6-day inhibitory effect of a single dose of ISS on subsequent allergen-induced eosinophil recruitment.

We also examined the time of onset of action of a single dose of ISS in inhibiting eosinophil recruitment. These studies demonstrated that a single dose of ISS had an initial onset of action in inhibiting eosinophil recruitment when administered as late as 1 day before termination of the experiment (ISS coadministered with OVA) (43% inhibition of BALF eosinophilia and 69% inhibition of lung tissue eosinophilia). This inhibitory effect was more prominent when ISS was administered 2 days before termination of the experiment (76% inhibition of BALF eosinophilia and 75% inhibition of lung tissue eosinophilia). Overall, these studies suggest that ISS-ODN inhibits airway eosinophilia by mechanisms that are both rapid in onset (onset of action within 24 h of ISS administration) as well as sustained (inhibitory effect sustained for at least 6 days after ISS administration).

ISS did not affect the number of BAL neutrophils, lymphocytes, or mononuclear cells. However, as there is not a significant increase in these cell types in BAL in models of allergen-induced

airway inflammation, this may not be the appropriate model to determine whether ISS effects these cell types.

Mucosal administration of ISS-ODN inhibits airway and lung eosinophilia

Not only systemic (i.p.) ISS-ODN administration, but also mucosal (i.e., i.n. or i.t.). ISS-ODN administration had a similar inhibitory effect on BALF and lung eosinophil accumulation (Fig. 6, A and B).

Effect of ISS on peripheral blood and bone marrow eosinophil numbers and eosinophil apoptosis

ISS not only inhibited eosinophilia in the airway (by 91%) and lung parenchyma (90%), but also inhibited blood eosinophilia (86%) (Fig. 4C), suggesting that ISS was exerting a significant effect on the bone marrow production of eosinophils (number of bone marrow eosinophils inhibited 58%) (Fig. 4D). OVA challenge increased the total number of nucleated cells in the bone marrow of M-ODN-treated mice ($19.8 \pm 9.2 \times 10^6$ cells/ml) ($p = 0.04$ versus ISS-ODN), whereas ISS-ODN treatment of OVA-challenged mice ($7.5 \pm 1.8 \times 10^6$ cells/ml) reduced the total number of bone marrow cells to a level similar to that noted in naive mice not treated with ODN ($4 \pm 0.3 \times 10^6$ cells/ml). ISS inhibited the total number of bone marrow eosinophils (M-ODN, $1812 \pm$

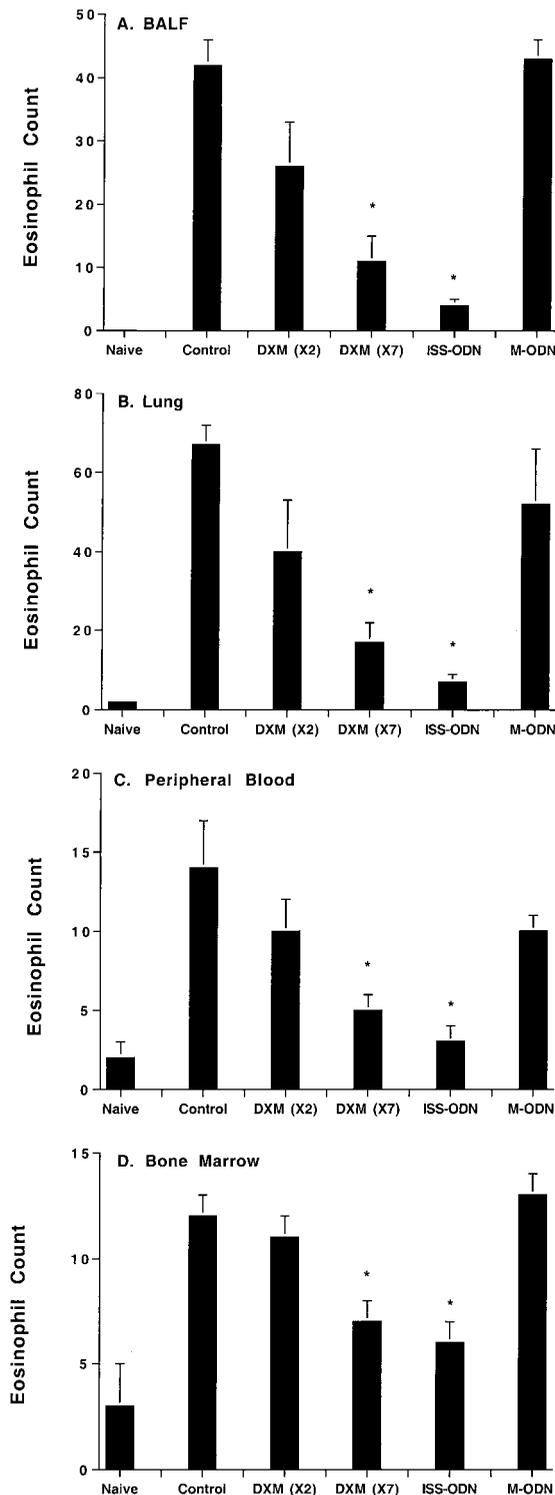


FIGURE 4. The effect of systemic ISS or corticosteroids on BALF, lung, and bone marrow eosinophil levels. Groups of mice were sensitized and challenged with OVA, or OVA sensitized and pretreated i.p. with either ISS-ODN, M-ODN, or corticosteroids (2 daily doses or 7 daily doses) starting before the first OVA inhalation challenge. The number of eosinophils in BALF (A), lung (B), blood (C), and bone marrow (D) were quantitated with DAB staining (magnification $\times 40$). Results are expressed as the mean \pm SE ($n = 8$ per group) and, for BALF, peripheral blood, and bone marrow, represent eosinophils as a percentage of total nucleated cells. The results for lung (#) represent the number of eosinophils per microscopic field. The ODN dose was $100 \mu\text{g}/\text{administration}$. The steroid dexamethasone (DXM) dose was $5 \text{ mg}/\text{kg}$. See Fig. 2 for details of the timing of intervention, sensitization, and challenge. PB, peripheral blood; BM, bone marrow. *, Denotes $p < 0.05$ versus the control group.

614×10^3 bone marrow eosinophils/ml versus ISS, $349 \pm 101 \times 10^3$ bone marrow eosinophils/ml, $p = 0.05$) and reduced the absolute number of peripheral blood eosinophils (M-ODN, $2815 \pm 995 \times 10^3$ eosinophils/ml versus ISS, $1390 \pm 44 \times 10^3$ eosinophils/ml, $p = 0.05$), but did not affect the total peripheral blood white blood cell count (M-ODN, $2.3 \pm 0.4 \times 10^4$ white blood cells/ μl versus ISS, $2.6 \pm 0.3 \times 10^4$ white blood cells/ μl).

The effect of ISS on reducing the number of eosinophils was not due to ISS directly inducing eosinophil apoptosis, as assessed in eosinophil apoptosis experiments in vitro. The percentage of apoptotic eosinophils after incubation for 18 h with ISS-ODN (51%) did not differ significantly from the percentage of apoptotic eosinophils after incubation with M-ODN (54%), or from the percentage of apoptotic eosinophils in untreated control cultures (55%). In contrast, positive and negative control experiments demonstrated that anti-Fas induced a significant degree of eosinophil apoptosis (87%), whereas GM-CSF protected eosinophils from apoptosis (5% apoptotic eosinophils).

Effect of ISS on the generation of IL-5

To investigate potential immunomodulatory mechanisms responsible for the ISS-induced inhibition of airway eosinophilia, the effect of ISS-ODN on IL-5 generation was evaluated. IL-5 induces eosinophil proliferation, differentiation, and resistance to apoptosis (1, 4), and genetically engineered elimination of IL-5 in mice in vivo dramatically reduces eosinophil numbers, as shown by the lack of eosinophils in IL-5 knockout mice (19). We evaluated whether inhibition of IL-5 production was responsible for ISS-ODN-mediated inhibition of bone marrow, peripheral blood, and airway eosinophilia. Systemic (i.p.) administration of ISS-ODN to OVA-sensitized and OVA-challenged mice reduced IL-5 production by 84%, while simultaneously inducing a 30-fold increase in IFN- γ production by OVA-stimulated CD4 $^+$ splenocytes (Fig. 5). The kinetics of ISS-induced inhibition of IL-5 correlated with the inhibitory effect of ISS on airway eosinophilia in vivo. Similar results, i.e., suppression of IL-5 and induction of IFN- γ by CD4 $^+$ splenocytes, were observed with mucosal ISS-ODN administration (i.n. and i.t.) (Fig. 7).

ISS inhibition of the generation of the eosinophil active cytokines (IL-5, GM-CSF, IL-3) is partially mediated by IL-12 and IFNs

We also monitored the effects of ISS-ODN on OVA/alum-sensitized, but nonchallenged mice. These OVA-sensitized mice provide an in vivo model to study whether ISS-ODN can inhibit the ability of an atopic mouse to release eosinophil active cytokines (IL-5, GM-CSF, IL-3). We postulated that administration of ISS-ODN alone (in the absence of Ag challenge) would stimulate the in vivo release of innate cytokines (IFN- α,β , IFN- γ , and IL-12), and that these cytokines would inhibit the secretion by T cells of eosinophil active cytokines (IL-5, GM-CSF, IL-3) without the induction of a subsequent Ag-specific Th1 response (no OVA Ag was administered by inhalation). In these studies, we evaluated OVA-stimulated spleen cell (derived from ISS-ODN-treated OVA-sensitized mice) production of IL-5, IL-3 (Table I), and GM-CSF (Table II), all of which are similar in their ability to induce eosinophil proliferation, and block eosinophil apoptosis through their activation of a common β -chain shared by these cytokine receptors (4). As shown in Table I, i.p. injection of ISS-ODN (1 day or 3 days before the in vitro cytokine assay) reduced secretion of IL-5 (83% inhibition group C versus A) and IL-3 (76% inhibition, group C versus A) by OVA-stimulated splenocytes without inducing any OVA-specific IFN- γ production. The experiments in Table I (OVA sensitization with no OVA challenge) and Fig. 5

Table I. *In vivo* ISS-ODN administration of OVA-sensitized mice inhibits IL-5 without induction of IFN- γ ^a

Group	Treatment	IL-3 (pg/ml)	IL-5 (pg/ml)	IFN- γ (pg/ml)
A	Control	1299 \pm 89	657 \pm 52	< 20
B	ISS-ODN (-1d)	309 \pm 26 ^b	112 \pm 18 ^b	< 20
C	ISS-ODN (-3d)	463 \pm 48 ^b	144 \pm 27 ^b	< 20
D	M-ODN (-1d)	964 \pm 81	508 \pm 77	< 20

^a Female BALB/c mice were sensitized to develop a Th2 response to OVA as detailed in Fig. 2, but without subsequent aerosol OVA challenge. Eight weeks after the last OVA/alum injection, mice were injected i.p. with 100 μ g of ODN, 1 day (-1d) or 3 days (-3d) prior to their sacrifice. The cytokine profiles of the supernatants derived from splenocytes incubated for 72 h with 100 μ g/ml of OVA were assayed by ELISA. Only baseline levels of IL-3, IL-5, and IFN- γ were detected in the supernatants of cultures incubated without OVA. No measurable levels of IL-4 and GM-CSF were detected in the various supernatants. Results are expressed as the mean \pm SE (n = 4 for each group).

^b Denotes p < 0.05, ISS-ODN-treated group vs control.

(OVA sensitization and OVA challenge) demonstrate that ISS can inhibit IL-5, GM-CSF, and IL-3 in OVA-sensitized mice in the presence or absence of OVA challenge. In contrast, ISS-treated mice require reexposure to OVA Ag to generate an OVA-specific Th1 response (induction of IFN- γ) (Figs. 5 and 7).

To address the role of innate cytokines (IL-12, IFNs) in mediating the *in vivo* inhibitory effects of ISS on the generation of IL-5, GM-CSF, and IL-3, we stimulated splenocytes *in vitro* with anti-CD3 Abs in the presence or absence of ISS-ODN and neutralizing Abs to IFN- α/β , IFN- γ , or IL-12 (Table III). *In vitro* incubation with ISS-ODN significantly inhibited anti-CD3-induced T cell production of IL-5 (47%), GM-CSF (49%), and IL-3 (47%) (Tables I and II). This inhibitory effect of ISS-ODN on eosinophil active cytokine release was partially mediated by the innate cytokines (IFNs and IL-12), as was shown in the related Ab neutralization studies (Table III). The *in vitro* stimulation of splenocytes with ISS-ODN and anti-CD3 Abs enhanced IFN- γ levels by threefold (data not shown). ISS stimulation neither inhibited IL-4 secretion nor increased IL-2 levels elicited by anti-CD3 stimulation (data not shown).

Investigations into the development of pulmonary eosinophilia and cytokine expression (IL-5 and IFN- γ) following a single ISS administration as compared with 7 days of corticosteroids

A single i.p. or i.n. ISS-ODN administration was as effective as seven daily doses of dexamethasone (5 mg/kg s.c.) in the suppression of airway eosinophilia (Figs. 4 and 6). A single dose of dexamethasone was not effective in inhibiting eosinophilic airway inflammation (data not shown), whereas two doses of dexamethasone had a partial effect on inhibiting eosinophil recruitment into the lung (Figs. 4 and 5). While both ISS and dexamethasone inhibited IL-5 generation (Figs. 5 and 7), only ISS was able to induce IFN- γ (Figs. 5 and 7).

Table II. *Effect of in vitro* ISS-ODN stimulation on anti-CD3-induced T cell cytokine production^a

Stimulation	IL-3 (pg/ml)	IL-5 (pg/ml)	GM-CSF (pg/ml)
Anti-CD3	2495 \pm 121	1553 \pm 120	1090 \pm 141
Anti-CD3/ISS-ODN	1183 \pm 217 ^b	730 \pm 230 ^b	535 \pm 118 ^b
Anti-CD3/M-ODN	4975 \pm 848	1795 \pm 116	1951 \pm 538

^a Splenocytes (5×10^6 /ml) were incubated with ISS-ODN or M-ODN (10 μ g/ml) at 37°C for 2 h and then added to anti-CD3-coated plates (5×10^5 cells/well) in triplicate. The cytokine profiles of the supernatants were assayed by ELISA. Results are expressed as the mean \pm SE (n = 4 per group).

^b Denotes p < 0.05, anti-CD3 plus ISS-ODN-treated group vs. anti-CD3-treated group.

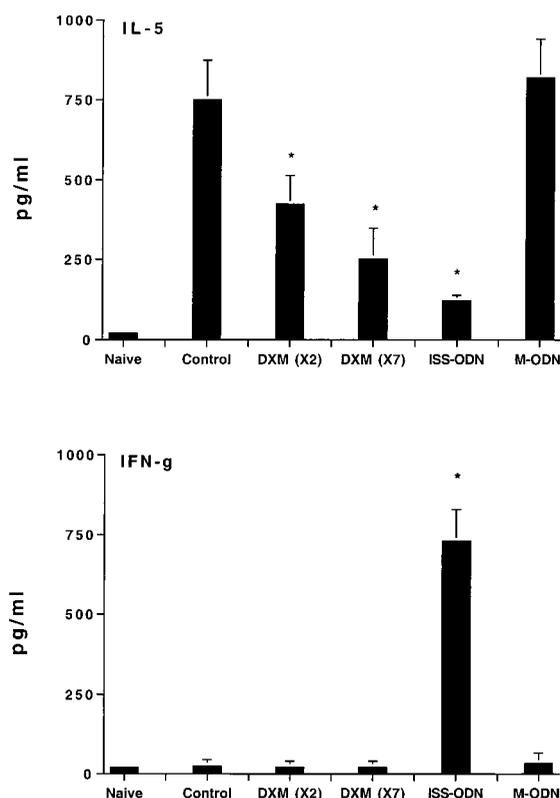


FIGURE 5. The effect of systemic ISS or corticosteroids on IL-5 and IFN- γ levels. Groups of mice were sensitized and challenged with OVA, or OVA sensitized and pretreated i.p. with either ISS-ODN, M-ODN, or corticosteroids (2 daily doses or 7 daily doses) starting before the first OVA inhalation challenge. The cytokine profiles of CD4⁺ splenocyte supernatants were assayed by ELISA from cells incubated for 72 h with 100 μ g/ml of OVA. Only baseline levels of IL-5 and IFN- γ were detected in the supernatants of cultures incubated without OVA. Results are mean \pm SE (n = 8 per group).

Discussion

In this study, we demonstrate that ISS inhibits airway eosinophilia and prevents the development of airway hyperresponsiveness. These observations are similar to those noted by Kline et al. (20) in a different mouse model of airway inflammation. In the present study, we have extended these observations by demonstrating 1) the novel mechanism by which ISS inhibits airway eosinophilia

Table III. *In vitro* ISS-ODN stimulation of anti-CD3-induced T cell cytokine production: effect of neutralizing Abs to innate cytokines (IL-12 and IFNs)^a

Stimulation	IL-3 (pg/ml)	IL-5 (pg/ml)	GM-CSF (pg/ml)
Anti-CD3/ISS-ODN	1183 \pm 217	730 \pm 230	535 \pm 118
Anti-CD3/ISS-ODN and anti-IL-12	1154 \pm 209	870 \pm 263	829 \pm 153 ^b
Anti-CD3/ISS-ODN and anti-IFN- γ	1169 \pm 214	2218 \pm 231 ^b	840 \pm 222 ^b
Anti-CD3/ISS-ODN and anti-IFN- α/β	1565 \pm 228 ^b	753 \pm 215	682 \pm 122 ^b

^a Splenocytes (5×10^6 /ml) were incubated with ISS-ODN or M-ODN (10 μ g/ml) at 37°C for 2 h and then added to anti-CD3-coated plates (5×10^5 cells/well) in triplicate, with or without neutralizing Abs to mouse IL-12, IFN- γ , and IFN- α (Bio-source). The cytokine profiles of the supernatants were assayed by ELISA. Results are expressed as the mean \pm SE (n = 4 per group).

^b Denotes p < 0.05, anti-CD3 plus ISS-ODN treated group vs anti-CD3 plus ISS-ODN plus cytokine neutralizing Ab-treated group.

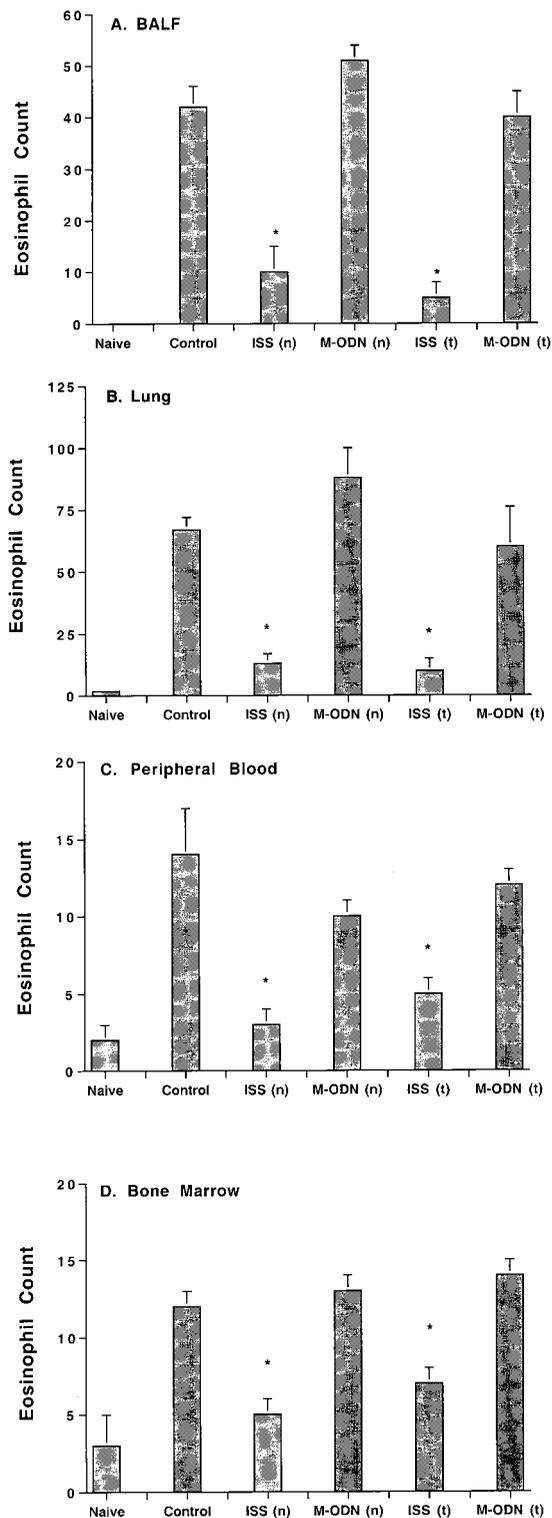


FIGURE 6. The effect of mucosal administration of ISS on BALF, lung, blood, and bone marrow eosinophil levels. Groups of mice were sensitized and challenged with OVA, or OVA sensitized and pretreated i.n. (n) or i.t. (t) with either ISS-ODN or M-ODN, starting before the first OVA inhalation challenge. The number of eosinophils in BALF (A), lung (B), blood (C), and bone marrow (D) were quantitated with DAB staining. Results are expressed as the mean \pm SE ($n = 8$ per group) and, for BALF, peripheral blood, and bone marrow, represent eosinophils as a percentage of total nucleated cells. The results for lung (#) represent the number of eosinophils per microscopic field. The ODN dose was 100 $\mu\text{g}/\text{administration}$. See Fig. 2 for details of the timing of intervention, sensitization, and challenge. PB, peripheral blood; BM, bone marrow; n, intranasal, t, intratracheal. *, Denotes $p < 0.05$ versus the control group.

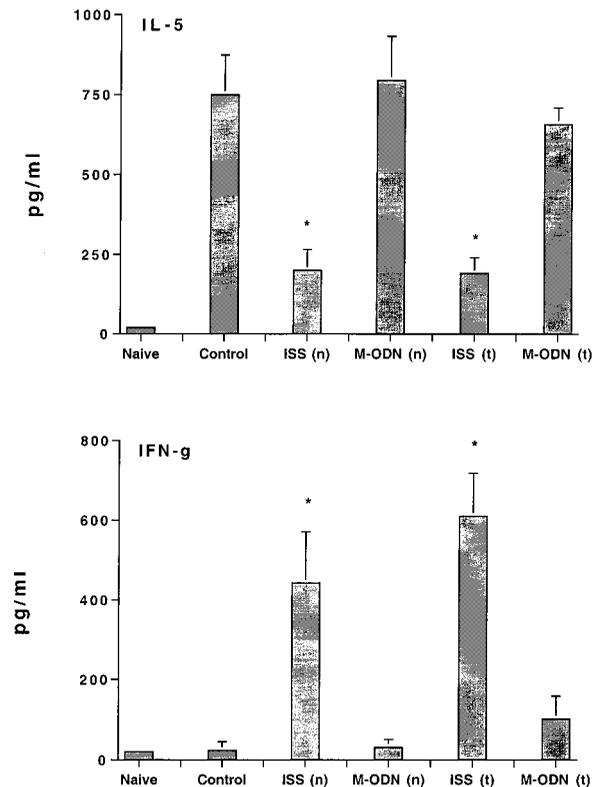


FIGURE 7. The effect of mucosal administration of ISS on IL-5 and IFN- γ levels. Groups of mice were OVA sensitized and OVA challenged, or OVA sensitized and pretreated i.n. (n) or i.t. (t) with either ISS-ODN or M-ODN, starting before the first OVA inhalation challenge. The cytokine profiles of CD4⁺ splenocytes were assayed by ELISA utilizing supernatants from cells incubated for 72 h with 100 $\mu\text{g}/\mu\text{l}$ of OVA. Only baseline levels of IL-5 and IFN- γ were detected in the supernatants of cultures incubated without OVA. Results are mean \pm SE ($n = 8$ per group). n, intranasal; t, intratracheal challenge. *, Denotes $p < 0.05$ versus the control group.

through inhibition of the production and release of eosinophils from the bone marrow; 2) that inhibition of bone marrow production of eosinophils was associated with a significant inhibition of IL-5, GM-CSF, and IL-3 production; 3) that ISS exerted this inhibitory effect on T cell cytokine production indirectly by stimulating monocytes/macrophages and NK cells to generate IL-12 and IFNs, as demonstrated in *in vitro* neutralizing Ab studies; 4) that the effect of ISS on reducing the number of tissue eosinophils was both immediate (onset within 1 day) and sustained (over 6 days), and was not due to ISS directly inducing eosinophil apoptosis; 5) that ISS was effective in inhibiting eosinophilic airway inflammation when administered either systemically or mucosally (i.e., i.n. or i.t.); 6) that a single administration of ISS (systemic or mucosal) inhibited airway eosinophilia as effectively as daily systemic administrations of corticosteroids for 7 days; and 7) that while both ISS and corticosteroids inhibited IL-5 generation, only ISS was able to induce IFN- γ (a cytokine that importantly biases the immune system to generate a Th1 and not a Th2 response to subsequently encountered allergens). Thus, systemic or mucosal administration of ISS before allergen exposure provides a novel form of active immunotherapy in allergic diseases.

The inhibitory effects of ISS are likely to be mediated by the innate cytokines derived from monocytes/macrophages and NK cells stimulated by ISS (6, 9, 10). In studies utilizing purified populations of mouse bone marrow-derived macrophages, we have

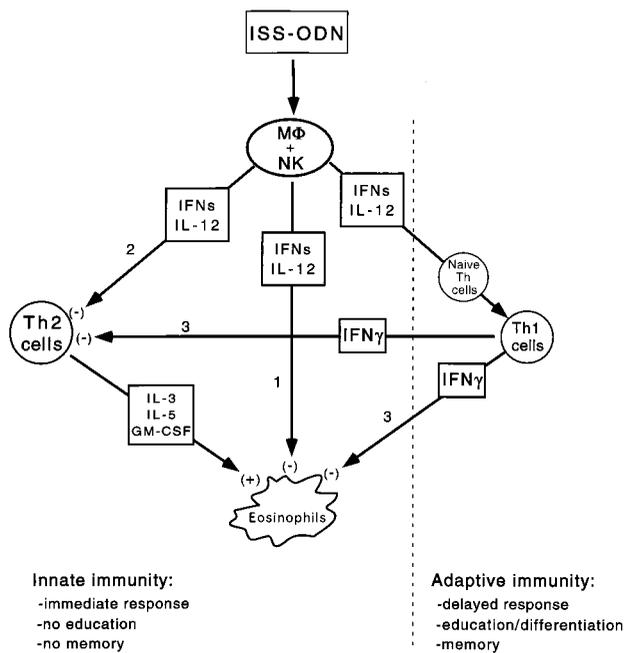


FIGURE 8. Mechanisms by which ISS-ODN suppress eosinophil accumulation in the lungs. ISS-ODN activates the innate immune system (macrophages and NK cells) to release IFNs (α , β , γ) and IL-12. These cytokines inhibit eosinophil recruitment and activation (step 1), as described (23–25). In addition, as shown in this study, these cytokines down-regulate the secretion by Th2 cells of IL-5, IL-3, and GM-CSF, cytokines that induce eosinophil proliferation in the bone marrow (step 2). The IFNs and IL-12 bias naive Th cells toward allergen-specific Th1 differentiation. Once generated, the Th1 cells secrete IFN- γ in response to allergen, which inhibit further Th2 activation and differentiation as well as eosinophil recruitment and activation (step 3) (26). Steps 1 and 2 occur within hours of exposure to ISS-ODN and are mediated by the innate immune system, while step 3 is the product of adaptive immunity. Furthermore, while steps 1 and 2 have transient antieosinophilic effects, step 3 leads to immunologic memory. Once established, allergen-specific Th1 responses can efficiently inhibit eosinophilic inflammation upon subsequent allergen exposure (step 3).

demonstrated recently that ISS induces these macrophages to release IL-12, but not IFN- γ .⁴ In additional studies,⁴ stimulation of cultured splenocytes derived from SCID mice with ISS induced the release of both IL-12 and IFN- γ . As purified populations of BALB/c-derived macrophages stimulated with ISS did not generate IFN- γ ,⁴ these studies with SCID mice suggest that NK cells may be the predominant source of IFN- γ in SCID mice stimulated with ISS. These⁴ and other studies (10, 21) suggest that ISS is able to stimulate both macrophages and NK cells to produce cytokines that modulate T cell cytokine production.

ISS-ODN administration, via activation of innate immunity, could inhibit pulmonary eosinophilia through at least three different, but additive mechanisms (Fig. 8). The first mechanism by which ISS can inhibit pulmonary eosinophilia is through an effect on T cell-derived cytokines important to the bone marrow generation of eosinophils. ISS exerts this inhibitory effect indirectly by stimulating monocytes/macrophages and NK cells to generate IL-12 and IFNs that subsequently inhibit T cell generation of IL-5, GM-CSF, and IL-3. The greater inhibition by ISS of peripheral blood eosinophilia (86% inhibition) compared with inhibition of bone marrow eosinophilia (58% inhibition) suggests that ISS may

have also inhibited release of eosinophils from the bone marrow. In this regard, IL-5 is known to induce release of eosinophils from the bone marrow (22), and inhibition of IL-5 generation by ISS could thus prevent bone marrow release of eosinophils. A second mechanism by which ISS-induced generation of IFNs and IL-12 could inhibit pulmonary eosinophilia is through an effect on eosinophil recruitment, as has previously been demonstrated with IL-12 (23, 24), IFN- α (25), and IFN- γ (26) in models of allergic inflammation and parasitic infection. A third eosinophil-inhibitory mechanism induced by ISS is the generation of an allergen-specific Th1 as opposed to a Th2 response. This would be important for long-term protection and immunologic memory. This ISS-induced OVA-specific Th1 response would generate IFN- γ , which further inhibits eosinophil accumulation by biasing naive T cells encountering Ag in an IFN- γ milieu to generate Th1 as opposed to Th2 responses to newly encountered allergens. The first two inhibitory mechanisms affecting pulmonary eosinophilia are most likely mediated by the innate immune response, and are therefore primarily immediate and Ag nonspecific in nature. In contrast, the third effect is mediated by an adaptive immune response and requires a longer period of time for differentiation and maturation of Ag-specific Th1 cells from naive CD4⁺ T cells (Fig. 8). Furthermore, while the first two mechanisms lead to a dramatic, but probably temporary reduction in eosinophil recruitment, the third mechanism is involved in the generation of immunologic memory that may prevent Th2 cell responses and eosinophil recruitment into the target organ (i.e., the lung) from developing following subsequent airway allergen challenge.

The effect of ISS on reducing the number of tissue eosinophils was both immediate (onset within 1 day) and sustained (over 6 days), and was not due to ISS directly inducing eosinophil apoptosis. Administration of ISS-ODN 6 days before the final OVA inhalation challenge was more effective in inhibiting pulmonary eosinophil infiltration than simultaneous delivery of ISS-ODN and OVA 1 day before the end of the experiment. Administration of ISS-ODN 6 days before the final OVA inhalation challenge also generated OVA-specific Th1 responses (induction of IFN- γ) and attenuated preexisting OVA-specific Th2 responses (i.e., reduction of IL-5, Figs. 5 and 7). The administration of ISS 1 day before, or together with the final OVA inhalation challenge was sufficient to inhibit pulmonary eosinophil recruitment as well as the generation of eosinophil active cytokines 24 h later. However, this method of ISS administration did not result in OVA-specific Th1 responses, probably because Ag-specific IFN- γ production generally requires more than 24–48 h.

The activation of the innate immune response by ISS-ODN could initially prevent the development of asthmatic symptoms by preventing early eosinophil accumulation, and later even bias the immune system to generate an allergen-specific Th1 response to any allergen subsequently encountered by the host in his own natural environment (Fig. 8). Thus, therapy with ISS has potential beneficial immunomodulatory effects on allergic inflammation not noted with corticosteroids. For example, therapy with corticosteroids, while effective in inhibiting IL-5 generation, did not induce IFN- γ (Fig. 5). The potential benefit of ISS therapy in comparison with corticosteroids in allergic disease would be the ability of ISS to alter the cytokine milieu of the host (IFN- γ) to favor generation of Th1 responses to subsequently encountered allergens.

A recent study demonstrated an inverse epidemiologic association between exposure to mycobacteria and the prevalence of atopic disorders, suggesting that the relatively recent decline in infections (e.g., tuberculosis) in developed countries is a factor underlying the increasing severity and prevalence of allergic diseases (27). Furthermore, infection of mice with *Mycobacterium*

⁴ E. Martin-Orozco, H. Kobayashi, M.-D. Nguyen, J. Van Uden, R. S. Kornbluth, and E. Raz. Activation of APCs by immunostimulatory DNA sequences. Submitted for publication.

bovis Bacillus Calmette Guerin has been shown to suppress allergen-induced airway eosinophilia (28). As previously mentioned, the ISS were initially identified and isolated from mycobacterial DNA (5, 6) and they appear at the expected frequency in many pathogenic bacteria and viruses, while they are underrepresented in the vertebrate genome (7). Thus, the natural exposure to ISS-enriched DNA from normal intracellular pathogens, through infection, may play a role in shaping the immune response away from an allergic phenotype and allergic responses following allergen challenge. As shown in this study, ISS-ODN can provide this immunomodulatory effect without the risk of inducing active infection.

In summary, ISS-ODN administration provides an alternative to the current practice of allergen protein desensitization, which has relatively low efficacy and high potential for significant side effects, including anaphylaxis (29, 30). ISS-ODN delivery via the systemic or mucosal route (i.n. or i.t.) in allergic patients may inhibit the allergic inflammatory responses in asthmatic lungs after natural allergen exposure. The future application of ISS-ODN therapy to human allergic diseases will depend on how well the inhibitory effects of ISS-ODN noted in murine models of experimental allergic airway inflammation translate to related human diseases.

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