Immunostimulatory oligonucleotides block allergic airway inflammation by inhibiting Th2 cell activation and IgE-mediated cytokine induction

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A single treatment with a CpG-containing immunostimulatory DNA sequence (ISS) given before allergen challenge can inhibit T helper type 2 cell (Th2)–mediated airway responses in animal models of allergic asthma; however, the mechanism of this inhibition remains largely undefined. Here, we demonstrate that airway delivery of ISS before allergen challenge in Th2-primed mice acts in two distinct ways to prevent the allergic responses to this challenge. The first is to prevent induction of cytokines from allergen-specific Th2 cells, as demonstrated by the nearly complete inhibition of Th2 cytokine production, Th2-dependent functional responses, and gene induction patterns. ISS inhibits the Th2 response by rendering lung antigen-presenting cells (APCs) unable to effectively present antigen to Th2 cells, but not to Th1 cells. This loss of APC function correlates with a reduced expression of costimulatory molecules, including programmed cell death ligand (PD-L)1, PD-L2, CD40, CD80, CD86, and inducible T cell costimulator, and of major histocompatibility complex class II on CD11c$^+$ APCs from the airways of ISS-treated mice. The second important action of ISS is inhibition of immunoglobulin E–dependent release of Th2 cytokines, especially interleukin 4, from basophils and/or mast cells in the airways of Th2-primed mice. Thus, inhibition by ISS of allergic responses can be explained by two novel mechanisms that culminate in the inhibition of the principal sources of type 2 cytokines in the airways.

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Abbreviations used: APTI, airway pressure-time index; BAL, bronchoalveolar lavage; ICOS, inducible T cell costimulator; IDO, indoleamine 2,3-dioxygenase; ISS, immunostimulatory DNA sequence(s); M-trp, 1-methyl-DL-tryptophan; ODN, oligodeoxynucleotide; PD-1, programmed cell death protein 1; PDC, plasmacytoid dendritic cell; PD-L, programmed cell death ligand; RW, ragweed pollen extract; TLR, Toll-like receptor.

CpG-containing immunostimulatory DNA sequences (ISS) have been shown to inhibit the major features of allergic asthma and airway inflammation. Studies performed by us and others have demonstrated that ISS inhibit the development of airway hyperresponsiveness, mucus production, and airway eosinophil infiltration in mouse asthma models (1–7). Treatment with one single systemic dose of ISS inhibits Th2 responses in the airways for a period of 4 wk, in spite of ongoing allergen challenges (5). ISS are effective when given before allergen challenge as a preventive measure (3, 5, 6) and have been demonstrated to reverse established disease (4, 8). In long-term mouse asthma models, ISS inhibit such lung-remodeling parameters as collagen deposition, goblet cell hyperplasia, and thickening of the epithelial basement membrane (9–11). Recently, similar effects of ISS have been reported in a monkey model for allergic asthma (12).

ISS activate the Toll-like receptor (TLR)-9 pathway (13) and promote the development of a Th1 cell response both in vitro and in vivo (14, 15). ISS activate cells of the innate immune system to generate cytokines, such as TNF-α, IL-12, IFN-α, and indirectly, IFN-γ (14, 15). One can envision that long-term treatment with ISS through induction of IL-12 and IFNs would lead to a rebalancing of Th1/Th2 responses, perhaps ultimately leading to complete inhibition of the Th2 response. A recent study in allergic rhinitis patients treated during the ragweed season with ISS conjugated to the dominant ragweed allergen *Amb a I* demonstrated increased levels of IFN-γ and reduced levels of IL-4 in nasal biopsies obtained 16 wk after the ragweed season, supporting this hypothesis (16).

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A key question, however, is how the immediate inhibitory effects of ISS on the Th2 response can be explained. A single treatment with ISS given just before allergen challenge can completely block the allergen-induced Th2 response. A direct effect on Th2 cells is unlikely, as T cells do not express TLR-9 (13). However, other modes of action are possible based on known activities of ISS. For example, cytokines or mediators induced by ISS could inhibit effector functions mediated by Th2 cytokines, such as IgE production, smooth muscle hyperreactivity, and eosinophilia. Studies investigating whether the immediate inhibitory effects of ISS are mediated by IL-12 or IFN-γ, however, give conflicting results (4, 17–19). Some studies demonstrate that ISS inhibition of allergen-induced eosinophil infiltration is partially dependent on IFN-γ (4), whereas others show that ISS inhibition of allergen-induced Th2 responses is independent of ISS-induced cytokines, such as IL-12 or IFN-γ (17–19). Alternatively, inhibition by ISS could result from actions on cell types required for Th2 activation, such as APCs. In this study, we sought to elucidate how ISS exert their immediate inhibitory effect on the allergic response and characterize more precisely which pathways and cellular events of the allergen-induced response are inhibited.

RESULTS

1018 ISS does not inhibit gene induction by IL-4 or IL-13

IL-4 and IL-13 play a central role in Th2 responses in the respiratory tract, inducing airway hyperresponsiveness, mucus overproduction, and eosinophil recruitment (20–22). Many biological activities mediated by IL-4 and IL-13 are known to be inhibited by cytokines inducible by ISS, such as IL-12, IFN-α, and IFN-γ (23–28), suggesting that ISS may inhibit the allergic airway response by antagonizing the actions of IL-4 and IL-13. To test this, naive BALB/c mice were treated intranasally with 20 μg 1018 ISS, control oligodeoxynucleotide (ODN), or saline, followed 1 d later by 5 μg IL-4 or IL-13, also given intranasally. Prior studies have shown optimal gene induction in the airways between 3 and 12 h after treatment at this dose of IL-4 or IL-13 (not depicted). The dose of 20 μg 1018 ISS was derived from previous experiments demonstrating optimal induction of ISS-inducible genes in the airways within a dose range of 5–20 μg 1018 ISS (not depicted). Lungs were harvested 6 h after treatment with cytokine and evaluated for mRNA expression. Treatment with either IL-4 or IL-13 induced increased mRNA expression levels for eotaxin, GOB-5, and FIZZ1 in the lung, and this was not inhibited by pretreatment with 1018 ISS (Fig. 1, A and B). This lack of effect of 1018 ISS was not due to inhibition of the actions of ISS by subsequent IL-4 or IL-13 administration, as genes known to be highly inducible by ISS, such as IP-10, MIG, and MCP-3, remained elevated after IL-4 or IL-13 treatment (Fig. 1 C and not depicted). In summary, the direct inhibition of IL-4– and IL-13–mediated functions does not appear to be the mechanism of action of ISS in this mouse asthma model.

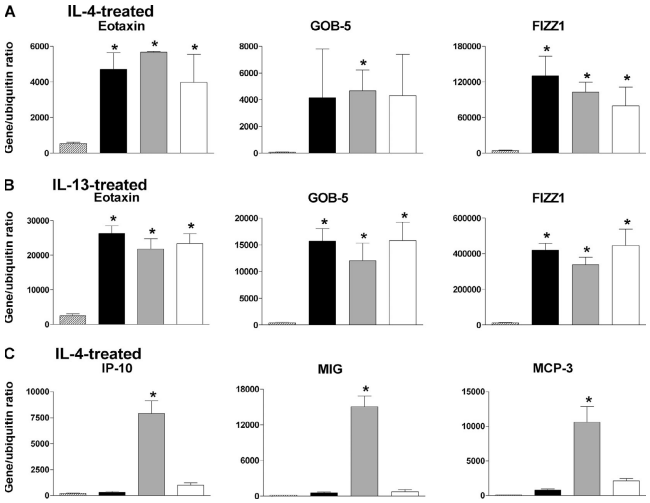

Figure 1. 1018 ISS does not inhibit gene induction by IL-4 or IL-13. (A–C) mRNA expression measured in whole lung is shown. Lungs were harvested 6 h after intranasal treatment with 5 μg IL-4 (A and C) or 5 μg IL-13 (B). 1 d before cytokine treatment was given, mice were treated intranasally with saline (black bars), 20 μg 1018 ISS (gray bars), or control ODN (white bars). Mice treated with saline only (and no cytokine) are represented by hatched bars. All data are expressed as gene/ubiquitin ratio (mean ± SEM; five mice per group), and * indicates a p-value of <0.05 when compared with the saline-only treated group. Increased levels of eotaxin, GOB-5, and FIZZ1 are observed after IL-4 and after IL-13 treatment, which cannot be inhibited by 1018 ISS treatment. Up-regulation of the ISS-inducible genes IP-10, MIG, and MCP-3 is observed after 1018 ISS treatment both in IL-4– (A) and IL-13–treated mice (not depicted).

1018 ISS does not prevent airway hyperresponsiveness induced by IL-13 administration to the lung

In addition to inducing STAT-6–regulated genes, IL-13 administration to the lung has also been reported to directly induce airway hyperresponsiveness to acetylcholine via a STAT-6–dependent mechanism (20, 29). To test whether 1018 ISS pretreatment could inhibit IL-13–induced airway hyperresponsiveness, mice were given three intratracheal doses of IL-13 on consecutive days (5 μg per day) as described previously (20). For this experiment, mice were given three (instead of one) intratracheal doses of IL-13 because, in our experience, this regimen induces a stronger, more consistent change in airway responsiveness than a single dose of IL-13. Before the IL-13 treatments, mice were given one intratracheal administration of 20 μg 1018 ISS, control ODN, or PBS. 1 d after the last IL-13 treatment, airway responsiveness to acetylcholine was measured. IL-13 caused a substantial increase in the airway pressure-time index (APTI; Fig. 2), indicating increased airway reactivity to acetylcholine, and this was unaffected by pretreatment with 1018 ISS (Fig. 2). In mice treated with control ODN and IL-13, the mean APTI was not significantly different from the PBS control.

Upon completion of the APTI measurements, lungs were harvested to confirm that 1018 ISS did not inhibit gene induction by the three-dose regimen of IL-13. As expected,

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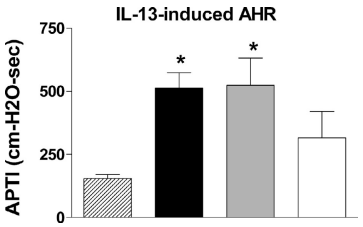

Figure 2. 1018 ISS does not prevent IL-13–induced airway hyperresponsiveness. Airway responsiveness to acetylcholine was measured in mice exposed to three intratracheal treatments with 5 μg IL-13. 1 d before the IL-13 treatment started, mice were treated intratracheally with PBS (black bar), 20 μg 1018 ISS (gray bars), or control ODN (white bar). Mice treated with PBS only (and no IL-13) are represented by the hatched bar. Airway responsiveness measurements were taken 1 d after the last IL-13 treatment. Airway responsiveness is defined by the time-integrated change in peak airway pressure or the APTI. Data shown are mean ± SEM of seven mice per group, and * indicates a p-value of <0.05 when compared with the PBS-only treated group. A significant increase in airway responsiveness is observed after IL-13 treatment. Treatment with 1018 ISS is not able to inhibit IL-13–induced airway hyperresponsiveness.

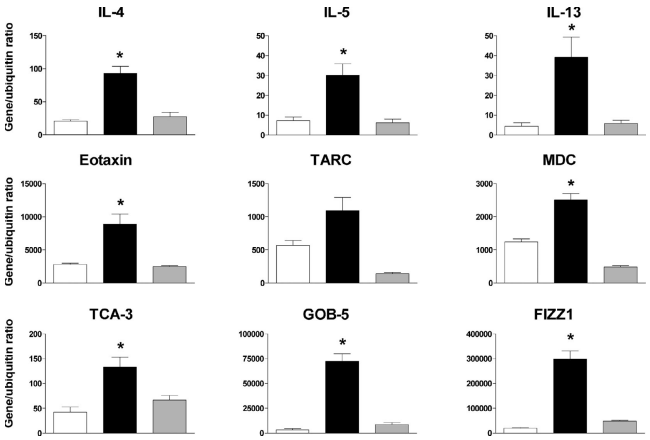

Figure 3. 1018 ISS prevents allergen-induced Th2 and Th2-dependent gene induction. mRNA expression measured in whole lung is shown. Mice were sensitized with RW and alum on days 0 and 3. Lungs were harvested 48 h after intratracheal challenge (day 10) with 200 μg RW in sensitized mice. 1 d before the RW challenge, mice were given PBS (black bars), 20 μg 1018 ISS (gray bars), or control ODN (not depicted) intratracheally. Mice challenged with PBS only (and not with RW) are represented by white bars. All data are expressed as gene/ubiquitin ratio (mean ± SEM; five mice per group), and * indicates a p-value of <0.05 when compared with the PBS-only challenged group. RW challenge in sensitized mice induces significant up-regulation of Th2 and Th2-dependent genes 48 h after challenge. Treatment with 1018 ISS completely inhibits the induction of these genes.

increased levels of eotaxin, GOB-5, and FIZZ1 mRNA expression were observed in IL-13–treated mice, and the levels of these genes were similar in the IL-13–treated/1018 ISS–pretreated mice (not depicted). Taken together, the experiments in Figs. 1 and 2 clearly demonstrate that pretreatment with doses of 1018 ISS capable of inhibiting antigen-induced Th2 responses (see Figs. 3 and 4 below) does not block any measurable lung response to IL-4 or IL-13.

1018 ISS prevents allergen-induced Th2 and Th2-dependent gene induction

The experiments described above demonstrate that the inhibition by ISS of antigen-driven Th2-mediated effector functions cannot be explained by direct ISS inhibition of those effector functions. This suggests that 1018 ISS is able to inhibit the activation of antigen-specific Th2 cells, although direct inhibition of polarized Th2 cells by ISS has not previously been reported. We assessed the activation of Th2 cells by antigen in situ by measuring increases in the Th2 cell cytokines IL-4, IL-5, and IL-13 at the protein and mRNA levels in the airways, and by measuring increases in the mRNA levels of genes directly dependent on IL-4 and IL-13 in the lung tissue. Mice were sensitized with ragweed pollen extract (RW) in alum (days 0 and 3) and challenged intratracheally (day 10) with 200 μg RW or PBS. 1 d before challenge (day 9), the mice were treated with 20 μg 1018 ISS, control ODN, or PBS. 48 h after RW challenge, lungs were harvested and mRNA levels of a panel of Th2 and Th2-dependent genes were evaluated using quantitative PCR. Challenge with RW induced a marked increase in IL-4, IL-5, and IL-13 mRNA as well as in the expression of Th2-dependent genes, such as the chemokines eotaxin, TARC, MDC, and TCA-3, and the goblet cell genes, GOB-5 and FIZZ1 (Fig. 3). Pretreatment with 1018 ISS, however, completely prevented increases in these Th2 and Th2-dependent genes in the lung (Fig. 3). Expression levels in the RW-challenged/1018 ISS–pretreated mice were reduced to the levels observed in control mice that were sensitized but challenged with PBS only. Treatment with control ODN caused no significant inhibition of allergen-induced gene expression (not depicted). These data clearly indicate that 1018 ISS prevents antigen-mediated activation of Th2 cells as measured directly in the lung.

1018 ISS inhibits allergen-induced airway hyperresponsiveness, eosinophilia, and Th2 cytokine production

To determine whether this inhibition of Th2 gene expression by 1018 ISS correlated with the inhibition of major features of asthma in this model, mice were sensitized, RW challenged, and treated with 1018 ISS 1 d before RW challenge as described in the preceding section. 48 h after RW challenge, airway responsiveness to acetylcholine was measured and airway inflammation was assessed by the number of eosinophils in the bronchoalveolar lavage (BAL) compartment. RW challenge in sensitized mice induced a significant increase in airway responsiveness when compared with control-challenged animals (Fig. 4, top left). As observed in similar experiments using OVA (5), pretreatment with 1018 ISS was able to significantly inhibit the development of airway hyperresponsiveness (49% inhibition), whereas pretreatment with control ODN had no effect. A similar result was obtained with BAL eosinophils. RW challenge induced infiltration of eosinophils into the BAL compartment, and

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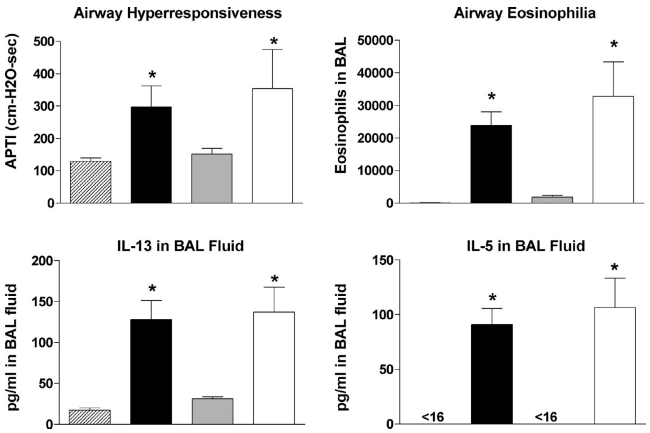

Figure 4. 1018 ISS inhibits allergen-induced airway hyperresponsiveness, eosinophilia, and Th2 cytokine production. Mice were sensitized with RW and alum (days 0 and 3) and challenged intratracheally on day 10 with 200 μg RW. 1 d before the challenge, the mice were treated via intratracheal administration of PBS (black bars), 20 μg 1018 ISS (gray bars), or control ODN (white bars). Mice challenged with PBS only (and not with RW) are represented by hatched bars. 2 d after challenge, airway responsiveness (top left) and the absolute number of eosinophils in the BAL (top right) were measured. In the same mice, IL-13 and IL-5 protein levels were determined in BAL fluid by ELISA (bottom left and right, respectively). Data shown are mean ± SEM of 10 mice per group, and * indicates a p-value of <0.05 when compared with the PBS-only challenged group. RW challenge in sensitized mice significantly increases airway responsiveness, the number of eosinophils, and IL-13 and IL-5 levels in BAL fluid. Treatment with 1018 ISS significantly inhibits all four of these features of allergic asthma.

pretreatment with 1018 ISS prevented the influx of these inflammatory cells (92% inhibition; Fig. 4, top right panel). RW challenge in sensitized mice also induced readily measurable IL-13 and IL-5 protein in the BAL fluid (Fig. 4, bottom left and right panels, respectively). Levels of both cytokines were significantly reduced by treatment with 1018 ISS (IL-13: 76% inhibition; IL-5: 82% inhibition), but not control ODN, confirming the inhibition of the mRNAs encoding these cytokines. In summary, it is clear that a single intranasal dose of 1018 ISS is able to inhibit antigen-dependent activation of Th2 cells in the lung as assessed by a number of physiological and molecular techniques.

APCs from the airways of 1018 ISS–treated mice do not activate Th2 cells in vitro

Based upon our current understanding of the biological activities of ISS, it is likely that the observed inhibition of Th2 cell activation is due to actions on TLR-9$^+$ APCs rather than on TLR-9$^-$ T cells. This was examined directly by testing the APC function of CD11c$^+$ cells derived from the lungs of mice treated with 1018 ISS and challenged with antigen. Mice were sensitized with OVA in alum (days 0 and 7). OVA was used instead of RW in these experiments as we had previously optimized conditions for stimulation of Th2 cells with APC from OVA-challenged

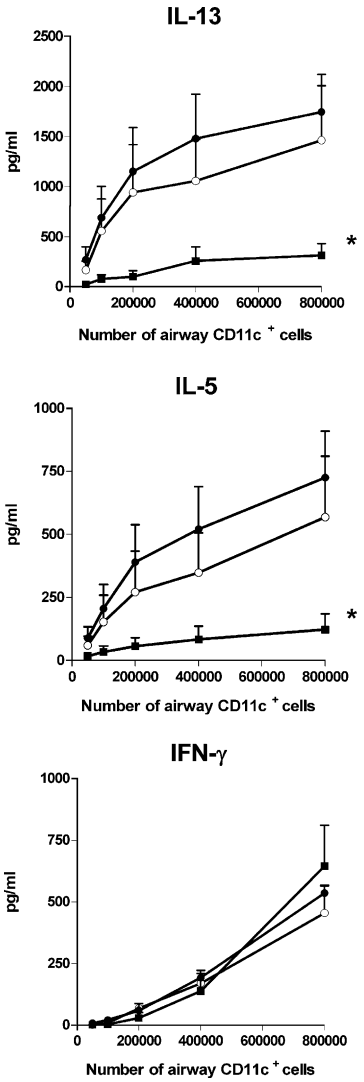

Figure 5. Antigen-presenting CD11c$^+$ cells from the airways of 1018 ISS–treated mice do not activate Th2 cells in vitro. IL-13, IL-5, and IFN-γ protein levels as measured by ELISA in culture supernatant are shown. A twofold dilution series of irradiated CD11c$^+$ cells from the airways (starting at 4×10^6 cells/ml) was incubated with a constant number of CD4$^+$/MEL14^{low} T cells (10^6 cells/ml) in 96 U-bottom wells for 72 h (total vol, 200 μl). The CD11c$^+$ cells were derived from the airways of sensitized and OVA-challenged mice treated in vivo with saline (black circles), 20 μg 1018 ISS (black squares), or control ODN (white circles). CD4$^+$/MEL14^{low} T cells were derived from the spleens of OVA-sensitized mice. Data shown are averaged from three separate experiments (mean ± SEM), and * indicates a p-value of <0.05 when compared with the response measured with CD11c$^+$ cells from OVA-challenged/saline-treated mice. CD11c$^+$ cells derived from OVA-challenged/saline-treated mice induce high levels of Th2 cytokines in the cultures. This is in sharp contrast to the low levels induced by CD11c$^+$ cells from OVA-challenged/1018 ISS–treated mice, demonstrating that these CD11c$^+$ cells prevent the activation of Th2 cells in vitro. IFN-γ levels are not changed, regardless of which in vivo pretreatment is applied.

mice. On day 13, mice were treated with 20 μg 1018 ISS, control ODN, or saline, and then on day 14 they were challenged with OVA aerosol. 24 h after OVA challenge,

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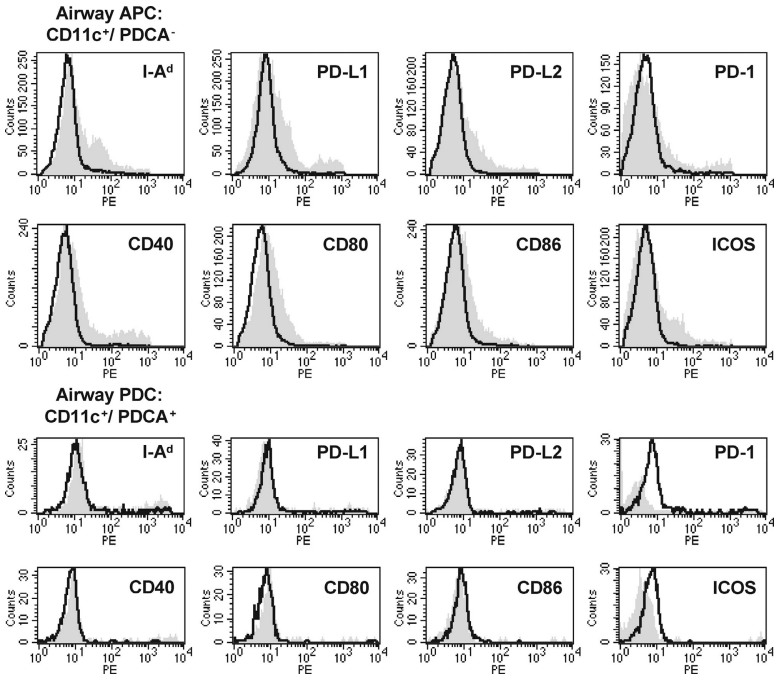

Figure 6. 1018 ISS prevents the induction of costimulatory molecules on airway APCs. CD11c$^+$ cells were derived from the airways of OVA-sensitized and -challenged mice treated in vivo with saline (shaded area) or 20 μg 1018 ISS (black line). Cells were stained with antibodies directed against CD11c, PDCA-1, B220, and a variety of costimulatory molecules, adhesion molecules, and MHC class II, including I-A^d, PD-L1, PD-L2, PD-1, CD40, CD80, CD86, and ICOS. Expression levels were analyzed in CD11c$^+$/PDCA-1$^-$ cells (airway APCs; top) and CD11c$^+$/PDCA-1$^+$ cells (airway PDCs; bottom). Airway APCs derived from OVA-challenged/1018 ISS–treated mice display lower expression levels of MHC class II and various costimulatory molecules than observed in OVA-challenged/saline-treated mice, whereas airway PDCs derived from 1018 ISS–treated mice display higher levels of PD-1 and ICOS compared with their saline-treated counterparts.

lungs were harvested and CD11c$^+$ cells were selected from the lung and irradiated. Our previous work has shown that 1 d after allergen challenge, the majority of cells presenting antigen in the airways are CD11c$^+$ (30). A dilution series of these CD11c$^+$ APCs was incubated with a constant number of CD4$^+$/MEL14^{low} T cells isolated from the spleens of OVA-sensitized mice. When CD11c$^+$ APCs were derived from allergen-challenged/saline-treated mice, high levels of IL-13 and IL-5 were induced, indicating efficient Th2 cell activation in these cultures (Fig. 5). In contrast, responses of both cytokines were greatly reduced at all APC doses when APCs were derived from allergen-challenged 1018 ISS–treated mice (inhibition at 8×10^5 APCs/2×10^5 T cells: 82 and 83%, respectively, for IL-13 and IL-5 when compared with saline-treated mice; Fig. 5). Although APCs from control ODN-treated mice were slightly less active than APCs from saline-treated mice, they were still very capable of stimulating Th2 cells in a dose-dependent manner. APCs from 1018 ISS-treated mice were not completely disabled as they were capable of stimulating an IFN-γ response from the relatively small Th1 cell population among the primed splenic T cells (Fig. 5). Our results demonstrate that 1018 ISS treatment in vivo alters the function of the airway APCs such that they prevent the activation of allergen-specific Th2 cells in vitro.

1018 ISS prevents induction of costimulatory molecules on airway APCs

In an attempt to elucidate why ISS treatment of allergic mice renders APCs in the airways unable to effectively activate Th2 cells, ISS-induced changes in the expression of molecules important for APC function were assessed. CD11c$^+$ cells derived from the airways of OVA-sensitized mice treated intranasally 1 d before allergen challenge with either saline or 1018 ISS were stained with antibodies to CD11c, B220, and the plasmacytoid dendritic cell (PDC)–specific antibody anti–PDCA-1, as well as with a variety of antibodies directed against costimulatory molecules, adhesion molecules, and MHC class II. CD11c$^+$/PDCA-1$^-$ cells from the airways of 1018 ISS–treated mice consistently expressed lower levels of MHC class II molecules on the cell surface when compared with CD11c$^+$/PDCA-1$^-$ cells from allergic mice treated with saline (Fig. 6, top). Interestingly, even though ISS are known inducers of dendritic cell maturation in vitro, expression of costimulatory molecules, including programmed cell death ligand (PD-L)1 and PD-L2, CD40, and inducible T cell costimulator (ICOS), was substantially lower on CD11c$^+$/PDCA-1$^-$ cells from 1018 ISS–treated mice when compared with the same cell population derived from the airways of allergic mice treated with saline (Fig. 6, top). Smaller differences were measured in the expression of

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CD80, CD86, or programmed cell death protein 1 (PD-1); however, CD11c$^+$/PDCA-1$^-$ cells from 1018 ISS–treated mice consistently expressed lower levels than those observed in saline-treated allergic mice. In allergic mice, the population of CD11c$^+$/PDCA-1$^-$ cells expressing the highest levels of costimulatory molecules also expressed B220, consistent with previous work describing CD11c$^+$/B220$^+$ cells as the predominant APCs in the airways (30). It is this population that demonstrates the greatest reduction in the expression of MHC class II and costimulatory molecules in 1018 ISS–treated mice. Thus, the overall pattern of reduced expression of molecules critical for antigen presentation provides support for the observation that CD11c$^+$ cells derived from 1018 ISS–treated mice are unable to activate Th2 cells in vitro and in vivo.

Concurrent with changes in the CD11c$^+$/PDCA-1$^-$ population, airway PDCs defined as CD11c$^+$/PDCA-1$^+$ cells derived from 1018 ISS–treated mice demonstrated a ninefold higher percentage of cells expressing the inhibitory molecule PD-1 compared with PDCs from saline-treated allergic mice (47% in 1018 ISS–treated mice vs. 5.3% in saline-treated mice; Fig. 6, bottom). In addition, PDCs from the airways of 1018 ISS–treated mice displayed increased levels of ICOS (25.2% in 1018 ISS–treated mice vs. 4.4% in saline-treated mice). No other differences in costimulatory molecule, adhesion molecule, or MHC class II expression were observed (Fig. 6, bottom). PD-1 acts as an important negative regulator of T cell responses (31), and it might be significant that this molecule is induced after in vivo treatment with 1018 ISS on TLR-9$^+$ PDCs in the airways. We speculate that activation of this inhibitory pathway may very well contribute to the inhibition of the Th2 response observed after ISS treatment.

1018 ISS inhibits IL-4 secretion and IL-4–mediated gene induction upon IgE cross-linking

Using the expression of such cytokines as IL-4 and IL-13 to measure the activation of Th2 cells in the lungs has a significant limitation. Allergic airways are rich in non–T cells capable of producing type 2 cytokines in response to allergen challenge. Previous work has shown that FcεRI-bearing cells, primarily mast cells and basophils, are the major source of IL-4 in the lung in the first few hours after allergen challenge, whereas at later times after challenge, IL-4 derives increasingly from activated Th2 cells (32). To determine whether ISS treatment was able to inhibit type 2 cytokine secretion from mast cells, basophils, and Th2 cells, we first investigated whether pretreatment with 1018 ISS was able to inhibit allergen-induced gene induction at an early time point after challenge. Mice were sensitized, treated with ISS, and challenged with RW as illustrated in Fig. 3, but the lungs were harvested at 6 h rather than 48 h after allergen challenge. In initial experiments (not depicted), many Th2-derived and Th2-dependent genes were highly induced as early as 6 h after challenge. In this series of experiments, all of the allergen-induced genes that were elevated at 48 h (Fig. 3) were observed to be significantly elevated by 6 h af-

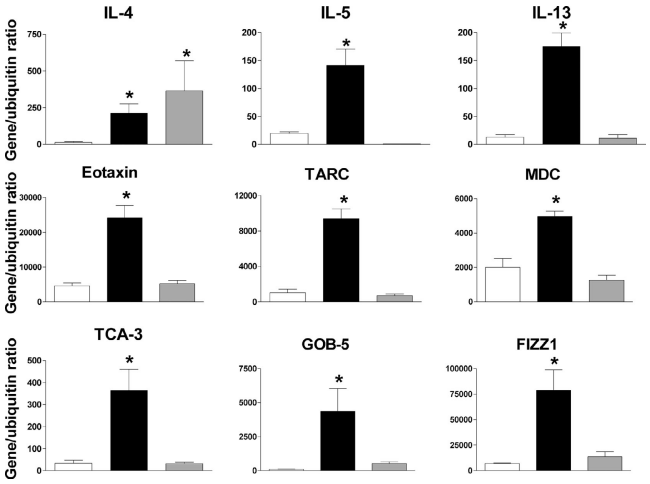

Figure 7. 1018 ISS prevents allergen-induced Th2 and Th2-dependent gene induction at an early time point after challenge. mRNA expression measured in whole lung is shown. Mice were sensitized with RW and alum on days 0 and 3. Lungs were harvested 6 h after intratracheal challenge (day 10) with 200 μg RW in sensitized mice. 1 d before the RW challenge, mice were given PBS (black bars), 20 μg 1018 ISS (gray bars), or control ODN (not depicted) intratracheally. Mice challenged with PBS only (and not with RW) are represented by white bars. All data are expressed as gene/ubiquitin ratio (mean ± SEM; five mice per group), and * indicates a p-value of <0.05 when compared with the PBS-only challenged group. RW challenge in sensitized mice induces significant up-regulation of Th2 and Th2-dependent genes 6 h after challenge. Pretreatment with 1018 ISS completely inhibits the induction of these genes, with the exception of IL-4.

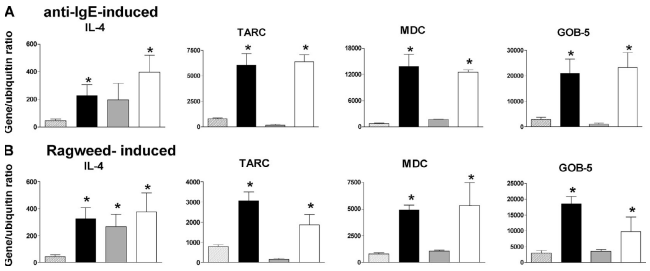

Figure 8. 1018 ISS also inhibits the induction of Th2-dependent genes after challenge with anti-IgE antibodies in sensitized mice. mRNA expression measured in whole lung is shown. Mice were sensitized with RW and alum on days 0 and 7. Lungs were harvested 6 h after intranasal challenge (day 14) of sensitized mice with 5 μg RW (A) or 50 μg anti-IgE antibodies (EM-95; B). 1 d before the RW or anti-IgE challenge, mice were given intranasal saline (black bars), 20 μg 1018 ISS (gray bars), or control ODN (white bars). Mice challenged with saline only (and not with RW or anti-IgE) are represented by hatched bars. All data are expressed as gene/ubiquitin ratio (mean ± SEM; five mice per group), and * indicates a p-value of <0.05 when compared with the saline-only challenged group. Intranasal challenge of sensitized mice with RW or anti-IgE antibodies up-regulates IL-4, TARC, MDC, and GOB-5 mRNA expression. Pretreatment with 1018 ISS completely prevents the induction of TARC, MDC, and GOB-5 mRNA expression.

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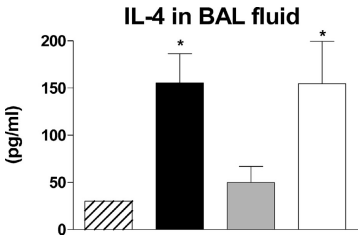

Figure 9. 1018 ISS prevents anti-IgE–induced IL-4 protein production in the BAL fluid of sensitized mice. IL-4 protein levels as measured by ELISA in BAL fluid are shown. Mice were sensitized with RW and alum on days 0 and 7. BAL fluid was harvested 6 h after intranasal challenge (day 14) with 50 μg anti-IgE antibodies (EM-95) in sensitized mice. 1 d before challenge, mice were treated intranasally with saline (black bar), 20 μg 1018 ISS (gray bar), or control ODN (white bar). Mice challenged with saline only (and not with anti-IgE) are represented by the hatched bar. Data shown are mean ± SEM of five mice per group, and * indicates a p-value of <0.05 when compared with the saline-only challenged group. Intranasal challenge of RW-sensitized mice with anti-IgE antibodies induces IL-4 protein production in BAL fluid, which is completely inhibited by 1018 ISS pretreatment.

ter allergen challenge in the group pretreated with saline (Figs. 7 and 8 A). In the 1018 ISS-treated mice, however, the induction of these genes was completely inhibited (Figs. 7 and 8 A), with the consistent exception of IL-4 mRNA. But as shown below, IL-4 protein secretion into BAL fluid is strongly inhibited by 1018 ISS at this time point.

The finding that early cytokine and chemokine induction is extensively inhibited by 1018 ISS treatment strongly suggests ISS inhibition of mast cell and basophil as well as T cell responses; however, gene expression at 6 h after allergen challenge does not conclusively demonstrate this as measurements at the early time point include both T and non–T cell sources of cytokine. To assess directly the effect of 1018 ISS on IgE-triggered pathways, mice primed with RW (days 0 and 7) were challenged (day 14) with anti-IgE instead of antigen. A subset of the genes induced by RW challenge (Fig. 8 A) was strongly induced with anti-IgE challenge: IL-4 and three of the most highly IL-4–inducible genes, TARC, MDC, and GOB-5 (Fig. 8 B). The IL-4–induced genes were very effectively inhibited by 1018 ISS pretreatment (Fig. 8 B); however, as observed in Figs. 7 and 8 A, IL-4 mRNA was not inhibited. As neither IL-13 protein nor mRNA levels were significantly elevated by anti-IgE challenge (not depicted), it was not obvious why the induction of genes regulated by IL-4 was inhibited, whereas IL-4 mRNA itself was not. One possible explanation was tested by measuring IL-4 protein recovered from the BAL fluid 6 h after anti-IgE challenge (Fig. 9). This resolved the paradox by showing that 1018 ISS was a potent inhibitor of induced IL-4 at the protein level, despite the lack of effect on IL-4 mRNA induction. Some FcεR1$^+$ cells have been shown to store IL-4 in granules for subsequent release (33), providing a possible explanation for the uncoupling of transcription and translation of IL-4 mRNA in this experimental setting.

DISCUSSION

CpG-containing ISS have shown significant promise for preventing allergic responses to pulmonary allergen challenge in both mouse (1–11) and primate (12) models of allergic asthma. They can be delivered systemically (1, 2, 4–6) or directly to the respiratory tract (1, 3, 6) and can inhibit not only acute responses to allergen, but also more chronic manifestations, such as airway remodeling (9–12). The response to a pulmonary allergen involves a series of distinct events mediated by a number of different cell types and cellular interactions, yet the events and cell types directly inhibited by ISS are poorly understood. In particular, the rapid and extensive, albeit transient, inhibition of acute Th2 responses caused by a single inhaled dose of ISS given before allergen challenge has yet to be explained.

Two distinct possible mechanisms underlying the immediate inhibition of the Th2 response by ISS were evaluated in this study. The first was inhibition by ISS of the effector functions mediated by the Th2 cell–dependent cytokines IL-4 and IL-13. Administration of IL-4 and IL-13 to the airways has been shown to induce many characteristics of allergic asthma, such as airway hyperresponsiveness, mucus production, and gene induction via a STAT-6–dependent mechanism (20–22, 29). Despite the known antagonism of IL-12, IFN-α, and IFN-γ on many IL-4– and IL-13–mediated activities (23–28), our data demonstrate clearly that this mechanism appears to contribute little to the inhibition of allergen challenge by ISS.

The second possible mechanism evaluated in our study was ISS-induced inhibition of Th2 cell activation. Th2 responses were measured directly in the lung by measuring the expression of Th2 and Th2-dependent genes in whole lung mRNA and confirming the expression of key cytokines by immunoassay of BAL fluid. ISS pretreatment resulted in extensive or complete inhibition of allergen-induced expression of the definitive Th2 cell genes, IL-4, IL-5, and IL-13, as well as all of the IL-4/IL-13–inducible genes we have tested. This direct evidence of Th2 cell inhibition was confirmed by a comparable inhibition of Th2-dependent airway hyperresponsiveness, eosinophilia, and Th2 cytokine proteins in the BAL fluid. These results constitute a comprehensive analysis of the T cell response to allergen challenge in the lung itself and are consistent with earlier studies that measured cytokines by ELISA and ELISPOT techniques (2–4).

FcεRI-bearing cells, such as mast cells and basophils, have been described to be a major source of IL-4 in the airways in the first few hours after allergen challenge, whereas at later times, IL-4 is increasingly derived from activated Th2 cells (32). The virtually complete inhibition of IL-4–induced genes and functions suggested that these non–T cell sources might also be inhibited by ISS. This could be a direct effect of ISS as it has been shown that mast cells and basophils are TLR-9$^+$ (34, 35) and respond to ISS stimulation with the production of IL-6 and TNF-α (36). This hypothesis was tested by challenge of allergic mice with anti-IgE antibodies rather than with allergen. Anti-IgE challenge induced IL-4

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secretion into the BAL fluid within 6 h, and this was indeed substantially inhibited by pretreatment with 1018 ISS. This was further confirmed by a profound reduction in the expression of the IL-4–inducible genes GOB-5, TARC, and MDC in the mice pretreated with 1018 ISS, indicating that biologically active IL-4 (and IL-13) had not been secreted. Although 1018 ISS did not significantly inhibit the early, mostly non–T cell–derived increase in IL-4 mRNA, it was clear that this IL-4 mRNA did not result in secreted IL-4 protein in ISS-treated mice. Taken together, these findings show that a single-dose pretreatment of ISS inhibits allergic airway responses by blocking the two key initial cellular events: cytokine release by mast cells and basophils by antigen cross-linking of IgE and cytokine production by allergen-activated Th2 cells. These actions are sufficient to explain the extensive inhibition—observed by us and many other groups—on the consequences of those initial cellular events: airway hyperresponsiveness, eosinophilia, goblet cell hyperplasia, mucus secretion, and airway remodeling (1–11).

One consequence of allergen-mediated IgE cross-linking may not, however, be affected by ISS treatment, degranulation, and release of such mediators as histamine and serotonin. Ikeda et al. (34) have reported that ISS does not inhibit the process of IgE-mediated degranulation, and we have confirmed this by measuring the immediate drop in body temperature after anti-IgE challenge. Challenge with anti-IgE as illustrated in Fig. 8 caused a transient reduction in rectal temperature from 38.1 ± 0.1°C before challenge to a minimum of 35.8 ± 0.1°C at 45 min postchallenge, consistent with an immediate response to IgE-mediated mast cell and basophil degranulation. A similar temperature drop was observed after anti-IgE challenge of sensitized/1018 ISS-treated mice, suggesting no inhibition of degranulation by ISS (Fig. S1, available at http://www.jem.org/cgi/content/full/jem.20050631/DC1). Chen et al. (37) have reported that IFN-α inhibits IL-3–primed IL-4 and IL-13 secretion from human basophils, whereas histamine and leukotriene C4 release was unaffected, providing additional evidence that cytokine and mediator release are regulated quite differently.

The nearly complete absence of allergen-induced Th2 cytokines combined with the absence of transcriptional and functional responses to those cytokines after ISS treatment demonstrated clearly that Th2 cell activation in the lung was inhibited by recent exposure to ISS. This was not a result of a change in the Th1/Th2 cell ratio as there was no evidence of a corresponding increase in the allergen-specific Th1 response. One possible mechanism for the failure of Th2 activation in ISS-treated mice could be inhibition of the migration of Th2 cells into the lung. This could account for part of the reduced Th2 response, particularly at the 48-h postchallenge time point. We have observed partial inhibition of $CD4^+$ T cell infiltration at 48 h in ISS-pretreated mice (not depicted), but this would not explain the virtually complete inhibition of Th2 responses we have observed as substantial numbers of antigen-specific Th2 cells reside in the lungs of primed mice, even before the first pulmonary challenge (30). The principal mechanism for Th2 inhibition in this model appears to be inhibition of the ability of lung APCs to activate differentiated Th2 cells. Antigen-presenting $CD11c^+$ cells derived from the lungs of sensitized and challenged mice treated in vivo with 1018 ISS failed to induce Th2 cell cytokine production in $CD4^+/MEL14^+$ T cell populations from primed spleen cells in vitro. This activity is not revealed in a complete in vitro system. The addition of 1018 ISS to the cultures of $CD4^+/MEL14^+$ T cells and antigen-presenting $CD11c^+$ cells derived from the airways of allergen-challenged mice did not inhibit Th2 cell cytokine production (not depicted). Thus, 1018 ISS needs to act in vivo in the presence of components of the lung microenvironment in order to have an inhibitory effect. This may explain why the ability of ISS to inhibit recall responses of fully polarized Th2 cells has not been reported previously.

We evaluated several potential mechanisms by which ISS may alter the function of $CD11c^+$ APCs. One of these was involvement of the rate-limiting enzyme of the tryptophan catabolism, indoleamine 2,3-dioxygenase (IDO). Increased levels of IDO lead to the enhanced degradation of the essential amino acid tryptophan, which in turn has been shown to inhibit T cell responses (38). ISS are capable of inducing increased levels of IDO in both lung epithelial cells and $CD11c^+$ dendritic cells (38, 39), making IDO a candidate by which $CD11c^+$ APCs could mediate the abrogation of Th2 cell activation that occurs after treatment with ISS. In a somewhat different model of murine asthma, inhibitors of IDO have been shown to reverse the protective effects of systemic ISS (39). In our models, we were able to measure a significant increase in pulmonary IDO mRNA levels after ISS treatment (Fig. S2, available at http://www.jem.org/cgi/content/full/jem.20050631/DC1); however, this did not result in significant IDO enzymatic activity (not depicted). Furthermore, subcutaneous implantation of slow-release pellets containing the IDO inhibitor 1-methyl-DL-tryptophan (M-trp) did not affect ISS-induced inhibition of the Th2 response (Fig. S3). Thus, in our models, IDO does not play a decisive role in the ISS-mediated inhibition of the Th2 response in the airways, and APCs of 1018 ISS–treated mice most probably use mechanisms other than IDO to down-regulate Th2 cell activation.

A second potential mechanism evaluated was an ISS-induced change in costimulatory molecule expression on the APCs. Costimulation is a critical component of APC function and various costimulatory molecules have important roles in stimulating effector T cell responses (for review see reference 31). We observed a drastic reduction in the overall expression of costimulatory molecules on $CD11c^+$ APCs from the airways of ISS-treated mice, including PD-L1, PD-L2, CD40, ICOS, CD80, CD86, and PD-1, accompanied by a clear reduction in the expression of MHC class II molecules, creating an overall picture of a much less competent APC. ISS may very likely induce these changes via a direct action on the APCs themselves as most APC subsets in the mouse express TLR-9 and respond to ISS (15). Alternatively, this might be

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regulated via indirect effects via other cell types. Of interest is our observation that in vivo ISS treatment induced the expression of the inhibitory molecule PD-1 on TLR-9$^+$ PDCs in the airways. Serebrisky et al. (4) investigated changes in costimulatory molecule mRNA expression levels in the airways after in vivo treatment with ISS. They observed that ISS-induced inhibition of the Th2 cell response was accompanied with an up-regulation in CD80 mRNA expression levels and decreased levels of CD86 mRNA. In contrast to our study, however, these changes were measured in whole lung.

In summary, we demonstrate that ISS do not act on cytokine-mediated effector pathways, but rather prevent Th2 cytokine secretion from both Th2 cells as well as IgE-activated cells, such as mast cells and basophils. In vivo treatment with ISS decreases the expression of MHC class II and costimulatory molecules on CD11c$^+$ APCs in the airways, resulting in APCs that fail to activate Th2 cells. Inhibition of both the Th2 and the IgE-mediated pathway enhances the prospects for successful therapeutic application of ISS in allergic asthma and reveals an exiting new mechanism of action of ISS.

MATERIALS AND METHODS

In vivo treatment with IL-4, IL-13, or ISS. Animal experiments described in our studies were performed at Northview Pacific Laboratories Inc. or at the animal facility of the Division of Immunobiology, Cincinnati Children's Hospital Medical Center, and all procedures applied were Institutional and Animal Care Use Committee–approved.

6–8-wk-old female BALB/c mice were anesthetized with isoflurane and treated intranasally with 5 μg IL-4 or IL-13 (R&D Systems) in 50 μl of pyrogenic-free saline. 1 d before IL-4 or IL-13 treatment, the mice were pretreated intranasally with 20 μg ISS or control ODN in 50 μl saline. The ISS and two control ODNs used in our experiments were 1018 ISS (5′-TGACTGTGAACGTTCGAGATGA), 1040 (5′-TGACTGTGAACCTTAGAGATGA), and C661 (5′-TGCTTGCAAGCTTGCAAGCA), respectively. All sequences used had <5 endotoxin units per mg of ODN as determined by Limulus amebocyte lysate assay (BioWhittaker). Sequences were synthesized as described previously (40). To induce IL-13–mediated airway hyperresponsiveness, mice were treated three times with 5 μg IL-13 on consecutive days (days 1–3) as described previously (20). Before the IL-13 treatments, one 20-μg ISS treatment (day 0) was administered. In this particular set of experiments, IL-13 and ISS were given intratracheally.

Ragweed sensitization and allergen or anti-IgE challenge. 6–8-wk-old female BALB/c mice were intraperitoneally sensitized with 150 μg defatted RW (*Ambrosia artemissifolia*; Greer Laboratories) and alum on days 0 and 3. On day 9, the mice were pretreated with 20 μg ISS, control ODN, or diluent control via intratracheal administration under ketamine/xylazine anesthesia. On day 10, the mice were intratracheally challenged with 200 μg RW or diluent control (41).

In a second series of experiments, a different sensitization was used. Mice were intraperitoneally sensitized with 10 μg RW in alum on days 0 and 7. On day 13, the mice were pretreated with 20 μg ISS, control ODN, or saline via intranasal administration. On day 14, these mice were challenged intranasally with 5 μg RW, 50 μg anti–mouse anti-IgE antibodies (EM95), or with saline. The anti–mouse anti-IgE antibodies were provided by F. Finkelman (University of Cincinnati, Cincinnati, OH).

Airway responsiveness measurements and assessment of airway inflammation. Airway responsiveness to intravenous acetylcholine was measured as described previously (42). In brief, 1 d after the last IL-13 treatment or 2 d after RW challenge, mice were anesthetized with sodium pentobarbital, intubated, and ventilated at a rate of 120 breaths/min with a constant tidal volume of 0.2 ml. After recording a stable baseline airway pressure, mice were paralyzed with decamethonium and injected intravenously with 50 μg/kg acetylcholine.

After airway responsiveness measurements, lungs were lavaged thoroughly with saline. BAL cells were separated from BAL fluid by centrifugation and counted. Cytospins were prepared and stained with Wright-Giemsa. Differential cell counts were obtained using oil immersion microscopy. At least 500 cells were counted per cytospin.

Isolation of T cells and airway APCs as well as flow cytometric analysis. Mice were intraperitoneally sensitized with 10 μg OVA (grade 5; Sigma-Aldrich) in alum on days 0 and 7. For T cell isolation, spleens were harvested 1 wk after sensitization (day 14). For isolation of airway allergen-presenting cells, sensitized mice were pretreated on day 13 with 20 μg ISS, control ODN, or saline via intranasal administration. 1 d later (day 14), the mice were challenged with 5 mg/ml of an OVA aerosol for 45 min. 24 h after challenge lungs were removed. Both the spleen and lungs were minced and incubated with 0.02 mg/ml DNase I (Sigma-Aldrich) and 1 mg/ml collagenase D (Roche) for 30 min at 37°C while shaking. Single cell suspensions were prepared, the red blood cells were lysed, and cell suspensions were thoroughly washed. For the spleen, the cells were first depleted for B220$^+$, CD8$^+$, and CD11b$^+$ cells using MACS microbeads and MACS LD columns (both from Miltenyi Biotec) according to the manufacturer's instructions. The remaining cells were then stained with fluorescently labeled anti-MEL14 (MEL-14; BD Biosciences) and anti-CD4 antibodies (GK1.5; BD Biosciences), and subsequently sorted for CD4$^+$/MEL14^{low} cells using a MoFlo high speed sorter (DakoCytomation). After sorting, the CD4$^+$/MEL14^{low} cells were washed and counted. For the lung, adherent cells were removed by incubating the cell suspension in a Petri dish for 1 h at 37°C. CD11c$^+$ cells were then selected from the nonadherent cell population using the appropriate MACS microbeads and MACS LD columns according to the manufacturer's instructions. The CD11c$^+$ cells were irradiated, washed, and counted.

Flow cytometric analysis of CD11c$^+$ cells isolated from the airways was performed using a BD FACSCalibur (BD Biosciences) after staining with monoclonal antibodies directed against a variety of costimulatory molecules, adhesion molecules, and MHC class II. Antibodies included anti-CD11c (HL-3), anti-B220 (RA3-6B2), anti–I-A^d (AMS-32.1), anti–PD-L1 (MIH5), anti–PD-1 (J43), anti-CD40 (3/23), anti-CD80 (16-10A1), anti-CD86 (GL1), anti-ICOS (7E.17G9; all from BD Biosciences), anti–PDCA-1 (Miltenyi Biotec), and anti–PD-L2 (TY25; eBioscience).

In vitro incubation of airway APCs and T cells. A twofold dilution series of CD11c$^+$ cells isolated from the airways (starting at 4×10^6 cells/ml) was incubated with a constant number of CD4$^+$/MEL14^{low} T cells (10^6 cells/ml) in 96 U-bottom wells for 72 h (total vol, 200 μl). As a positive control, wells containing CD11c$^+$ cells, CD4$^+$/MEL14^{low} T cells, and OVA (250 μg/ml) were included. The CD11c$^+$ cells were derived from the airways of OVA-sensitized and -challenged mice pretreated in vivo with saline, 20 μg 1018 ISS, or control ODN. CD4$^+$/MEL14^{low} T cells were derived from the spleens of OVA-sensitized mice. After 72 h of incubation, supernatant was harvested and tested for the presence of IL-13, IL-5, and IFN-γ cytokines by ELISA.

Cytokine measurement in BAL fluid or culture supernatant. BAL fluids from individual mice were lyophilized and resuspended in 200 μl saline. Levels of IL-4, IL-5, IL-13, and IFN-γ protein were measured in BAL fluid samples or culture supernatants using ELISA kits (R&D Systems) according to the manufacturer's instructions. The lower detection levels were 8, 16, and 8 pg/ml for the IL-4, IL-5, and IL-13 ELISA, respectively. IFN-γ protein levels were assayed by matched antibody pairs (BD Biosciences) used according to the manufacturer's instructions. The lower detection level of this ELISA was 13 pg/ml.

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Quantitation of gene expression by real-time PCR. The protocol for the analysis of gene expression has been detailed elsewhere (43). In brief, snap-frozen mouse lungs were homogenized in RLT buffer (QIAGEN) using a polytron homogenizer. Total RNA was extracted via the RNeasy Mini Column Protocol (QIAGEN). Of each RNA sample, 5 μg was incubated with DNase I (Boerhinger) in the presence of RNAsin (Promega) and subsequently converted into cDNA using oligo dT (Promega), random hexamers, and SuperScript RT II (Invitrogen). cDNA was diluted 1:100, and PCR was conducted using QuantiTect SYBR green PCR master mix (QIAGEN) combined with primer pairs (Operon) or QuantiTect probe PCR master mix and PDAR primer pairs with labeled probe (Applied Biosystems). PCR reactions were performed using a sequence detector (GeneAmp 5700; Applied Biosystems). PCR amplification of the housekeeping gene ubiquitin was performed for each sample, allowing for normalization of data. All data are expressed as a gene/ubiquitin ratio.

Data analysis. Data were analyzed with a one-way analysis of variance followed by a Bonferroni multiple comparisons test. When data groups demonstrated unequal standard deviations or were not normally distributed, a Kruskal-Wallis nonparametric analysis of variance was performed followed by a Dunn's multiple comparisons test. ELISA data from the culture supernatants were analyzed with a two-way analysis of variance followed by a Bonferroni posttest. Analyses were performed with GraphPad InStat, and $P < 0.05$ was considered significant for all statistical analyses.

Online supplemental material. Fig. S1 shows the change in body temperature measured before and at various time points after intranasal challenge with anti-IgE antibodies in RW-sensitized mice. 1 d before the challenge, mice were treated intranasally with either saline or 1018 ISS. These data demonstrate that challenge of RW-sensitized mice with anti-IgE antibodies significantly decreases the body temperature either in the absence or presence of 1018 ISS pretreatment. Fig. S2 shows IDO mRNA levels measured in whole lung after intranasal treatment with saline, 1018 ISS, or control ODN in naive or Th2-primed mice. Significant IDO expression is observed after the treatment of naive and allergic mice with 1018 ISS. Fig. S3 shows IL-5 and IL-13 protein levels in BAL fluid measured both in the RW and in the OVA asthma model. Mice were sensitized as described above, and on day 12, slow-release pellets containing the IDO inhibitor M-trp were inserted under the dorsal skin. Mice were treated with 1018 ISS and challenged with allergen as described above, and BAL fluid was harvested 24 h after challenge. These data demonstrate that treatment with 1018 ISS significantly inhibits the Th2 response in the airways, which is not prevented by concurrent treatment with M-trp.

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