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J Immunol 2004; 173:7556-7564; ;
doi: 10.4049/jimmunol.173.12.7556
<http://www.jimmunol.org/content/173/12/7556>

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The American Association of Immunologists, Inc.,
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Print ISSN: 0022-1767 Online ISSN: 1550-6606.



Immunostimulatory DNA Reverses Established Allergen-Induced Airway Remodeling¹

Cho Jae Youn, Marina Miller, Kwang Je Baek, Ji Won Han, Jyothi Nayar, Sook Young Lee, Kirsti McElwain, Shauna McElwain, Eyal Raz, and David H. Broide²

To determine whether immunostimulatory sequences of DNA (ISS) can reverse established airway remodeling, mice that had developed airway remodeling following 3 mo of repetitive OVA challenges, were treated with ISS for 1–3 mo. Systemic administration of ISS to mice that had already developed established airway remodeling significantly reduced the degree of airway collagen deposition (assessed by lung collagen content, peribronchial trichrome staining, and immunostaining with anticollagen type III and type V Abs). ISS reduced bronchoalveolar lavage and lung levels of TGF- β 1 and reduced the number of TGF- β 1-positive eosinophils and TGF- β 1-positive mononuclear cells recruited to the airway. *In vitro* studies demonstrated that ISS inhibited TGF- β 1 expression by macrophages (RAW 264.7 cell line and bone marrow-derived macrophages). In addition, ISS significantly reduces lung levels of expression of the chemokine thymus- and activation-regulated chemokine, as well as the number of peribronchial CD4⁺ lymphocytes that express Th2 cytokines that promote peribronchial fibrosis. Overall, these studies demonstrate that ISS can reverse features of airway collagen deposition by reducing levels of lung TGF- β 1, as well as by reducing levels of the chemokine thymus- and activation-regulated chemokine and the numbers of peribronchial CD4⁺ lymphocytes that drive the ongoing Th2 immune response. *The Journal of Immunology*, 2004, 173: 7556–7564.

Immunostimulatory sequences of DNA (ISS)³ are being investigated as potential therapeutic agents in asthma based on studies in mouse models demonstrating that ISS can prevent (1–6) as well as reverse (6–8) acute allergen-induced eosinophilic inflammation and airway hyperreactivity. In addition, ISS reduces airway inflammation, mucus expression, and viral load in mice challenged with respiratory syncytial virus, a virus associated with triggering episodes of asthma (9). The mechanism by which ISS inhibits airway inflammation in mouse models of asthma is incompletely understood. The immune response to ISS is characterized by the expression of cytokines including IFNs (α , β , and γ), IL-6, IL-10, and IL-12 (10). ISS also up-regulates the expression of Th1 cytokine receptors such as the IFN- γ receptor (11). In addition to inducing Th1 responses, ISS also inhibits expression of Th2 cytokines in the lung including IL-4 (2, 12), IL-5 (1), IL-9 (12), and IL-13 (13), while also reducing levels of expression of Th2 cytokine receptors such as the IL-4R (11). The immunomodulatory effect of ISS is due to its effect on cells that express TLR-9 (14) the receptor for ISS, in particular dendritic cells, as well as macrophages, and B cells.

In mouse models ISS is effective in inhibiting Th2-mediated eosinophilic inflammation in the lung when administered either systemically or delivered directly to the mucosa of the airway (1). A single systemic dose of ISS inhibits Th2 responses to OVA

inhalation challenge for at least 4 wk (5). ISS is as effective as corticosteroids in preventing (1), or reversing (7), eosinophilic inflammation and airway hyperreactivity in mouse models. More recently, studies in our (13) and other laboratories (15) have demonstrated that prophylactic administration of ISS can prevent airway remodeling when administered to mice before starting inhalation challenge with allergen. Prophylactic ISS administration significantly prevents features of airway remodeling including peribronchial fibrosis, as well as the allergen-induced increased thickness of the peribronchial smooth muscle layer (13). ISS administration not only prevented the development of these structural features of airway remodeling, but also inhibited the development of airway hyperreactivity, eosinophilic inflammation, airway mucus expression, and reduced levels of profibrotic growth factors including TGF- β and IL-13 (13). In this study we have investigated whether ISS can reverse established airway remodeling, as opposed to the previous studies that demonstrated ISS can prevent airway remodeling (13, 15). In prior studies examining whether ISS can prevent airway remodeling we started the therapeutic intervention with ISS before the onset of the first of a series of 3 mo of repetitive OVA challenges (13). In contrast, in this study we first instituted the 3 mo of repetitive OVA challenges to induce airway remodeling, and at that time point instituted ISS therapy to determine whether airway remodeling can be reversed. Although a variety of pathologic features comprise airway remodeling in asthma (16), we have focused in this study in particular on examining the effect of ISS on reversing established peribronchial collagen deposition in the lung.

Materials and Methods

Induction of chronic pulmonary eosinophilic inflammation

Female BALB/c mice ($n = 16$ mice/group; The Jackson Laboratory, Bar Harbor, ME), 8–10 wk of age, were immunized s.c. on days 0, 7, 14, and 21 with 25 μ g of OVA (grade V; Sigma-Aldrich, St. Louis, MO) adsorbed to 1 mg of alum (Sigma-Aldrich) in 200 μ l of normal saline. Intranasal OVA challenges (20 ng/50 μ l in PBS) were administered on days 27, 29, and 31 under isoflurane (Vedco, St. Joseph, MO) anesthesia. Intranasal

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Received for publication January 5, 2004. Accepted for publication October 15, 2004.

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¹ This study was supported by National Institutes of Health Grants AI 33977 and AI 38425 (to D.B.) and National Institutes of Health Grant AI 40682 (to E.R.).

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³ Abbreviations used in this paper: ISS, immunostimulatory sequence; TARC, thymus- and activation-regulated chemokine; ODN, oligodeoxynucleotide; BAL, bronchoalveolar lavage; PAS, periodic acid Schiff; MBP, major basic protein.

well as the total number of peribronchial cells staining positive for either MBP and F4/80 in ISS treated or control mice. Results are expressed as the total number of peribronchial cells staining positive for TGF- β 1/bronchiole, and as the total number of peribronchial cells staining positive for either MBP or F4/80/bronchiole, of bronchioles 150–200 μ m of internal diameter. At least 10 bronchioles were counted in each slide.

Effect of ISS on macrophage TGF- β 1 and IL-6 expression in vitro

Previous studies have not reported whether ISS modulates macrophage TGF- β 1 expression that was the focus of these experiments. In these experiments, 5×10^5 macrophage RAW 264.7 cells were incubated with LPS 100 ng/ml in triplicate for 24 h in the presence or absence of ISS or mutated ODN (10 μ g/ml). Supernatants were collected and assayed for TGF- β 1 by ELISA. As a control, we examined the effect of ISS on RAW 264.7 cell line expression of IL-6 (measured by ELISA), which several groups have demonstrated is induced by ISS (19). We also performed experiments assessing the effect of ISS on macrophage TGF- β 1 expression using purified populations of macrophages derived from the culture of mouse bone marrow using culture methods previously described in this laboratory (20).

Effect of ISS on levels of lung thymus- and activation-regulated chemokine (TARC) expression and peribronchial CD4⁺ T lymphocyte accumulation

As T lymphocytes produce cytokines such as IL-5 and IL-13 that contribute to airway remodeling (16, 17), we measured levels of lung expression of the T lymphocyte chemoattractant TARC, as well as the number of peribronchial CD4⁺ lymphocytes. Previous studies have not examined whether ISS effects the lung expression of chemokines such as TARC that bind to CCR4 receptors on Th2 cells, as well as whether ISS effects the number of peribronchial CD4⁺ lymphocytes in the remodeled airway.

Measurement of TARC. Lungs were homogenized as described, and the lung supernatant was assayed for TARC by ELISA.

Number of peribronchial CD4⁺ lymphocytes. Serial sequential lung sections were processed for CD4 immunohistochemistry in a similar manner as already described for lung MBP immunostaining. The primary monoclonal anti-CD4 Ab was obtained from Santa Cruz Biotechnology. Results are expressed as the number of peribronchial cells staining positive for CD4 per bronchiole of bronchioles 150–200 μ m of internal diameter. At least 10 bronchioles were counted in each slide.

Effect of ISS on macrophage TARC expression in vitro

As TARC is expressed by macrophages we measured levels of TARC in supernatants derived from RAW 264.7 cells stimulated with LPS 100 ng/ml in triplicate for 24 h in the presence or absence of ISS or mutated ODN (10 μ g/ml).

Effect of ISS on area of peribronchial α -smooth muscle actin immunostaining

Lung sections for α -smooth muscle actin immunostaining were processed in a similar manner as described for lung collagen immunostaining. The primary anti- α -smooth muscle actin mAb was obtained from Sigma-Aldrich. The area of peribronchial α -smooth muscle actin immunostaining was outlined and quantified using a light microscope attached to an image analysis system as earlier described.

Effect of ISS on airway mucus expression

To quantitate the level of mucus expression in the airway, the number of PAS-positive and PAS-negative epithelial cells in individual bronchioles were counted as previously described in this laboratory study (13, 17). At least 10 bronchioles were counted in each slide. Results are expressed as the percentage of PAS-positive cells per bronchiole that is calculated from the number of PAS-positive epithelial cells per bronchus divided by the total number of epithelial cells of each bronchiole.

Statistical analysis

Results in the different groups of mice were compared by ANOVA using the nonparametric Kruskal-Wallis test followed by posttesting using Dunn's multiple comparison of means. All results are presented as mean \pm SEM. A statistical software package (Graph Pad Prism, San Diego, CA) was used for the analysis. Values of $p < 0.05$ were considered statistically significant.

Results

Effect of ISS on reversing peribronchial collagen deposition

Mice exposed to repetitive OVA challenge for 3 mo had a significant increase in the levels of peribronchial fibrosis compared with non-OVA-challenged mice as assessed by several parameters including lung collagen (Fig. 2A), trichrome stain (B), as well as collagen III (C), and collagen V (D) immunostaining. The non-OVA-challenged mice in all experiments now described were sensitized to OVA as described in *Materials and Methods*. Mice challenged with OVA for 3 mo, and then followed for an additional 1 mo, or for 3 mo without further OVA challenges maintained the increase in peribronchial fibrosis compared with non-OVA-challenged mice at 1 mo of follow up, and at 3 mo of follow up (Fig. 2, A–D). Although levels of peribronchial trichrome staining and collagen V immunostaining were still significantly elevated at 1 and 3 mo following discontinuation of OVA challenges, these levels did decline following discontinuation of OVA challenges (Fig. 2, B and D). In contrast, there was no decline in levels of lung collagen or collagen III when OVA challenges were discontinued (Fig. 2, A and C).

To determine whether ISS could reverse established peribronchial fibrosis, ISS treatment was only started after completion of 3 mo of OVA challenges. When ISS therapy was instituted at this time point for 1 or 3 mo, ISS significantly reduced the level of peribronchial fibrosis including lung collagen (Fig. 2A), trichrome stain (B), as well as collagen III (C) and collagen V (D) immunostaining.

Fibroblasts have not thus far been reported to express TLR-9. To determine whether ISS could directly modulate fibroblast function via TLR-9 on fibroblasts we performed RT-PCR studies to detect whether TLR-9 is expressed by fibroblasts using a fibroblast cell line. RT-PCR studies demonstrated that the fibroblast cell line PA317 did not express TLR-9 the receptor for ISS (data not shown). Control mouse bone marrow-derived mast cells strongly expressed TLR-9 as previously described in this laboratory study (12).

Effect of ISS on lung TGF- β 1 expression

As the fibroblast cell line did not express TLR-9, we examined whether ISS reduced expression of TGF- β 1 a growth factor associated with peribronchial fibrosis (16, 20). We measured levels of TGF- β 1 in BAL fluid, lung, as well as the number of peribronchial cells expressing TGF- β 1. Levels of BAL TGF- β 1 (Fig. 2E), lung TGF- β 1 (F), and the number of peribronchial TGF- β 1-positive cells (G) were significantly increased in mice exposed to repetitive OVA challenge for 3 mo compared with non-OVA-challenged mice. One month after the 3 mo of OVA challenges were discontinued, BAL levels of TGF- β 1 (Fig. 2E), lung levels of TGF- β 1 (F), and the number of peribronchial TGF- β 1-positive cells (G) were still significantly elevated compared with non-OVA-challenged mice, although levels had declined from the time of the last OVA challenge. At 3 mo after the OVA challenges were discontinued, BAL levels of TGF- β 1 (Fig. 2E), and the number of peribronchial TGF- β 1-positive cells (G) were still significantly elevated compared with non-OVA-challenged mice, although levels had declined from the time of the last OVA challenge. Lung levels of TGF- β 1 (Fig. 2F) were no longer elevated 3 mo after OVA challenges were discontinued.

Institution of ISS treatment after completion of the OVA challenges significantly reduced the BAL levels of TGF- β 1 (Fig. 2E), lung levels of TGF- β 1 (F), and the number of peribronchial TGF- β 1-positive cells (G) in mice following 1 mo of ISS treatment.

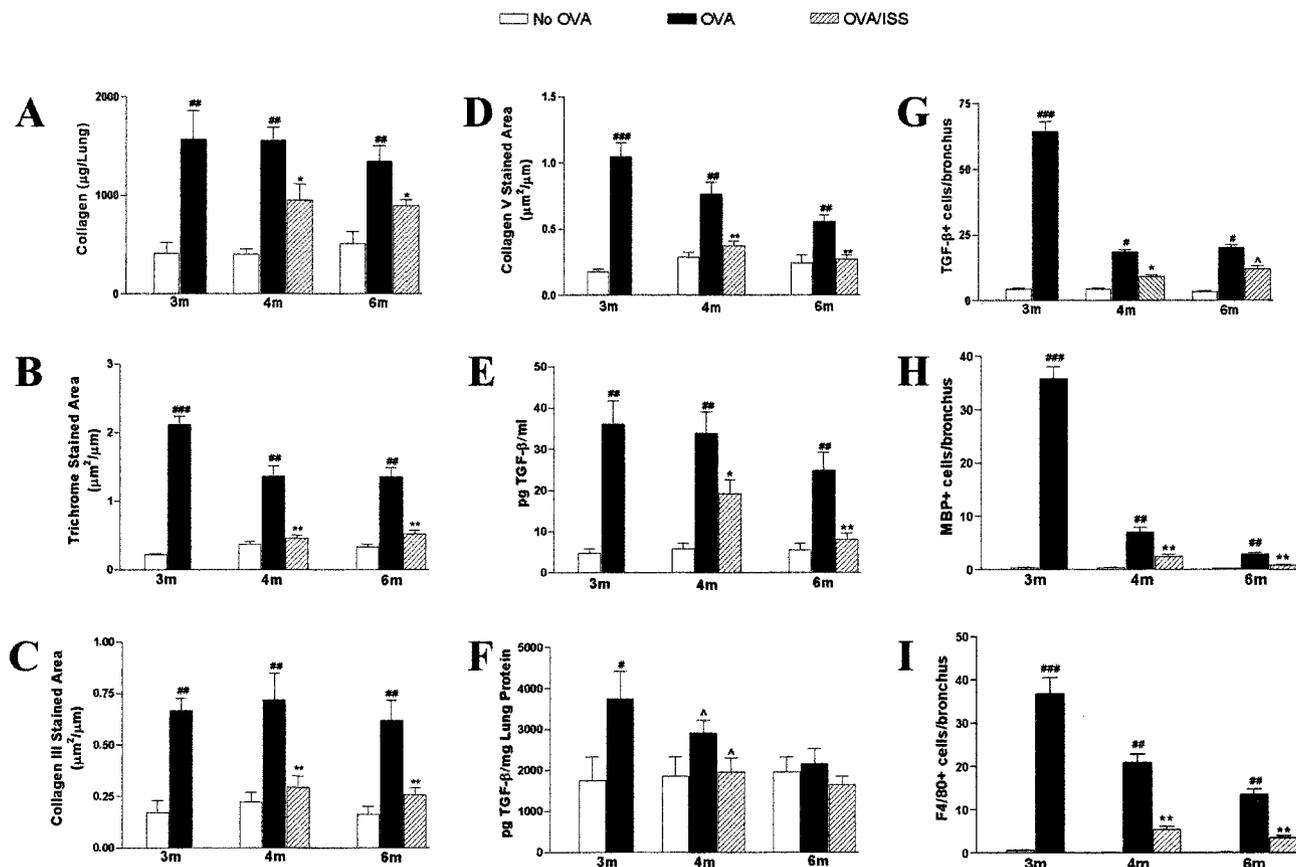


FIGURE 2. ISS reduces peribronchial fibrosis. *A–D*, Repetitive OVA challenge for 3 mo (3m) in untreated mice induced a significant increase in the levels of lung collagen (*A*), peribronchial trichrome stained area (*B*), as well as peribronchial collagen III (*C*), and collagen V immunostaining (*D*) compared with non-OVA-challenged mice (#, $p = 0.01$; ##, $p = 0.001$; ###, $p = 0.0001$) ($n = 16$ mice/group). Mice challenged with OVA for 3 mo, and then followed for an additional 1 mo (4m), or 3 mo (6m) without further OVA challenges continued to have a significant increase in the levels of lung collagen (*A*), peribronchial trichrome stained area (*B*), as well as peribronchial collagen III (*C*), and collagen V immunostaining (*D*) compared with non-OVA-challenged mice. Institution of ISS treatment after 3 mo of OVA challenges for 1 mo (4m) or 3 mo (6m) significantly reduced the level of lung collagen (*A*), peribronchial trichrome stained area (*B*), as well as peribronchial collagen III (*C*), and collagen V immunostaining (*D*) (*, $p = 0.01$; **, $p = 0.001$; ***, $p = 0.0001$). *E–I*, Repetitive OVA challenges for 3 mo induced significantly increased levels of BAL TGF- $\beta 1$ (*E*), lung TGF- $\beta 1$ (*F*), as well as the number of peribronchial TGF- $\beta 1^+$ (*G*), MBP $^+$ (*H*), and F4/80 $^+$ (*I*) cells compared with non-OVA-challenged mice (#, $p = 0.01$; ##, $p = 0.001$; ###, $p = 0.0001$). Institution of ISS treatment after 3 mo of OVA challenges, for 1 mo (4m) or 3 mo (6m), also significantly reduced the levels of BAL TGF- $\beta 1$ (*E*), lung TGF- $\beta 1$ (*F*), as well as the number of peribronchial TGF- $\beta 1^+$ (*G*), MBP $^+$ (*H*), and F4/80 $^+$ (*I*) cells (*, $p = 0.05$; *, $p = 0.01$; **, $p = 0.001$; ***, $p = 0.0001$).

Similar declines in BAL levels of TGF- $\beta 1$ (Fig. 2*E*), and the number of peribronchial TGF- $\beta 1$ -positive cells (*G*) were noted following 3 mo of ISS treatment.

Mechanisms by which ISS inhibits TGF- $\beta 1$ expression

ISS can theoretically reduce levels of lung TGF- $\beta 1$ by either decreasing the total number of lung cells that express TGF- $\beta 1$ (e.g., the number of MBP $^+$ eosinophils, or F4/80 $^+$ mononuclear cells) in the airway, and/or by decreasing levels of expression of TGF- $\beta 1$ by individual cells in the airway. We therefore examined whether ISS reduced the number of TGF- $\beta 1$ -positive cells in vivo, and whether it directly inhibited macrophage TGF- $\beta 1$ expression in vitro.

Effect of ISS on the number of MBP $^+$ and F4/80 $^+$ peribronchial cells

In previous studies, using double immunostaining to TGF- $\beta 1$ and MBP or F4/80, we have demonstrated that mononuclear cells and eosinophils are the predominant peribronchial cellular source of TGF- $\beta 1$ following 3 mo of repetitive OVA challenge (17). We therefore determined the effect of ISS on reducing the total number

of peribronchial mononuclear cells and eosinophils, as well as the effect of ISS on reducing the number of peribronchial TGF- $\beta 1$ -positive cells. Repetitive OVA challenge for 3 mo in untreated mice induced a significant increase in the number of MBP $^+$ peribronchial cells (35.7 ± 2.3 vs 0.3 ± 0.1 peribronchial MBP $^+$ cells) (OVA vs no OVA; $p = 0.0001$) (Fig. 2*H*), and F4/80 $^+$ peribronchial cells (36.7 ± 3.8 vs 0.5 ± 0.1 peribronchial F4/80 $^+$ cells) (OVA vs no OVA; $p = 0.0001$) (*I*) compared with non-OVA challenged mice. After discontinuing OVA challenges at 3 mo, the number of peribronchial eosinophils declined to a greater degree than did the number of peribronchial mononuclear cells (Fig. 2, *H* and *I*). However, mice challenged with OVA for 3 mo, and then followed for up to 3 mo, without further OVA challenges maintained a significant increase in the number of peribronchial MBP $^+$ and F4/80 $^+$ cells compared with non-OVA-challenged mice at 1 mo of follow up ($p = 0.001$; MBP, Fig. 2*H*) ($p = 0.001$; F4/80; *I*), and at 3 mo of follow up ($p = 0.001$; MBP, *H*) ($p = 0.001$; F4/80, *I*). Institution of ISS treatment at the time of discontinuing OVA challenges significantly reduced the number of peribronchial MBP $^+$ and F4/80 $^+$ cells in mice following 1 mo of ISS treatment

($p = 0.001$; MBP, Fig. 2H; $p = 0.001$; F4/80, I), as well as following 3 mo of ISS treatment ($p = 0.001$; MBP, H; $p = 0.001$; F4/80, I).

ISS induced a significant decrease in the total number of peribronchial cells that express TGF- β 1 (TGF- β 1-positive cells per bronchus), as well as a parallel decline in the combined total number of eosinophils and mononuclear cells per bronchus. Following 3 mo of OVA challenges, therapy with ISS for 1 mo, compared with treatment with diluent, reduced both the number of peribronchial TGF- β 1 positive cells (9.1 ± 0.6 vs 18.5 ± 1.0 peribronchial TGF- β 1-positive cells per bronchus) ($p = 0.001$), and the total combined number of peribronchial eosinophils plus mononuclear cells (5.8 ± 1.4 vs 27.9 ± 1.9 MBP⁺ and F4/80⁺ cells per bronchus) ($p = 0.001$).

Effect of ISS on macrophage TGF- β 1 and IL-6 levels

To determine whether ISS inhibits release of TGF- β 1 by macrophages, macrophages (RAW 264.7 cell line) were incubated in vitro in the presence or absence of ISS. Supernatants were assayed for both TGF- β 1, and as a control IL-6 a cytokine known to be regulated by ISS. Incubation of macrophages with ISS induced a significant release of IL-6 into the supernatant (628.2 ± 2.4 vs 9.2 ± 1.6 pg/ml IL-6) (ISS vs medium; $p = 0.008$) (Fig. 3A), whereas ISS did not induce macrophages to release significant amounts of TGF- β 1 compared with cells incubated in medium alone (B) (ISS vs medium). A mutated ODN did not induce significant IL-6 or TGF- β 1 release from macrophages (mutated ODN vs medium) (Fig. 3, A and B). Although ISS induced macrophages to release significantly more IL-6 than TGF- β 1, LPS differed from ISS in that it stimulated both IL-6 release from macrophages (1066.9 ± 53.8 vs 9.2 ± 1.6 pg/ml IL-6) (LPS vs medium; $p = 0.001$) (Fig. 3A), and TGF- β 1 release from macrophages (279.0 ± 52.4 vs 68.6 ± 18.9 pg/ml TGF- β 1) (LPS vs medium; $p = 0.001$) (B). To determine whether ISS modulated TGF- β 1 expression by macrophages, macrophages were incubated with LPS to release TGF- β 1 in the presence or absence of ISS. ISS significantly inhibited LPS-stimulated macrophage release of TGF- β 1 (45.1 ± 18.5 vs 279.2 ± 52.4 pg/ml TGF- β 1) (LPS plus ISS vs LPS; $p = 0.001$) (Fig. 3B), reducing TGF- β 1 to levels similar to that noted in macrophages incubated with medium (45.1 ± 18.5 vs 68.6 ± 18.9 pg/ml TGF- β 1) (LPS plus ISS vs medium) (B). In contrast, ISS had a slight effect on inhibiting IL-6 release from LPS stimulated macrophages (LPS plus ISS vs LPS; $p = 0.05$) (Fig. 3A).

Similar to the results obtained in experiments examining ISS-mediated inhibition of TGF- β 1 release from a macrophage cell line, ISS also inhibited the LPS-induced release of TGF- β 1 from bone marrow-derived macrophages (52.3 ± 13.8 vs 86.4 ± 10.9 pg/ml TGF- β 1) (LPS plus ISS vs LPS; $p = 0.05$) (Fig. 3C).

Effect of ISS on reversing area of peribronchial α -smooth muscle actin immunostaining

In addition to determining whether ISS reversed peribronchial collagen deposition, we also investigated whether ISS reversed the allergen induced increase in the area of peribronchial α -smooth muscle actin immunostaining. Repetitive OVA challenge for 3 mo in untreated mice induced a significant increase in peribronchial α -smooth muscle actin immunostaining compared with non-OVA-challenged mice (1.28 ± 0.06 vs 0.30 ± 0.02 $\mu\text{m}^2/\mu\text{m}$) (OVA vs no-OVA; $p = 0.0001$) (Fig. 4). Mice challenged with OVA, and then followed without further OVA challenges maintained a significant increase in the area of peribronchial α -smooth muscle actin immunostaining compared with non-OVA-challenged mice at 1 mo of follow up ($p = 0.001$), as well as at 3 mo of follow up ($p = 0.001$) (Fig. 4). However, discontinuation of OVA challenges for

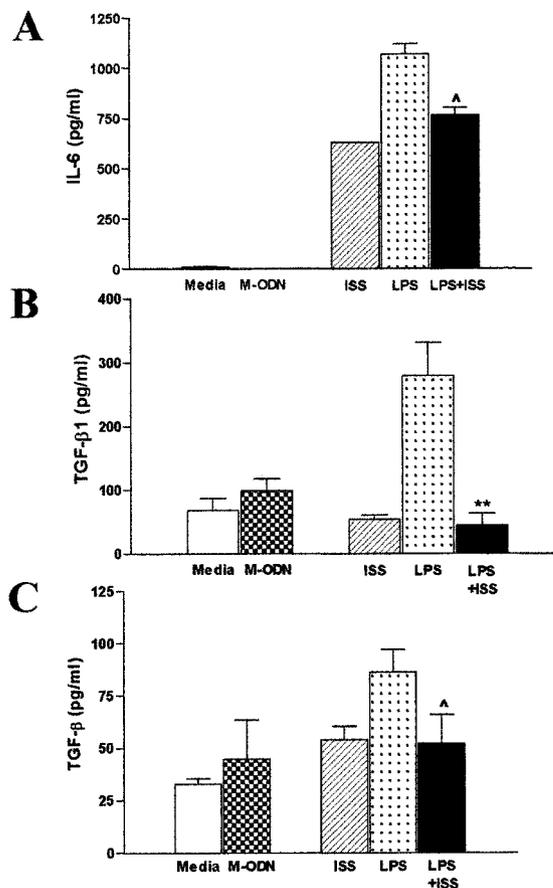


FIGURE 3. Effect of ISS on macrophage cytokine expression in vitro. A, Expression by macrophage cell line RAW 264.7. To determine whether ISS modulates release of IL-6 by macrophages, macrophages (RAW 264.7 cell line) were incubated in vitro with LPS ($10 \mu\text{g}/\text{ml}$) in the presence or absence of ISS ($10 \mu\text{g}/\text{ml}$) for 24 h ($n = 3$). Addition of ISS to macrophages induced significant release of IL-6 into the supernatant (ISS vs medium) ($p = 0.008$). A control mutated ODN (M-ODN) did not induce macrophage IL-6 release (mutated ODN vs medium). LPS, like ISS, induced significant release of IL-6 by macrophages (LPS vs medium) ($p = 0.001$). ISS had a slight effect on inhibiting IL-6 release from LPS-stimulated macrophages (ISS + LPS vs LPS) (*, $p = 0.05$). B, Effect of ISS on TGF- β 1 expression by macrophage cell line RAW 264.7. To determine whether ISS inhibits release of TGF- β 1 by macrophages, macrophages (RAW 264.7 cell line) were incubated in vitro with LPS ($10 \mu\text{g}/\text{ml}$) in the presence or absence of ISS ($10 \mu\text{g}/\text{ml}$) for 24 h ($n = 3$). Neither ISS nor mutated ODN induced macrophages to release significant amounts of TGF- β 1 compared with cells in medium alone. LPS differed from ISS in that it stimulated significant TGF- β 1 release from macrophages (LPS vs medium) ($p = 0.001$). ISS significantly inhibited LPS-stimulated macrophage release of TGF- β 1 (LPS + ISS vs LPS) ($p = 0.001$), reducing TGF- β 1 to levels similar to that noted in unstimulated macrophage supernatants. A mutated ODN control had a slight, but statistically insignificant effect on LPS-stimulated macrophage production of TGF- β 1 (LPS + M-ODN vs LPS) ($p = \text{not significant}$). (*, $p = 0.05$; *, $p = 0.01$; **, $p = 0.001$; ***, $p = 0.0001$). C, Effect of ISS on TGF- β 1 expression by bone marrow-derived macrophages. To determine whether ISS inhibits release of TGF- β 1 by macrophages, bone marrow-derived macrophages were incubated in vitro with LPS ($10 \mu\text{g}/\text{ml}$) in the presence or absence of ISS ($10 \mu\text{g}/\text{ml}$) for 24 h ($n = 3$). Neither ISS nor mutated ODN induced macrophages to release significantly more TGF- β 1 than cells in medium alone. LPS differed from ISS in that it stimulated TGF- β 1 release from macrophages (LPS vs medium) ($p = 0.05$). ISS significantly inhibited LPS-stimulated macrophage release of TGF- β 1 (LPS + ISS vs LPS) ($p = 0.05$). (*, $p = 0.05$; *, $p = 0.01$; **, $p = 0.001$; ***, $p = 0.0001$).

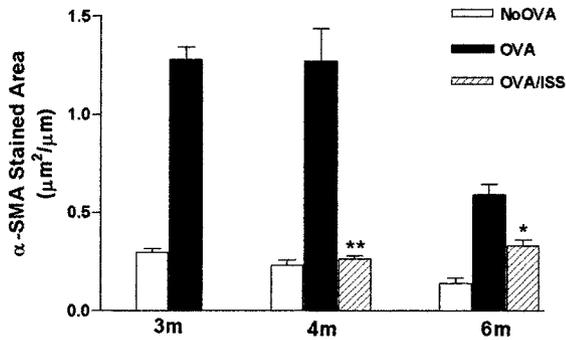


FIGURE 4. ISS reduces levels of peribronchial α -smooth muscle actin expression. Repetitive OVA challenge for 3 mo (3m) in untreated mice induced a significant increase in the area of peribronchial α -smooth muscle actin immunostaining compared with non-OVA-challenged mice ($p = 0.0001$). Mice challenged with OVA for 3 mo, and followed without further OVA challenges continued to have a significant increase in the area of peribronchial α -smooth muscle actin immunostaining compared with non-OVA-challenged mice at 1 mo of follow up (4m) ($p = 0.001$). At 3 mo of follow up, the area of peribronchial α -smooth muscle actin immunostaining was still significantly increased compared with non-OVA-challenged mice (6m) ($p = 0.001$), but was significantly less than that noted following 1 mo of discontinuing OVA challenges. Institution of ISS treatment after 3 mo of OVA challenges significantly reduced the area of peribronchial α -smooth muscle actin immunostaining in mice following 1 mo of ISS treatment (4m) ($p = 0.0001$), as well as following 3 mo of ISS treatment (6m) ($p = 0.01$). $n = 16$ mice/group (\cdot , $p = 0.05$; $*$, $p = 0.01$; $**$, $p = 0.001$; $***$, $p = 0.0001$).

3 mo following the 3 mo OVA challenge period significantly reduced levels of peribronchial α -smooth muscle actin immunostaining (Fig. 4).

Institution of ISS treatment after completion of OVA challenges significantly reduced the area of peribronchial α -smooth muscle actin immunostaining in mice following 1 mo of ISS treatment (0.26 ± 0.02 vs $1.27 \pm 0.17 \mu\text{m}^2/\mu\text{m}$; $p = 0.001$) (Fig. 4), as well as following 3 mo of ISS treatment (0.33 ± 0.03 vs $0.59 \pm 0.05 \mu\text{m}^2/\mu\text{m}$; $p = 0.01$) (Fig. 4).

ISS inhibits lung TARC expression and the accumulation of CD4⁺ peribronchial cells

As previous studies have demonstrated that ISS inhibits expression of Th2 cytokines (1, 2, 12, 13) but does not act directly on T lymphocytes, we investigated whether one mechanism by which ISS may inhibit the expression of Th2 cytokines is through inhibition of expression of T cell chemoattractants (e.g., TARC) and resultant inhibition of recruitment of CD4⁺ lymphocytes to the airway.

Effect of ISS on levels of lung TARC

Levels of lung TARC were significantly increased in mice exposed to repetitive OVA challenge for 3 mo compared with non-OVA-challenged mice (652.1 ± 35.2 vs 59.9 ± 16.9 pg TARC/mg lung protein) (OVA vs no-OVA; $p = 0.01$) (Fig. 5A). Mice challenged with OVA and then followed for an additional 1 mo ($p = 0.05$) (Fig. 5A), or 3 mo ($p = 0.05$) (A) maintained an increase in the lung levels of TARC compared with non-OVA-challenged mice. However, the levels of lung TARC at 1 and 3 mo after discontinuing OVA challenges were significantly less than that noted following 3 mo of OVA challenges (Fig. 5A).

Institution of ISS treatment after the completion of the OVA challenges significantly reduced the lung levels of TARC in mice following 1 mo of ISS treatment (129.1 ± 15.9 vs 240.0 ± 23.5 pg

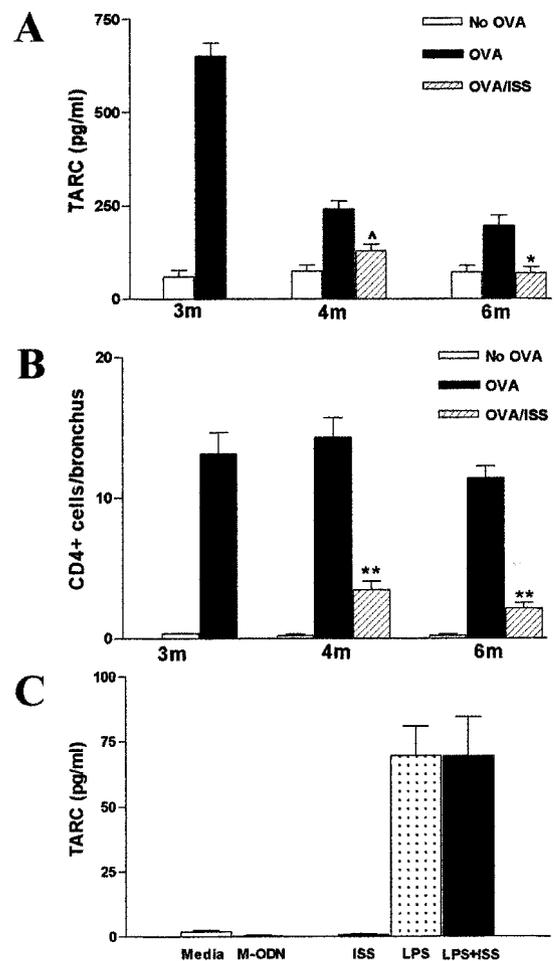


FIGURE 5. ISS reduces levels of TARC and the number of peribronchial CD4⁺ positive cells. **A**, Effect of ISS on levels of TARC. Levels of lung TARC were significantly increased in mice exposed to repetitive OVA challenge for 3 mo (3m) compared with non-OVA-challenged mice ($p = 0.01$). Mice challenged with OVA for 3 mo, and then followed for an additional 1 mo (4m) ($p = 0.05$) or 3 mo (6m) ($p = 0.05$), continued to have an increase in lung levels of TARC compared with non-OVA-challenged mice. Institution of ISS treatment after 3 mo of OVA challenges significantly reduced the lung levels of TARC in mice following 1 mo of ISS treatment (4m) ($p = 0.04$), as well as after 3 mo of ISS treatment (6m) ($p = 0.01$). $n = 16$ mice/group (\cdot , $p = 0.05$; $*$, $p = 0.01$; $**$, $p = 0.001$; $***$, $p = 0.0001$). **B**, Effect of ISS on the number of peribronchial CD4⁺ cells. Repetitive OVA challenge for 3 mo (3m) in untreated mice induced a significant increase in the number of CD4⁺ peribronchial cells ($p = 0.001$) compared with non-OVA-challenged mice. Mice challenged with OVA for 3 mo, and then followed without further OVA challenges continued to have a significant increase in the number of peribronchial CD4⁺ cells compared with non-OVA-challenged mice at 1 mo of follow up (4m) ($p = 0.001$), or at 3 mo of follow up (6m) ($p = 0.001$). Institution of ISS treatment after 3 mo of OVA challenges significantly reduced the number of peribronchial CD4⁺ cells in mice following 1 mo of ISS treatment (4m) ($p = 0.001$), as well as following 3 mo of ISS treatment (6m) ($p = 0.001$). $n = 16$ mice/group (\cdot , $p = 0.05$; $*$, $p = 0.01$; $**$, $p = 0.001$; $***$, $p = 0.0001$). **C**, Effect of ISS on macrophage TARC expression in vitro. To determine whether ISS inhibits expression of TARC by macrophages, macrophages (RAW 264.7 cell line) were LPS-stimulated in vitro for 24 h in the presence or absence of ISS ($n = 3$). Neither ISS nor mutated ODN (M-ODN) alone induced expression of TARC by macrophages. LPS stimulated significant TARC release from macrophages (LPS vs medium) ($p = 0.001$). ISS had no inhibitory effect on LPS-induced TARC release from macrophages.

TARC/mg lung protein; $p = 0.04$) (Fig. 5A), as well as after 3 mo of ISS treatment (69.1 ± 15.4 vs 196.0 ± 27.8 pg TARC/mg lung protein; $p = 0.01$) (A).

Effect of ISS on the accumulation of CD4⁺ peribronchial cells

Repetitive OVA challenge for 3 mo in untreated mice induced a significant increase in the number of CD4⁺ peribronchial cells compared with non-OVA-challenged mice (13.2 ± 1.5 vs 0.3 ± 0.1 peribronchial CD4⁺ cells) (OVA vs no OVA; $p = 0.001$) (Fig. 5B). Interestingly, mice challenged with OVA for 3 mo, and then followed for up to 3 mo, without further OVA challenges maintained the increase in the number of CD4⁺ peribronchial cells compared with non-OVA-challenged mice at 1 mo of follow up ($p = 0.001$) (Fig. 5B), and at 3 mo of follow up ($p = 0.001$) (B). Institution of ISS treatment after completion of OVA challenges significantly reduced the number of peribronchial CD4⁺ cells in mice following 1 mo of ISS treatment (3.4 ± 0.6 vs 14.3 ± 1.4 peribronchial CD4⁺ cells; $p = 0.001$) (Fig. 5B), as well as following 3 mo of ISS treatment (2.1 ± 0.4 vs 11.4 ± 0.9 peribronchial CD4⁺ cells; $p = 0.001$) (B).

Effect of ISS on macrophage TARC expression

To determine whether ISS inhibits expression of TARC by macrophages, macrophages (RAW 264.7 cell line) were stimulated *in vitro* in the presence or absence of ISS. Neither ISS nor mutated ODN alone induced expression of TARC by macrophages (Fig. 5C). LPS stimulated significant TARC release from macrophages (69.7 ± 11.2 vs 2.0 ± 0.8 pg/ml TARC) (LPS vs unstimulated; $p = 0.001$) (Fig. 5C). ISS had no inhibitory effect on LPS induced TARC release from macrophages (Fig. 5C).

Effect of ISS on airway mucus expression

Repetitive OVA challenge for 3 mo in untreated mice induced a significant increase in the percentage of airway epithelium that stained positive with PAS compared with non-OVA-challenged mice (39.6 ± 1.8 vs $0.3 \pm 0.01\%$ PAS-positive cells per bronchus) (OVA vs non-OVA; $p = 0.0001$) (Fig. 6). Mice challenged with

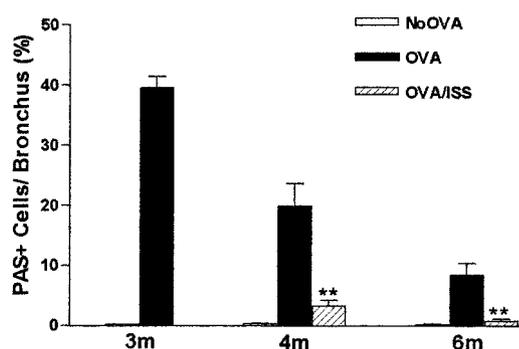


FIGURE 6. ISS reduces levels of airway mucus expression. Repetitive OVA challenge for 3 mo (3m) in untreated mice induced a significant increase in the percentage of airway epithelium that stained positive with PAS compared with non-OVA-challenged mice ($p = 0.0001$). Mice challenged with OVA for 3 mo, and then followed without further OVA challenges continued to have a significant increase in the percentage of airway epithelium, which stained positive with PAS compared with non-OVA-challenged mice at 1 mo of follow up (4m) ($p = 0.001$), or at 3 mo of follow up (6m) ($p = 0.001$). Institution of ISS treatment after 3 mo of OVA challenges significantly reduced the percentage of airway epithelium that stained positive with PAS in mice following 1 mo of ISS treatment (4m) ($p = 0.002$), as well as following 3 mo of ISS treatment (6m) ($p = 0.001$). $n = 16$ mice/group. (C, $p = 0.05$; *, $p = 0.01$; **, $p = 0.001$; ***, $p = 0.0001$).

OVA and then followed without further OVA challenges maintained a significant increase in the percentage of airway epithelium that stained positive with PAS compared with non-OVA-challenged mice at 1 mo of follow up ($p = 0.001$), or at 3 mo of follow up ($p = 0.001$) (Fig. 6).

Institution of ISS treatment after completion of the OVA challenges significantly reduced the percentage of airway epithelium that stained positive with PAS in mice following 1 mo of ISS treatment (3.3 ± 1.0 vs $19.9 \pm 3.8\%$ PAS-positive cells per bronchus; $p = 0.002$) (Fig. 6), as well as following 3 mo of ISS treatment (0.8 ± 0.4 vs $8.5 \pm 2.0\%$ PAS positive cells per bronchus; $p = 0.001$) (Fig. 6).

Discussion

In this study, we have used a model of allergen-induced airway remodeling to demonstrate that ISS can reverse features of airway remodeling that have already been induced. In particular ISS reverses peribronchial collagen deposition as assessed by measuring total lung collagen, peribronchial trichrome staining, as well as peribronchial collagen III and collagen V immunostaining. ISS may theoretically inhibit fibroblast collagen expression through either direct effects on fibroblasts and/or through indirect effects on cells that generate mediators, which regulate fibroblast collagen synthesis. In this study we demonstrate that ISS may indirectly inhibit fibroblast collagen synthesis through its effects on macrophages (reducing TGF- β 1 expression by macrophages), as well as by reducing the number of TGF- β 1 positive eosinophils and mononuclear cells recruited to the airway. In addition, ISS significantly reduces levels of expression of the chemokine TARC, as well as the number of peribronchial CD4⁺ lymphocytes. CD4⁺ lymphocytes are likely to play an important role in orchestrating the airway remodeling response by generating Th2 cytokines such as IL-5 (17) and IL-13 (16), which have been implicated in airway remodeling. ISS induced reduction in the number of peribronchial CD4⁺ lymphocytes is therefore likely to contribute to reducing peribronchial fibrosis by reducing levels of cytokines such as IL-5 and IL-13 that contribute to fibrosis. Previous studies from this laboratory have demonstrated that 3 mo of OVA challenge is associated with significantly increased levels of expression of Th2 cytokines including IL-4 (12), IL-5 (17), IL-9 (12), and IL-13 (13), and that ISS administered prophylactically for 3 mo, inhibits Th2 cytokine expression (12, 13). Overall, this study demonstrates that ISS can reverse features of airway collagen deposition, and that this reversal is associated with significant inhibitory effects on lung TGF- β 1 expression. However, we cannot exclude additional direct effects of ISS on lung fibroblasts to inhibit collagen synthesis. The absence of TLR-9 expression (as assessed by RT-PCR) on a fibroblast cell line suggests that ISS may not exert direct effects on fibroblasts, but as fibroblasts are heterogeneous cells further studies with primary lung fibroblasts are needed to exclude this possible direct inhibitory effect of ISS on lung fibroblast collagen synthesis.

In humans, the basement membrane of airway epithelium is comprised of two layers, the basal lamina (referred to as the true basement membrane and of normal thickness in asthma) and the lamina reticularis (comprised of collagen type I, type III, type V, and fibronectin), which is thickened in asthma (16). As peribronchial deposition of type III and type V collagen is a characteristic feature of remodeling in asthma, we were interested to determine whether ISS could not only prevent deposition of collagen as demonstrated in previous studies (12, 15), but was also able to reverse the collagen already deposited in the remodeled airway. In this study we demonstrate that ISS can reverse allergen-induced peribronchial collagen deposition of types III and type V collagen by

reducing accumulation of peribronchial CD4⁺ cells that drive ongoing eosinophil and macrophage accumulation, expression of TGF- β , and collagen deposition. Although discontinuation of OVA challenge at 3 mo is associated with some reduction in collagen deposition over the subsequent 3 mo follow up period, ISS significantly accelerates the reduction in peribronchial collagen deposition. This study therefore suggests that although allergen avoidance can reduce levels of peribronchial collagen deposition, ISS administration is significantly better than allergen avoidance in reversing peribronchial collagen deposition. Stopping exposure to allergen also leads to reduced levels of peribronchial inflammatory cells (eosinophils, mononuclear cells, but not CD4⁺ cells), reductions in levels of TGF- β (lung TGF- β more than BAL TGF- β) and reductions in collagen deposition (collagen III rather than collagen V), suggesting that switching off the inflammatory response reduces ongoing collagen deposition whether it is due to avoidance of allergen (a small reduction in fibrosis), or due to ISS (more effective and therefore a larger reduction in fibrosis). At present it is not clear whether stopping exposure to allergen activates all the same pathways (and to the same degree) as that induced by ISS to reduce levels of peribronchial collagen deposition.

One potential mechanism by which ISS can reduce fibroblast collagen synthesis is through an effect on cell types that secrete TGF- β 1, a cytokine that stimulates fibroblasts to synthesize collagen (21). ISS reduces the number of peribronchial cells expressing TGF- β 1, suggesting that ISS either inhibits the expression of TGF- β 1 and/or modulates the number of TGF- β 1-positive cells in the airway. To address whether ISS can modulate cellular TGF- β 1 expression, we performed in vitro studies with macrophages, which express TLR-9 to determine whether ISS could modulate levels of TGF- β 1 expression by macrophages. Although previous studies have demonstrated that ISS induces macrophages to express cytokines such as IL-6, TNF, and IL-12 (19), these studies have not focused on whether ISS modulates macrophage TGF- β 1 expression. Our in vitro studies demonstrated that ISS directly inhibits macrophage TGF- β 1 expression, suggesting that ISS may inhibit levels of lung TGF- β 1 by reducing the levels of TGF- β 1 expressed by peribronchial macrophages. Thus, ISS may reduce levels of TGF- β 1 through several mechanisms including inhibiting macrophage TGF- β 1 expression, as well as by reducing the number of peribronchial cells that express TGF- β 1 (mononuclear cells, eosinophils). Indeed, our studies demonstrated that ISS induced a parallel decline in the total number of peribronchial TGF- β 1-positive cells, as well as the total number of MBP⁺ and F4/80⁺ peribronchial cells that are significant sources of TGF- β 1. In studies with IL-5 deficient mice we have previously demonstrated that there is a significant reduction in both the number of MBP⁺ eosinophils and TGF- β 1-positive cells detected in the airway of mice subjected to repetitive OVA challenge for 3 mo (17), suggesting that eosinophils are a significant source of TGF- β 1 in this model of airway remodeling. The mechanism by which ISS reduces the number of peribronchial eosinophils is likely due to decreased production in the number of bone marrow eosinophils (1) and decreased trafficking of eosinophils into the lung, rather than increased apoptosis of eosinophils as ISS does not directly induce apoptosis of eosinophils in vitro (1).

In this study, we also made the novel observation that ISS reduces the number of peribronchial CD4⁺ lymphocytes. As CD4⁺ lymphocytes do not express the TLR-9 receptor, this reduction in the number of CD4⁺ lymphocytes is unlikely to be due to a direct effect of ISS on CD4⁺ lymphocytes. As the chemokines TARC binds to CCR4 receptors on Th2 lymphocytes, we explored whether ISS modulated levels of expression of this chemokine. Mouse models of allergic airway inflammation have shown that

neutralization of TARC cannot only inhibit T cell and eosinophil infiltration into the lung but can also inhibit bronchial hyperresponsiveness (22). In addition, BAL fluid from asthmatic subjects after allergen challenge contains significantly increased levels of TARC (23). Interestingly, ISS induced a significant decrease in levels of lung TARC that was associated with a significant reduction in peribronchial CD4⁺ T lymphocytes. As CD4⁺ lymphocytes orchestrate the inflammatory response including the recruitment of TGF- β 1⁺ eosinophils, ISS-mediated reduction in the number of peribronchial CD4⁺ lymphocytes will also contribute to reductions in TGF- β 1 levels. As TARC is expressed by macrophages, epithelium, and T lymphocytes (22, 23), we explored whether ISS directly modulated levels of TARC expression by macrophages. In vitro ISS did not inhibit TARC release by macrophages. Therefore, ISS mediated reductions in TARC levels in vivo could either be due to direct effects of ISS on mononuclear cells (less likely based on our in vitro studies), or due to effects of ISS on other TARC-expressing cells such as epithelial cells. In addition, ISS may modulate levels of TARC in vivo through effects on mediators that subsequently effect TARC production by these cells and/or inhibit recruitment of TARC-producing cells. Immunostaining lung sections with an Ab to TARC demonstrated increased staining of peribronchial mononuclear cells in OVA-challenged mice, with significant reductions in TARC staining in ISS treated mice (data not shown). In addition to ISS having effects on CD4⁺ T cell recruitment to the lung, we have recently demonstrated that ISS can induce Th2 cell death in the lung through induction of IDO (24). In these studies, OVA specific in vitro generated Th2 cells (DO11.10) were adoptively transferred into SCID mice that were challenged acutely with OVA (this in vivo SCID mouse model is deficient in T regulatory cells) (24). Results from these studies demonstrated that ISS induction of IDO in the lung plays a key role in mediating inhibition of Th2 cell responses using an acute OVA Ag challenge protocol. This effect of ISS as an inducer of Th2 cell death was inhibited by coadministration of an IDO inhibitor (24). These adoptive transfer experiments of Th2 cells in SCID mice indicate that ISS induction of IDO, rather than ISS induction of T regulatory cells, is a key mechanism mediating inhibition of Th2 cell responses in this acute adoptive transfer OVA model. In the chronic model of airway remodeling, the reductions in levels of lung TGF- β induced by ISS suggest that ISS is not inducing TGF- β expressing T regulatory cells. However, further studies are needed to determine whether T regulatory cells are induced by ISS in the chronic model of airway remodeling.

In summary, in this study we have demonstrated that systemic administration of ISS to mice that had already developed established airway remodeling significantly reduced the degree of airway collagen deposition. The ability of ISS to reduce lung collagen expression in vivo may be mediated through its effects on both reducing TGF- β 1 expression by macrophages, as well as by reducing the number of TGF- β 1-positive eosinophils and mononuclear cells recruited to the airway. In addition, ISS significantly reduces levels of expression of the chemokine TARC, as well as the number of peribronchial CD4⁺ lymphocytes further reducing signals for peribronchial recruitment of TGF- β 1 positive eosinophils.

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