

Immunostimulatory DNA inhibits allergen-induced peribronchial angiogenesis in mice

Sook Young Lee, MD,^{a,b} Jae Youn Cho, MD, PhD,^a Marina Miller, MD, PhD,^a Kirsti McElwain, BS,^a Shauna McElwain, BS,^a Pragada Sriramara, PhD,^c Eyal Raz, MD,^a and David H. Broide, MBChB^a La Jolla, Calif, and Seoul, Korea

Background: Airway remodeling in asthma is associated with angiogenesis.

Objective: We have examined whether immunostimulatory sequences of DNA (ISSs) inhibit allergen-induced airway angiogenesis and expression of angiogenic cytokines in a mouse model of airway remodeling.

Methods: Mice sensitized to ovalbumin were challenged repetitively with ovalbumin for three months to develop airway remodeling and angiogenesis. Levels of angiogenesis were compared in ISS-treated and control mice.

Results: Mice challenged with ovalbumin developed significantly increased levels of peribronchial angiogenesis (increase in the number of CD31⁺ peribronchial small blood vessels) and an increase in the peribronchial vascular area as assessed by image analysis. Ovalbumin-induced peribronchial angiogenesis was associated with increased bronchoalveolar lavage levels of vascular endothelial growth factor (VEGF) and an increase in the number of peribronchial cells expressing VEGF. Treatment of mice with ISS before repetitive ovalbumin challenge significantly reduced the levels of peribronchial angiogenesis as well as the levels of bronchoalveolar lavage VEGF and the number of peribronchial cells expressing VEGF. ISS is unlikely to act directly on endothelial cells to inhibit angiogenesis because lung endothelial cells did not express Toll receptor 9, the receptor for ISS as assessed by RT-PCR. *In vitro* studies demonstrated that ISS inhibited macrophage expression of VEGF.

Conclusion: The ability of ISS to inhibit angiogenesis *in vivo* is likely to be mediated by several mechanisms, including ISS reducing the number of peribronchial inflammatory cells that express VEGF, ISS inhibiting expression of T_H2 cytokines such as IL-13 that promote VEGF expression, and direct effects of ISS on macrophages to inhibit VEGF expression. (J Allergy Clin Immunol 2006;117:597-603.)

Key words: Asthma, remodeling, angiogenesis, VEGF, ISS, TLR-9

Abbreviations used

BAL: Bronchoalveolar lavage

IDO: Indoleamine, 2,3-dioxygenase

ISS: Immunostimulatory sequences of DNA

TLR-9: Toll receptor 9

VEGF: Vascular endothelial growth factor

Airway remodeling in asthma is associated with several structural changes including the development of subepithelial fibrosis, smooth muscle hypertrophy/hyperplasia, goblet cell hyperplasia, and increased vascularity.¹ The characterization of the extent of angiogenesis in asthma has been the subject of several recent studies. Overall, studies in subjects with asthma provide evidence of peribronchial expression of angiogenic cytokines and their receptors, as well as evidence of a significant increase in the extent of the peribronchial vasculature.²⁻⁸ For example, immunohistochemical studies demonstrate an increased number of airway submucosal vessels in patients with severe asthma compared with both patients with mild asthma and subjects without asthma.³ Subjects with asthma also exhibit higher levels of expression of angiogenic cytokines including vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), and angiogenin immunoreactivity in the airway submucosa, which is associated with significantly more vessels and greater vascular area than observed in control subjects.²⁻⁸ Furthermore, the degree of airway vascularity is inversely correlated with airway caliber and airway responsiveness.² Colocalization studies have revealed that the angiogenic factor-positive cells in the airway are predominantly CD34⁺ cells, eosinophils, and macrophages.² The functional significance of vascular changes of the airway wall in asthma is as yet unknown. However, studies in the skin of VEGF transgenic mice have demonstrated that angiogenic vessels exhibit a proadhesive function for circulating leukocytes,⁹ suggesting that angiogenic vessels may contribute to recruitment of circulating leukocytes and thus to airway inflammation. In addition, studies have demonstrated that angiogenic vessels exhibit increased permeability, which may contribute to airway wall edema in asthma.³

Angiogenesis is a complex process by which new blood vessels arise from the pre-existing vasculature.³ The switch from the normal quiescent vasculature to angiogenesis may be induced by several cytokines that act either directly on endothelial cells (eg, vascular endothelial growth factor;

From ^athe Department of Medicine, University of California San Diego; ^bKangnam St Mary's Hospital, Seoul; and ^cthe La Jolla Institute for Molecular Medicine.

Disclosure of potential conflict of interest: E. Raz has stock in Dynavax. The rest of the authors have no conflict of interest to disclose.

Supported by National Institutes of Health grant AI 38425 (Dr Broide), National Institutes of Health grant AI 40682, and Dynavax (Dr Raz).

Received for publication April 1, 2005; revised September 24, 2005; accepted for publication November 3, 2005.

Available online January 27, 2006.

Reprint requests: David Broide, MBChB, University of California San Diego, Basic Science Building, Room 5090, 9500 Gilman Drive, La Jolla, CA 92093-0635. E-mail: dbroide@ucsd.edu.

0091-6749/\$32.00

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doi:10.1016/j.jaci.2005.11.008

VEGF),¹⁰ or by cytokines (eg, IL-13) that induce expression of angiogenic cytokines.¹¹ Angiogenic cytokines induce the migration and proliferation of endothelial cells in existing vessels to form capillary tubes and recruit other cell types to stabilize new blood vessels.¹⁰ VEGF is a key mediator of vascular development in embryogenesis and also plays an important role in angiogenesis associated with tumors¹⁰ and chronic inflammation.³ Increased levels of expression of VEGF have been reported in asthmatic airways, and VEGF levels correlate with the increase in airway vessel number.² IL-13 is another cytokine expressed in asthma that has the potential to play a role in allergen induced angiogenesis. *In vitro* IL-13 stimulates VEGF production from smooth muscle cells,¹¹ whereas *in vivo* IL-13 transgenic mice exposed to hyperoxia develop significant increased levels of lung VEGF expression and lung angiogenesis.¹² Because IL-13 is expressed in asthma, IL-13 may also play a similar role in allergen induced angiogenesis by inducing VEGF expression.

We have recently demonstrated that immunostimulatory sequences of DNA (ISSs) containing a cytosine phosphorothioate guanosine (CpG) motif can prevent¹³ as well as reverse¹⁴ structural features of allergen induced airway remodeling including the development of peribronchial fibrosis and the increased thickness of the smooth muscle layer. The mouse model of allergen-induced airway remodeling we have developed resembles airway remodeling in human asthma in that it is associated with the T_H2 cytokine-dependent development of peribronchial fibrosis, smooth muscle hypertrophy, and mucus expression.¹⁵ Recent studies suggest that ISS mediates its inhibitory effects on allergen-induced T_H2 immune responses by binding to Toll receptor 9 (TLR-9) receptors and induction of indoleamine, 2,3-dioxygenase (IDO).¹⁶ Administration of a pharmacologic inhibitor of IDO to mice treated with ISS inhibited both IDO activity and the antiallergic effect of ISS, suggesting that IDO and/or its metabolites are important in mediating the antiallergic effect of ISS.¹⁶ In this study, we have used a mouse model of airway remodeling to investigate whether allergen challenge (acute versus chronic) induces another feature of airway remodeling (ie, peribronchial angiogenesis), and whether ISS can inhibit the allergen induced expression of angiogenic cytokines and the development of airway angiogenesis.

METHODS

Ovalbumin sensitization and repetitive ovalbumin challenge protocol

Female BALB/c mice (16 mice/group are used in each *in vivo* experiment presented in the Results and Figures 1, 2, 3, and 5; Jackson Laboratory, Bar Harbor, Me) 8-10 weeks of age were sensitized by means of subcutaneous injection of 25 μ g ovalbumin, grade V; Sigma, St Louis, Mo) emulsified in 1 mg aluminum hydroxide (Aldrich, Milwaukee, Wis) in a total volume of 200 μ L PBS on days 0, 7, 14, and 21, as previously described.¹³⁻¹⁵ Intranasal ovalbumin challenges (20 ng/50 μ L in PBS) were administered on days 26,

29, and 31 under isoflurane (Vedco, Inc, St Joseph, Mo) anesthesia. Intranasal ovalbumin challenges were then repeated twice a week for 3 months as previously described in this laboratory.¹³⁻¹⁵ Age-matched and sex-matched control mice were sensitized but not challenged with ovalbumin. Mice were killed 24 hours after the final ovalbumin challenge, and bronchoalveolar lavage (BAL) fluid and lungs were analyzed. In selected experiments, mice were killed on day 32, twenty-four hours after completion of the acute ovalbumin challenge protocol. All animal experimental protocols were approved by the University of California San Diego Animal Subjects committee.

Therapeutic intervention with ISS

To determine whether ISS inhibits allergen-induced lung angiogenesis, different groups of mice were administered intraperitoneal endotoxin-free (<1 ng/mg DNA) phosphorothioate ISS-oligodeoxynucleotide (ODN) (5'-TGACTGTGAACGTTTCGAGATGA-3'; Trilink, San Diego, Calif; 100 μ g in 100 μ L sterile, endotoxin-free PBS), or diluent control starting 1 day before the first intranasal ovalbumin challenge on day 27, and then continuing every other week 1 day before intranasal challenges for 3 months. Previous studies in our laboratory have demonstrated that ISS, but not mutated ODN, inhibits ovalbumin-induced eosinophilic inflammation, airway remodeling, and airway hyperreactivity when administered 1 day before ovalbumin challenge, and that this inhibitory effect lasts at least 4 weeks.¹³

Processing of lung for image analysis

Lungs in the different groups of mice were equivalently inflated with an intratracheal injection of a similar volume of 4% paraformaldehyde solution (Sigma) to preserve the pulmonary architecture. The inflated lungs were embedded in paraffin and stained with hematoxylin and eosin or processed for immunohistochemistry to detect peribronchial blood vessels or VEGF-positive cells. Lungs from the different experimental groups were processed as a batch for either histologic staining or immunostaining under identical conditions. Stained and immunostained slides were all quantified under identical light microscope conditions, including magnification (20 $\times$), gain, camera position, and background illumination. The quantitative histologic and image analysis of all coded slides was performed by researchers blinded to the coding of all of the slides.

Quantitation of the number of small, medium, and large airway blood vessels

Peribronchial blood vessels were quantified by counting the number of small, medium, and large blood vessels in the peribronchial space. Peribronchial blood vessels were identified by immunostaining lung sections in the different groups of mice with an anti-platelet endothelial cell adhesion molecule Ab (rat antimouse CD31 mAb; BD Pharmingen, San Diego, Calif) using the immunoperoxidase method as previously described in this laboratory.¹³ CD31 has been shown to be a highly sensitive endothelial cell marker.¹⁷ Although the CD31 mAb also stains mononuclear cells, CD31⁺ blood vessels can readily be distinguished from individual CD31⁺ mononuclear cells by the lumen present in blood vessels and absent in mononuclear cells. Blood vessels can also be distinguished by their content of red blood cells. The number of small (<15 μ m), medium (16-35 μ m), and large (>35 μ m) diameter CD31 positive blood vessels in the peribronchial space (the area between the epithelial basement membrane and the base of the alveolar attachments) in at least 10 bronchi (150-200 μ m of internal diameter) was counted by using a light microscope (Leica DMLS; Leica Microsystems Inc, Buffalo, NY) attached to an image analysis system (Image-Pro Plus; Media Cybernetics, Bethesda, Md). The bronchioles were chosen randomly in lung sections on each slide

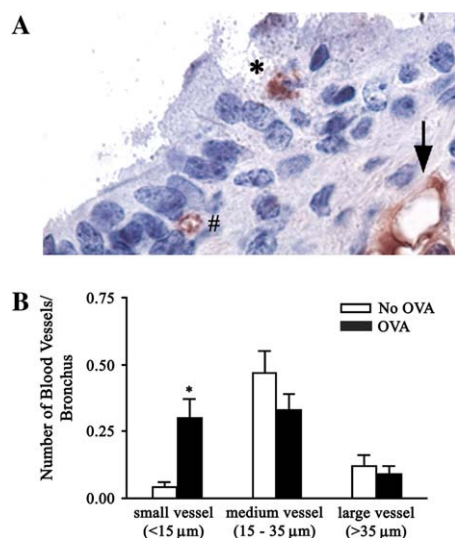


FIG 1. Angiogenesis following ovalbumin (OVA) challenge. **A**, Examples are depicted of PECAM-positive peribronchial small blood vessels 6 μm (arrow) and 1 μm (#) in diameter. A PECAM-positive mononuclear cell (*) lacks the lumen of the blood vessels. **B**, OVA challenge increased the number of small blood vessels/bronchus ($P = .05$). There was no difference in medium and large blood vessels/bronchus (OVA versus no-OVA). *, $P = .05$.

by moving in a predetermined sequence in all slides until at least 10 bronchioles were chosen.

Vascular area of peribronchial space (% peribronchial vascularity)

The area of the peribronchial space that is vascular was calculated from the area of the outlined blood vessels within the peribronchial space using an image analysis program. The total area internal to each blood vessel endothelial basement membrane was used in calculating the total blood vascular area in at least 10 bronchi. Results of the area of the outlined blood vessels within the peribronchial area are expressed as the percentage peribronchial vascularity (% peribronchial vascularity).

Number of peribronchial blood vessels per square micrometer

In initial experiments (Fig 1), we expressed the results of the number of CD31⁺ blood vessels as the number of blood vessels/bronchus (the area around each bronchus being the area between the epithelial basement membrane and the base of the alveolar attachments). We also quantified the number of blood vessels per square micrometer of peribronchial space area by calculating the area of the outlined peribronchial space in each bronchus ($8062 \pm 555 \mu\text{m}^2$ per bronchus) and expressed the results as the number of small blood vessels/ μm^2 peribronchial space (Figs 2 and 3). Both methods gave similar results.

Endothelial cell TLR-9 expression

Endothelial cell TLR-9 expression was determined by RT-PCR using a mouse lung endothelial cell line (LEISVO) and a mouse bone marrow derived endothelial cell line (STR-12).¹⁸ Total cellular RNA was isolated from the endothelial cell lines or a control mouse macrophage cell line, RAW 264.7, known to express TLR-9, using TRIzol reagent (Life Technologies, Gaithersburg, Md) as previously described in this laboratory.¹⁴ The expression of TLR-9 was assessed

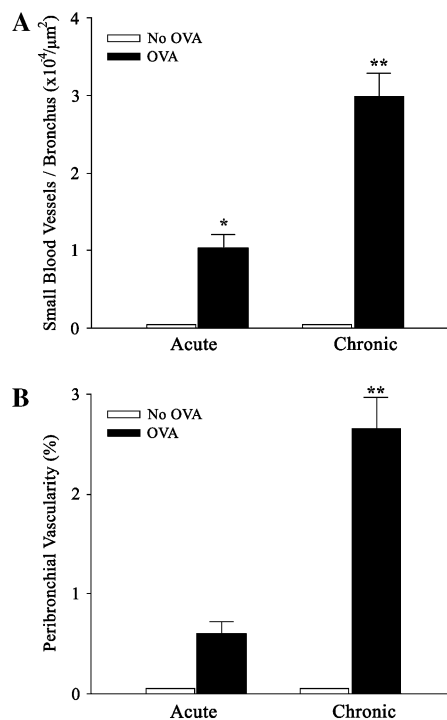


FIG 2. Effect of acute versus chronic ovalbumin (OVA) challenge on angiogenesis. **A**, Chronic OVA challenge increased the number of small peribronchial blood vessels (chronic OVA versus no-OVA, $P = .01$; chronic OVA versus acute OVA, $P = .001$), as did acute OVA challenge (acute OVA versus no-OVA, $P = .05$). **B**, Chronic OVA challenge increased the area of peribronchial vascularity (chronic OVA versus no-OVA, $P = .01$; chronic OVA versus acute OVA, $P = .01$). *, $P = .05$; **, $P = .01$.

with the following sense and antisense oligonucleotide sequence primers: sense primer, 5'-AGG CTG TCA ATG GCT CTC AGT T-3'; antisense primer, 5'-TGA ACG ATT TCC AGT GGT ACA AGT-3'.¹⁴ The PCR products (611 bp) were electrophoresed in a 1.5% agarose gel and were visualized with ethidium bromide.

Effect of ISS on VEGF expression

Effect of ISS on macrophage VEGF expression in vitro. Purified populations of macrophages were obtained by adherence using BAL from naive mice as previously described.¹⁹ In *in vitro* experiments, 5×10^5 mouse BAL macrophages were incubated with LPS (100 ng/mL) in triplicate for 24 hours in the presence or absence of ISS (10 $\mu\text{g}/\text{mL}$). Supernatants were collected and assayed for VEGF by ELISA.

Effect of ISS on BAL VEGF levels. After instillation of 1 mL sterile PBS through the trachea into the lung, BAL fluid was withdrawn. Levels of VEGF were assayed in BAL fluid by ELISA.

Effect of ISS on VEGF expression by peribronchial cells. Lung sections were processed for immunohistology as previously described¹³⁻¹⁵ using a primary mAb directed against VEGF (Santa Cruz Biotechnology, Santa Cruz, Calif). The area of peribronchial VEGF cellular staining was outlined and quantified by using a light microscope attached to an image analysis system as previously described.¹³⁻¹⁵ Results are expressed as the area of peribronchial VEGF staining per micrometer length of basement membrane of bronchioles 150-200 μm of internal diameter. At least 10 bronchioles were counted in each slide.

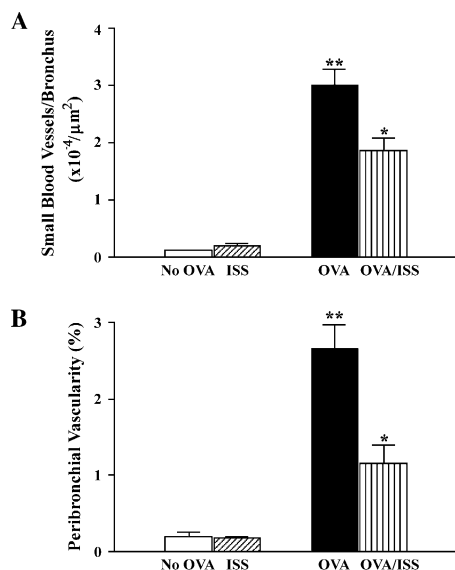


FIG 3. Effect of ISS on angiogenesis. **A**, Chronic ovalbumin (OVA) challenge increased the number of small peribronchial blood vessels (chronic OVA versus no-OVA; $P = .01$). ISS reduced small peribronchial blood vessel number ($P = .05$; ISS + OVA versus OVA). **B**, Chronic OVA challenge increased the area of peribronchial vascularity (chronic OVA versus no-OVA; $P = .001$). ISS reduced the area of peribronchial vascularity ($P = .01$; ISS + OVA versus OVA). *, $P = .05$; **, $P = .01$.

Measurement of lung IL-13 expression

Because IL-13 can also induce VEGF expression and angiogenesis,^{11,12} we measured the concentrations of IL-13 in lung tissue by ELISA using methods for lung cytokine analysis previously described in this laboratory.¹³⁻¹⁵

Measurement of BAL eosinophilic inflammation

To ensure that the dose of ISS used in this 3-month study was optimal for detecting inhibition of angiogenesis, we measured the effect of administration of ISS on levels of BAL eosinophilic inflammation, an end point known to be inhibited by ISS.¹³⁻¹⁵ BAL cells were cytospun onto microscope slides and stained with modified Wright-Giemsa to enumerate the number of eosinophils as previously described.¹³⁻¹⁵ At least 400 cells were examined on each slide.

Statistics

Results in the different groups of mice were compared by means of ANOVA with the nonparametric Kruskal-Wallis test, followed by posttesting with the Dunn multiple comparison of means. A statistical software package (In Stat, San Diego, Calif) was used for the analysis. P values less than .05 were considered statistically significant. All results are presented as means $\pm$ SEMs.

RESULTS

Effect of chronic ovalbumin challenge on the number of small, medium, and large peribronchial blood vessels

Initial studies were performed to determine the number of small, medium, and large peribronchial blood vessels in

mice that had undergone repetitive ovalbumin challenge for 3 months, a time point we have previously shown to be associated with other remodeling end points (subepithelial fibrosis and increased smooth muscle thickness).¹³⁻¹⁵ Repetitive ovalbumin challenge induced a significant increase in the number of peribronchial small blood vessels compared with nonovalbumin-challenged mice (0.30 ± 0.07 vs 0.04 ± 0.02 small blood vessels/bronchus; chronic ovalbumin versus nonovalbumin; $P = .05$; Fig 1, A and B). In contrast with the increase in the number of small blood vessels in mice repetitively challenged with ovalbumin, neither the number of medium-sized blood vessels (Fig 1, B) nor the number of large-sized blood vessels (Fig 1, B) was significantly different in chronic ovalbumin-challenged mice compared with nonovalbumin-challenged mice.

Comparison of acute versus chronic ovalbumin challenge on the number of peribronchial small blood vessels

These studies were performed to determine whether the number of peribronchial small blood vessels were different in mice that had undergone either an acute ovalbumin challenge (3 intranasal ovalbumin challenges in 5 days and mice killed 24 hours after the last intranasal ovalbumin challenge) or chronic ovalbumin challenge (repetitive ovalbumin challenge for 3 months). The number of small blood vessels per square micrometer of peribronchial space area was significantly greater in chronic ovalbumin-challenged mice compared with acute ovalbumin-challenged mice (2.99 ± 0.30 vs 1.03 ± 0.18 small blood vessels per $10^4 \mu\text{m}^2$ peribronchial space; chronic ovalbumin versus acute ovalbumin; $P = .001$; Fig 2, A). Although acute ovalbumin challenge did not increase the number of peribronchial blood vessels as much as the chronic ovalbumin challenge, acute ovalbumin-challenged mice had significantly more peribronchial small blood vessels compared with the number of small blood vessels in non-ovalbumin-challenged mice (1.03 ± 0.18 vs 0.12 ± 0.02 small blood vessels per $10^4 \mu\text{m}^2$ peribronchial space; acute ovalbumin versus nonovalbumin; $P = .05$; Fig 2, A).

Effect of acute versus chronic ovalbumin challenge on peribronchial vascularity

The vascular area of the peribronchial space (% peribronchial vascularity) was calculated from the area of outlined blood vessels in the peribronchial space using image analysis. Repetitive ovalbumin-challenged mice had a significant increase in the area of blood vessels within the peribronchial space compared with nonovalbumin-challenged mice ($2.65\% \pm 0.31\%$ vs $0.14\% \pm 0.08\%$ peribronchial vascularity; chronic ovalbumin versus non-ovalbumin; $P = .001$; Fig 2, B). The area of blood vessels within the peribronchial space was also significantly greater in chronic ovalbumin-challenged mice compared with acute ovalbumin-challenged mice ($2.65\% \pm 0.31\%$ vs $0.61\% \pm 0.11\%$ peribronchial vascularity; chronic ovalbumin versus acute ovalbumin; $P = .001$; Fig 2, B).

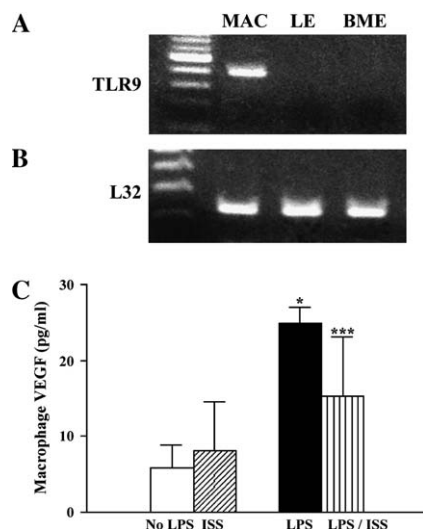


FIG 4. Evaluation of TLR-9 expression by endothelial cells and macrophages. **A**, RT-PCR demonstrated TLR-9 expression by mouse macrophage (MAC), but not lung endothelial cell lines (LE) or bone marrow (BME) endothelial cell lines. **B**, Housekeeping gene L32. **C**, Incubation of BAL macrophages with LPS induced release of VEGF into the supernatant (LPS versus no LPS; $P = .05$). This was inhibited by ISS (LPS versus LPS + ISS; $P = .05$). *, $P = .05$; ***, $P = .001$.

Effect of ISS on the number of peribronchial small blood vessels and peribronchial vascularity

Mice treated with ISS and repetitively challenged with ovalbumin had significantly fewer peribronchial small blood vessels compared with ovalbumin challenged mice not treated with ISS (1.85 ± 0.23 vs 2.99 ± 0.30 small blood vessels/ $10^4 \mu\text{m}^2$ peribronchial space; ISS + ovalbumin versus ovalbumin; $P = .05$; Fig 3, A).

Mice treated with ISS and repetitively challenged with ovalbumin had significantly less peribronchial vascularity compared with ovalbumin-challenged mice not treated with ISS ($1.16\% \pm 0.22\%$ vs $2.65\% \pm 0.31\%$ peribronchial vascularity; ISS + ovalbumin versus ovalbumin; $P = .01$; Fig 3, B).

Treatment with ISS of ovalbumin-sensitized mice not challenged with ovalbumin did not induce an increase in the number of small blood vessels/bronchus or peribronchial vascularity (Fig 3, A and B) or increase the number of large blood vessels (0.12 ± 0.04 vs 0.13 ± 0.02 large blood vessels/bronchus; no ovalbumin vs no ovalbumin + ISS).

Evaluation of TLR-9 expression by endothelial cells

To determine whether ISS could be inhibiting angiogenesis through a direct effect on endothelial cells, we examined whether endothelial cells express TLR-9, the receptor for ISS.²⁰ RT-PCR studies demonstrated that 2 different mouse endothelial cell lines (LEISVO, a lung endothelial cell line, and STR-12, a bone marrow-derived endothelial cell line) did not express TLR-9 (Fig 4, A and

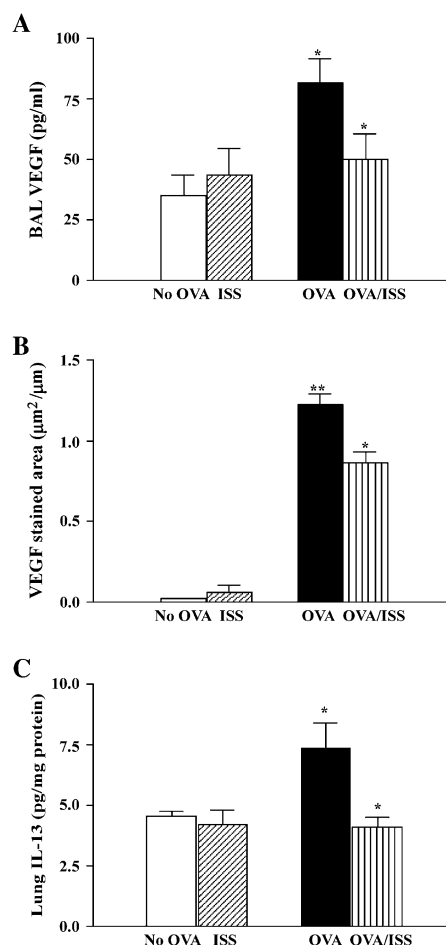


FIG 5. Effect of ISS on VEGF expression *in vivo*. **(A)** Chronic ovalbumin (OVA) challenge increased BAL VEGF levels (OVA versus no-OVA; $P = .05$) as well as **(B)** peribronchial VEGF immunostained area (OVA versus no-OVA; $P = .001$), and **(C)** lung IL-13 levels (OVA versus no-OVA; $P = .05$). ISS reduced BAL VEGF ($P = .05$; ISS + OVA versus OVA), VEGF immunostained area (ISS + OVA versus OVA; $P = .01$), and lung IL-13 ($P = .05$; ISS + OVA versus OVA). *, $P = .05$; **, $P = .01$.

B). A control macrophage cell line strongly expressed TLR-9 (Fig 4, A and B).

Effect of ISS on VEGF expression

Effect of ISS on macrophage VEGF expression in vitro. Incubation of BAL macrophages with LPS induced significant release of VEGF into the supernatant (LPS versus no LPS; $P = .05$; Fig 4, C). Incubation of macrophages with ISS and LPS significantly inhibited VEGF release into the supernatant (LPS versus LPS + ISS; $P = .05$; Fig 4, C).

Effect of ISS on BAL VEGF levels in vivo as assessed by ELISA. Repetitive ovalbumin challenge induced a significant increase in BAL VEGF levels compared with nonovalbumin-challenged mice (81.7 ± 9.6 vs 35.1 ± 8.1 pg/mL VEGF; chronic ovalbumin versus no ovalbumin; $P = .05$; Fig 5, A). ISS-treated mice repetitively challenged with ovalbumin had significantly less BAL VEGF

compared with ovalbumin-challenged mice not treated with ISS (49.8 ± 10.5 vs 81.7 ± 8.1 pg/mL VEGF; ISS + ovalbumin versus ovalbumin; $P = .05$; Fig 5, A).

Effect of ISS on peribronchial cellular VEGF expression as assessed by immunohistochemistry and image analysis. Repetitive ovalbumin challenge induced a significant increase in the levels of peribronchial cellular expression of VEGF compared with nonovalbumin-challenged mice (1.22 ± 0.07 vs 0.02 ± 0.01 VEGF immunostained area μm^2 per μm ; chronic ovalbumin versus no ovalbumin; $P = .001$; Fig 5, B). ISS-treated mice repetitively challenged with ovalbumin had significantly less peribronchial cellular expression of VEGF compared with ovalbumin-challenged mice not treated with ISS (0.86 ± 0.07 vs 1.22 ± 0.07 VEGF immunostained area μm^2 per μm ; ISS + ovalbumin versus ovalbumin; $P = .01$; Fig 5, B).

Effect of ISS on lung IL-13 expression

Repetitive ovalbumin challenge induced a significant increase in lung IL-13 levels compared with nonovalbumin-challenged mice (7.4 ± 1.0 vs 4.5 ± 0.2 pg IL-13/mg lung protein; chronic ovalbumin versus nonovalbumin; $P = .05$; Fig 5, C). ISS-treated mice repetitively challenged with ovalbumin had significantly less lung IL-13 compared with ovalbumin-challenged mice not treated with ISS (4.1 ± 0.4 vs 7.4 ± 1.0 pg IL-13/mg lung protein; ISS + ovalbumin versus ovalbumin; $P = .05$; Fig 5, C).

Effect of ISS on BAL eosinophils

Mice sensitized and challenged repetitively with ovalbumin for 3 months developed significant BAL eosinophilia ($15.0 \pm 3.4 \times 10^4$ BAL eosinophils) compared with nonovalbumin-challenged mice ($0.06 \pm 0.03 \times 10^4$ BAL eosinophils; $P = .001$). Systemic ISS treatment every other week, started before the first intranasal ovalbumin challenge, in mice challenged repetitively with ovalbumin for 3 months also significantly reduced levels of BAL eosinophilia ($3.5 \pm 0.6 \times 10^4$ BAL eosinophils; $P = .01$) compared with mice repetitively challenged with ovalbumin that had not been treated with ISS.

DISCUSSION

In this study, we have demonstrated that repetitive allergen challenge in mice can induce expression of angiogenic cytokines as well as peribronchial angiogenesis, a feature of the remodeled airway in asthma. Mice subjected to repetitive allergen challenge develop structural features of remodeling, including increased peribronchial fibrosis,¹³⁻¹⁵ increased thickness of the smooth muscle layer,¹³⁻¹⁵ and, as demonstrated in this study, peribronchial angiogenesis. As with other features of airway remodeling, repetitive allergen challenge induces higher levels of angiogenesis compared with acute allergen challenge. In addition, we have demonstrated that a therapeutic intervention with ISS inhibits the expression of angiogenic cytokines as well as the development of peribronchial angiogenesis. Thus, ISS is able to prevent and reverse many of the structural features of airway remodeling in mice,

including peribronchial fibrosis, the increased thickness of the smooth muscle layer, and angiogenesis.¹³⁻¹⁵ The mechanism by which ISS inhibits allergen induced angiogenesis is unlikely to be a result of a direct effect of ISS on endothelial cells, because we have demonstrated that endothelial cells do not express TLR-9, the receptor for ISS.²⁰ Rather, ISS is likely to inhibit angiogenesis by reducing levels of angiogenic cytokines induced by repetitive allergen challenge. Support for this mechanism of inhibition of angiogenesis by ISS is derived from our studies demonstrating that ISS reduces levels of angiogenic cytokines, including VEGF and IL-13 expression *in vivo*. Several mechanisms are therefore likely to contribute to ISS reducing angiogenesis *in vivo*, including (1) ISS reducing peribronchial accumulation of inflammatory cells that express VEGF (mononuclear cells,³ eosinophils,²¹ mast cells²²), (2) ISS directly inhibiting VEGF expression by macrophages, and (3) ISS inhibiting expression of cytokines such as IL-13 that induce expression of VEGF by macrophages.¹¹ In this study, we have demonstrated that ISS has direct effects on inhibiting macrophage VEGF expression, that ISS inhibits IL-13 expression, and that ISS reduces the levels of peribronchial cell VEGF expression. Previous studies have demonstrated that CpG DNA induces the murine macrophage cell line J774A to express VEGF (as assessed by either RT-PCR or immunostaining macrophages with an anti-VEGF Ab) and induces angiogenesis in the cornea of mice.²³ The differences in results in the 2 studies are likely a result of differences in the methods and the mouse models used. In this study, we used primary macrophages rather than a macrophage cell line to investigate VEGF expression *in vitro*.²³ The *in vivo* mouse models also differed significantly in the 2 studies in terms of the site of ISS injection (i.p. versus into the cornea), the presence or absence of tissue inflammation before ISS injection (inflammatory cells express VEGF), and the organ being studied for extent of angiogenesis (lung versus cornea). In this study, multiple systemic doses of ISS were administered over a 3-month period to mice that developed significant increased lung levels of VEGF expression and T_H2 inflammation in response to allergen challenge, whereas studies showing increased angiogenesis with CpG DNA have investigated the effect of administering a single dose of ISS into the cornea of a naive mouse expressing no VEGF or inflammation in the cornea.²³

The potential importance of VEGF to allergen-induced angiogenesis has been suggested from studies of transgenic mice engineered to overexpress VEGF in airway epithelium.²⁴ These VEGF transgenic mice have significantly increased levels of airway angiogenesis, supporting an important role for VEGF in the development of airway angiogenesis. After allergen inhalation, VEGF could either be released from cells directly responding to allergen (eg, macrophages³ or mast cells²²) or be released from cells not directly responding to allergen such as epithelial cells,²⁵ eosinophils,²¹ and smooth muscle cells.¹¹ IL-13 is a mediator released during episodes of T_H2-mediated inflammation that could also contribute to angiogenesis

through induction of VEGF expression. For example, *in vitro* IL-13 can induce VEGF production by airway smooth muscle cells¹¹ and macrophages,¹² whereas *in vivo* IL-13 transgenic mice have high levels of VEGF expression in the airway.¹² Immunohistology experiments in this study using an anti-VEGF Ab suggested that peribronchial inflammatory cells such as macrophages and eosinophils were the major cellular sources of VEGF after repetitive ovalbumin challenge (data not shown).

In summary, we have demonstrated that allergen challenge can induce peribronchial angiogenesis and expression of angiogenic cytokines. Repeated exposure to allergen induces higher levels of peribronchial angiogenesis than acute exposure to allergen. Previous studies have demonstrated that ISS can inhibit structural features of allergen-induced remodeling, including peribronchial collagen deposition and the increased thickness of the smooth muscle layer.¹³ In this study, we demonstrate that ISS can prevent the development of an additional feature of airway remodeling, peribronchial angiogenesis. The mechanism by which ISS exerts this inhibitory effect on the development of peribronchial angiogenesis is likely to be mediated by several pathways, including inhibition of macrophage expression of VEGF, inhibition of recruitment of peribronchial inflammatory cells that express VEGF, and inhibition of cytokines such as IL-13 that induce VEGF expression. Further studies are needed to determine whether the ability of ISS to inhibit angiogenesis has functional effects in asthma in terms of reducing airway edema and adhesion of leukocytes in angiogenic vessels.

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