

Accumulation of Peribronchial Mast Cells in a Mouse Model of Ovalbumin Allergen Induced Chronic Airway Inflammation: Modulation by Immunostimulatory DNA Sequences¹

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Few peribronchial mast cells are noted either in the lungs of naive mice or in the lungs of OVA-sensitized mice challenged acutely with OVA by inhalation. In this study, we demonstrate that OVA-sensitized mice exposed to repetitive OVA inhalation for 1–6 mo have a significant accumulation of peribronchial mast cells. This accumulation of peribronchial mast cells is associated with increased expression of the Th2 cell-derived mast cell growth factors, including IL-4 and IL-9, but not with the non-Th2 cell-derived mast cell growth factor, stem cell factor. Pretreating mice with immunostimulatory sequences (ISS) of DNA containing a CpG motif significantly inhibited the accumulation of peribronchial mast cells and the expression of IL-4 and IL-9. To determine whether mast cells express Toll-like receptor-9 (TLR-9; the receptor for ISS), TLR-9 expression by mouse bone marrow-derived mast cells (MBMNCs) was assessed by RT-PCR. MBMNCs strongly expressed TLR-9 and bound rhodamine-labeled ISS. However, incubation of MBMNCs with ISS *in vitro* neither inhibited MBMNC proliferation nor inhibited Ag/IgE-mediated MBMNC degranulation, but they did induce IL-6. Overall these studies demonstrate that mice exposed to repetitive OVA challenge, but not acute OVA challenge, have an accumulation of peribronchial mast cells and express increased levels of mast cell growth factors in the lung. Although mast cells express TLR-9, ISS does not directly inhibit mast cell proliferation *in vitro*, suggesting that ISS inhibits accumulation of peribronchial mast cells *in vivo* by indirect mechanism(s), which include inhibiting the lung expression of Th2 cell-derived mast cell growth factors. *The Journal of Immunology*, 2003, 171: 4860–4867.

Cross linking of high affinity IgE receptors on mast cells by inhaled allergen triggers the rapid release of proinflammatory mediators that can induce smooth muscle contraction and mucus secretion, which are cardinal features of asthma (1, 2). Several studies have demonstrated an increased number of mast cells in the airways of human asthmatics (3–8), which correlates significantly with airway hyperreactivity (AHR)³ and the severity of asthma (6). Recent studies have also demonstrated an increased number of mast cells in airway smooth muscle in asthmatics compared with controls (8), suggesting a potential direct interaction of mast cells and smooth muscle cells in inducing airway hyperreactivity as well as smooth muscle remodeling (hypertrophy, hyperplasia, and differentiation into myofibroblast). The increased number of airway mast cells in asthma is associated with evidence of ongoing mast cell degranulation in the airways of asthmatics who are symptomatic (9). Although mast cells play a well-established central role in the immediate response to inhaled allergen (1, 2), mast cells also have the potential to play an important

role in chronic asthma. The potential of mast cells to play a role in chronic asthma and airway remodeling is suggested from studies demonstrating an important role for mast cells in tissue fibrosis, including pulmonary fibrosis (10, 11). Several mast cell-derived mediators are candidates for playing a role in mediating airway remodeling based on their demonstrated ability *in vitro* to influence fibroblast proliferation or collagen synthesis (12–14). For example, both histamine and tryptase stimulate fibroblast proliferation as well as collagen synthesis (12–14). In addition TGF- β , a key cytokine-regulating fibrosis, is also generated by mast cells (15). Coculture of mast cells and fibroblasts *in vitro* results in both fibroblast proliferation and mast cell proliferation, again suggesting a role for mast cells in fibrosis (16).

The potential importance of mast cells to AHR and inflammation has also been studied extensively in mice exposed to acute OVA challenge. Studies of AHR and eosinophilic inflammation in mast cell-deficient mice demonstrate an important role for mast cells in AHR to methacholine (17). Mast cell-deficient WBB6F₁-W/W^v mice sensitized to OVA without adjuvant, followed by exposure to repetitive intranasal Ag challenge, have less AHR and airway eosinophilia compared with congenic +/+ controls (18, 19). Furthermore, reconstitution of mast cell-deficient WBB6F₁-W/W^v mice with bone marrow-derived mast cells re-establishes AHR to aerosolized Ag, establishing the importance of mast cells in acute Ag-induced bronchial hyperreactivity (18). The importance of the mast cell in chronic as opposed to acute mouse models of asthma has not been as extensively investigated. Our laboratory⁴ and others (20) have developed mouse models in which repetitive

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³ Abbreviations used in this paper: AHR, airway hyperreactivity; ISS, immunostimulatory sequence; MBMNC, mouse bone marrow-derived mast cell; Penh, enhanced pause; DAB, 3,3'-diaminobenzidine; M-ODN, mutated oligodeoxynucleotide; MFI, mean fluorescence intensity; SCF, stem cell factor.

⁴ J. Y. Cho, M. Miller, K. J. Baek, J. W. Han, J. Nayar, M. Rodriguez, S. Y. Lee, K. McElwain, S. McElwain, E. Raz, and D. H. Broide. Immunostimulatory DNA inhibits TGF- β expression and airway remodeling. *Submitted for publication*.

allergen administration is associated with sustained lung Th2 responses for up to 6 mo of allergen challenge. Because Th2-derived cytokines such as IL-4 (21, 22) and IL-9 (23–26) are known to serve as mast cell growth factors, we sought to determine whether repetitive airway allergen challenge is associated with the accumulation of peribronchial mast cells as well as the expression of these lung mast cell growth factors. In addition, because our laboratory (27) and others (28, 29) have demonstrated that immunostimulatory DNA sequences (ISS) containing CpG-rich motifs down-regulate the Th2 inflammatory response in mouse models of asthma, we sought to determine whether ISS could inhibit both the accumulation of peribronchial mast cells and the expression of mast cell growth factors in a murine model of repetitive airway allergen challenge for periods of 1–6 mo.

Materials and Methods

Mice

Female BALB/c mice (Harlan Laboratories, Indianapolis, IN; $n = 12$ mice/group) were used for *in vivo* experiments when they reached 8–10 wk of age. Mouse bone marrow-derived mast cells (MBMNCs) were generated from the bone marrow of BALB/c mice for *in vitro* experiments. All animal experimental protocols were approved by the University of California at San Diego Animal Subjects Committee.

Acute and chronic OVA challenge protocols

In both the acute and chronic Ag challenge experimental protocols, mice 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 aluminum hydroxide (Sigma-Aldrich) in 200 μ l of PBS, as previously described in our laboratory (27).

In the acute protocol, intranasal OVA challenges (20 μ g in 50 μ l of PBS) were administered on days 26, 28, 30, and 35, with mice being sacrificed on day 36. In the chronic protocol, mice completed the same intranasal OVA challenges on days 26, 28, and 30 as in the acute protocol and then continued to receive twice weekly intranasal OVA challenges for an additional 1, 3, or 6 mo. To facilitate intranasal administration of OVA, mice were briefly anesthetized with isoflurane (Isosol TM; Abbott Laboratories, North Chicago, IL).

Administration of ISS (50 μ g in 200 μ l of PBS i.p.) in the acute Ag challenge protocol was started on day 24 before the first intranasal OVA challenge on day 26 and repeated on day 34 before the final OVA challenge on day 35. In the chronic Ag challenge protocol, the first dose of ISS (50 μ g in 200 μ l of PBS i.p.) was also administered on day 24 before the first intranasal OVA challenge on day 26. The same dose of ISS was subsequently administered i.p. every other week until the chronic OVA protocol was terminated after 1, 2, 3, and 6 mo of repetitive airway OVA challenge. Previous studies in our laboratory have demonstrated that ISS inhibits OVA-induced eosinophilic inflammation and AHR when administered 1 day before OVA challenge (27) and that this inhibitory effect lasts at least 4 wk (30).

Determination of airway responsiveness *in vivo*

Airway responsiveness was analyzed on the day of sacrifice using a single-chamber, whole-body plethysmograph obtained from Buxco (Troy, NY), as previously described (27). The enhanced pause (Penh), a dimensionless value that correlates well with pulmonary resistance measured by conventional two-chamber plethysmography in ventilated mice in this laboratory (31) and other laboratories (32), was used to detect airway responsiveness.

In the chronic Ag exposure protocol, airway responsiveness was assessed on the day of sacrifice. Mice were exposed for 3 min to nebulized PBS to establish baseline Penh values, and they were subsequently exposed to increasing concentrations of nebulized methacholine (Sigma-Aldrich) in PBS using an Aeroson ultrasonic nebulizer (DeVilbiss, Somerset, PA). After each nebulization, recordings were taken for 3 min. The Penh values measured during each 3-min sequence were averaged, and In Stat (San Diego, CA) software was used to perform linear regression analysis of the results to determine the provocative dose of methacholine required to cause a 200% increase in baseline Penh values (PC200) for each mouse.

Enumeration of peribronchial lung mast cells

On the day of sacrifice, mouse lungs were tied off at the trachea with surgical suture and were preserved in 10% buffered formalin (Sigma-

Aldrich, St. Louis, MO) before being embedded in paraffin. The paraffin-embedded lungs were sectioned at 5 μ m onto microscope slides. The paraffin was removed from the lung sections using alcohol gradients and Citrosolv (Fisher, Pittsburgh, PA). The lung sections were stained with 1% toluidine blue (Sigma-Aldrich) to detect metachromatic mast cell cytoplasmic granules as previously described in this laboratory (33).

Light microscopy was used to count the number of mast cells per airway in lung sections of naive mice, as well as mice acutely or chronically exposed to OVA Ag challenge. To standardize the quantification of peribronchial mast cells, airways were characterized by size using an image analysis system as large (125–250 μ m), medium (60–125 μ m), or small (<60 μ m) by measuring the diameter of the long axis of the airway. The peribronchial area was defined as the circumferential area extending from the luminal surface of the airway epithelium to 8 μ m beneath the basement membrane of the airway epithelium in small airways, to 15 μ m beneath the basement membrane of the airway epithelium in medium-sized airways, and to 20 μ m beneath the basement membrane of the airway epithelium in large airways. The distance beneath the basement membrane chosen to define the peribronchial space was based on pilot experiments demonstrating the area in which the majority of non-mast cell peribronchial inflammatory cells were recruited after Ag challenge. The number of mast cells per airway were counted in 10 randomly selected airways of each size in each mouse.

In addition to detecting mast cells with toluidine blue staining, anti-IgE immunostaining was performed on selected lung sections using a rat anti-mouse IgE mAb. Endogenous peroxidase activity was quenched by incubation of in-lung sections for 30 min in 0.3% H₂O₂ in methanol. The lung sections were then incubated overnight with the primary Ab (rat anti-mouse IgE mAb; BD Pharmingen, San Diego, CA) at a concentration of 1/20 at 4°C. After washing with PBS, the lung sections were incubated with the biotinylated secondary Ab (mouse anti-rat IgG1/2a; BD Pharmingen), at a concentration of 1/100 for 30 min. The lung sections were then sequentially incubated with ABC reagent (Avidin, biotinylated HRP; Pierce, Rockford, IL), 3,3'-diaminobenzidine (DAB) buffer (DAB substrate kit; Vector Laboratories, Burlingame, CA), and DAB substrate reagent before briefly counterstaining with hematoxylin.

Lung cytokine analysis

The concentrations of IL-4, IL-9, and stem cell factor were assayed in lung tissue by ELISA, as previously described in our laboratory (34). Lungs homogenized in lysis buffer (0.5% Triton X-100, 150 mM NaCl, 15 mM Tris, 1 mM CaCl₂, and 1 mM MgCl₂) were centrifuged at 10,000 $\times g$ for 20 min. After the lung supernatant was passed through a 0.8- μ m pore size filter, the lung supernatant was assayed for cytokines by ELISA (R&D Systems, Minneapolis, MN). The sensitivity of the IL-4 assay was 7.8 pg/ml, the IL-9 assay was 61 pg/ml, and the stem cell factor assay was 31 pg/ml.

ISS and rhodamine-labeled ISS

Endotoxin-free (<1 ng/mg DNA) phosphorothioate ISS (5'-TGACTGTGAACGTTCCGAGATGA-3') and mutated oligodeoxynucleotides (M-ODN) (5'-TGACTGTGAAGGTTGGAGATGA-3') (Trilink, San Diego, CA) were synthesized as previously described (27) for use in *in vitro* and *in vivo* experiments. For *in vitro* experiments, rhodamine was conjugated to the 5' end of ISS or M-ODN by Solulink (San Diego, CA) to provide fluorescently labeled ISS (5'-rhodamine-ISS-3') or M-ODN (5'-rhodamine-M-ODN-3') for studies of ISS binding to mast cells.

MBMNCs

MBMNCs were generated from the bone marrow of BALB/c mice. The bone marrow cells were cultured in medium containing 20% WEHI supernatant as a source of IL-3, as previously described in this laboratory (35). After 3 wk, the viability of MBMNCs in the bone marrow culture was >95% as assessed by trypan blue staining, and the purity of MBMNCs was >95% as assessed by toluidine blue staining (35).

Expression of Toll-like receptor-9 (TLR-9) by MBMNCs assessed by RT-PCR

MBMNC TLR-9 mRNA expression was determined by RT-PCR. Total cellular RNA was isolated from MBMNCs using TRIzol reagent (Life Technologies, Gaithersburg, MD) as previously described in this laboratory (36). As a negative control, naive CD4⁺ T cells derived from mouse spleen were positively selected using a MACS CD4⁺ T cell isolation kit (Miltenyi Biotec, Auburn, CA) according to the manufacturer's instructions. The expression of TLR-9 was conducted with the following sense and anti-sense oligonucleotide sequence primers: sense primer, 5'-AGG

CTG TCA ATG GCT CTC AGT T-3'; antisense primer, 5'-TGA ACG ATTTC AGT GGT ACA AGT-3' (37). PCR amplification was conducted in a 50- μ l reaction volume containing 50 pmol of each primer, 50 mM KCl, 20 mM Tris-HCl, 1.5 mM MgCl₂, 0.2 mM of each dNTP, 1 μ l of formamide, and 1 U of *Taq* polymerase (BRL, Gaithersburg, MD). The reaction mixture was denatured at 95°C for 5 min, followed by 30 cycles of 95°C for 30 s and 60°C for 30 s and extended at 72°C for 30 s followed by an extension of 8 min at 72°C. The PCR products (611 bp) were electrophoresed in a 1.5% agarose gel and were visualized with ethidium bromide.

Effect of ISS on MBMMC proliferation in vitro

To assess whether ISS directly influences mast cell proliferation in vitro, 1×10^5 MBMMCs were cultured in triplicate for 1 or 7 days in a 96-well plate in the presence or absence of ISS (1–10 μ g/ml) and 20% WEHI supernatant. After 1 or 7 days in culture, wells containing MBMMCs were washed and aspirated with a pipette three times to recover all of the MBMMCs in each well as previously described in this laboratory (38). The total number of MBMMCs in each well was counted using a light microscope.

Binding of rhodamine-labeled ISS to MBMMCs

To assess whether mast cells can bind ISS, MBMMCs (5×10^5 /ml) were incubated with ISS conjugated to rhodamine (2 μ M) or as a negative control with 2 μ M rhodamine alone for 6 h at 37°C, and the binding was visualized using fluorescence confocal microscopy. To determine whether pretreatment with unlabeled ISS would inhibit the rhodamine-labeled ISS from binding to MBMMCs, MBMMCs were pretreated with 10 μ M unlabeled ISS 30 min before rhodamine-labeled ISS was added to the MBMMCs. At the end of the 6-h incubation period, MBMMCs were aspirated, washed with cold PBS/BSA, cytospun onto slides that were fixed with 1% paraformaldehyde in PBS, and were examined by confocal microscopy.

Confocal microscopy

The mean fluorescence intensity (MFI) of 10 randomly selected fields of MBMMCs was measured using a DeltaVision deconvolution microscope system (Applied Precision, Issaquah, WA). A charge-coupled device (Roper Scientific, Tucson, AZ) mounted on a Nikon microscope (Melville, NY) was used to capture images from the slides, using a rhodamine filter and a 20 $\times$ lens with 1 s of exposure time. The data sets were analyzed using SoftWorx software (Applied Precision) on a Silicon Graphics Octane workstation (Mountain View, CA).

Effect of ISS on Ag/IgE-mediated MBMMC mediator release

In these experiments, 1×10^6 MBMMCs were cultured in triplicate for 4–6 h in the presence or absence of ISS (1–10 μ g/ml). MBMMCs were then sensitized with anti-DNP IgE (1 μ g/ 10^6 MBMMCs) for 30 min, washed, and challenged with DNP-BSA Ag (200 ng) at 37°C for 20 min in Tyrodes buffer containing 1.8 mM CaCl₂ and 1 mM MgCl₂ (38). Levels of histamine in cell pellets and supernatants were measured 20 min after Ag stimulation, whereas supernatant IL-6 levels were measured 12 h after Ag stimulation. Histamine and IL-6 were measured with immunoassays having a sensitivity of 0.5 pg/ml and 7.8 pg/ml, respectively.

Statistical analysis

Statistical analysis was performed with ANOVA, and individual groups were compared using an unpaired Mann-Whitney test. All statistical analyses were performed with a statistical software package (In Stat). Values of $p < 0.05$ were considered statistically significant. Results are expressed as the mean $\pm$ SEM unless otherwise indicated.

Results

Accumulation of peribronchial mast cells after chronic Ag exposure

Peribronchial mast cells were quantitated using toluidine blue staining (Fig. 1A) in lung sections of mice challenged with OVA allergen for varying time periods. As depicted in Fig. 1, the majority of the accumulated mast cells were intraepithelial (~90%), with the remainder being subepithelial. Preliminary studies demonstrated that the number of toluidine blue-positive staining cells (Fig. 1A) were equivalent to the number of cells that stained positive for IgE (Fig. 1B), and therefore only the number of toluidine blue-positive cells were quantitated. Peribronchial mast cells were

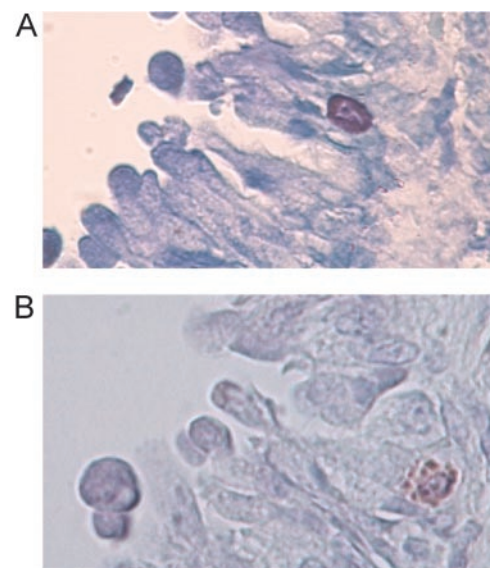


FIGURE 1. Airway mast cell detection. A, Toluidine blue (1%) was used to detect mast cells in lung tissue. A toluidine blue-positive mast cell is present in the airway epithelial layer. "L" indicates airway lumen. B, Anti-IgE immunostaining was used to detect IgE-positive cells in the airway. "L" indicates airway lumen.

not detected in unchallenged mice (0 ± 0 mast cells per large, medium, and small airways; $n = 8$ mice/group). A very small number of peribronchial mast cells were occasionally detected after acute OVA challenge (0.05 ± 0.04 mast cells per large airway and 0 ± 0 mast cells per medium and small airways; $n = 8$ mice/group).

In contrast with acute OVA challenge, repetitive OVA challenge induced a significant increase in the number of peribronchial mast cells after 1, 2, 3, and 6 mo of airway Ag challenge in the large, medium, and small airways (Fig. 2). The number of peribronchial mast cells in the large airways were significantly increased after repetitive OVA challenge for 1 mo (10.5 ± 1.0 vs 0 ± 0 mast cells per airway; $p = 0.0001$ compared with 1 mo non-OVA-challenged mice), repetitive OVA challenge for 2 mo (8.7 ± 1.0 vs 0.02 ± 0.02 mast cells per airway; $p = 0.0001$ compared with 2 mo non-OVA-challenged mice), repetitive OVA challenge for 3 mo (9.5 ± 1.1 vs 0.02 ± 0.02 mast cells per airway; $p = 0.0002$ compared with 3 mo non-OVA-challenged mice), and repetitive OVA challenge for 6 mo (7.3 ± 0.6 vs 0.03 ± 0.03 mast cells per airway; $p = 0.0001$ compared with 6 mo non-OVA-challenged mice) (Fig. 2A). Similar results were noted in the medium and small airways (Fig. 2, B and C). The increase in the number of peribronchial mast cells peaked after 1 mo of repetitive OVA challenge, but was still significantly elevated after 6 mo of repetitive OVA challenge (Fig. 2).

ISS reduces airway mast cell numbers during chronic Ag exposure

Mice pretreated with ISS before repetitive OVA challenges had fewer peribronchial mast cells in airways of all sizes compared with OVA-challenged mice that had not received ISS. ISS-treated mice repetitively challenged with OVA had a significantly lower number of peribronchial mast cells in the large airways after 1 mo of repetitive OVA challenge (6.8 ± 0.8 vs 10.5 ± 1.0 mast cells per airway; ISS + OVA vs OVA; $p = 0.006$), after 2 mo of OVA challenge (5.5 ± 0.7 vs 8.7 ± 1.0 mast cells per airway; ISS + OVA vs OVA; $p = 0.006$), after 3 mo of OVA challenge (5.5 ± 0.8 vs 9.5 ± 1.1 mast cells per airway; ISS + OVA vs OVA; $p =$

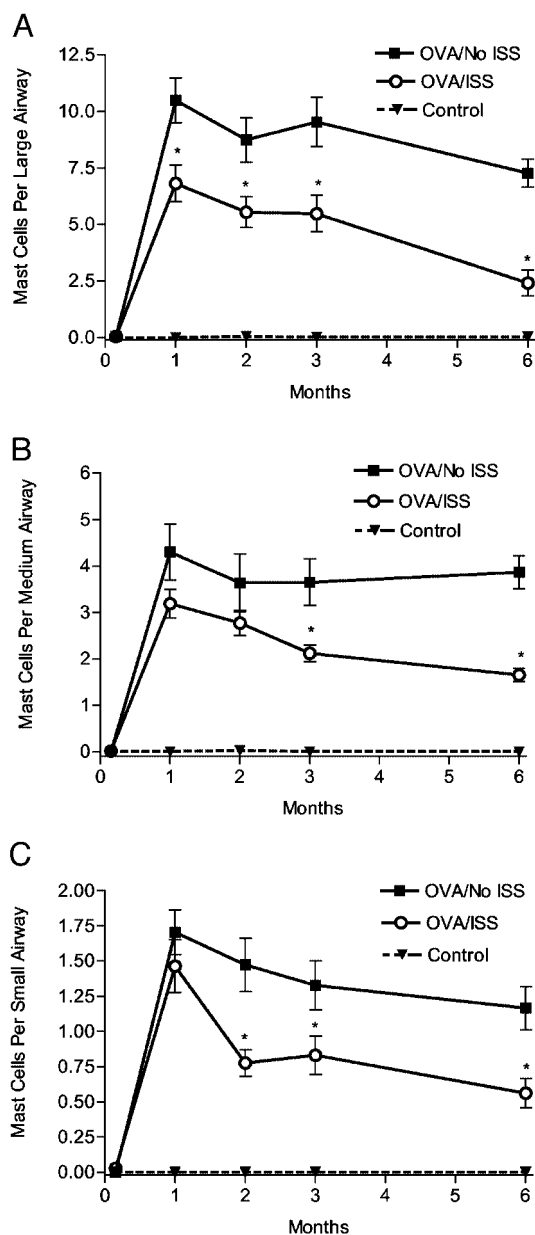


FIGURE 2. Chronic airway OVA exposure induces accumulation of peribronchial mast cells: modulation by ISS treatment. *A*, Large airways. Repetitive OVA Ag challenge (OVA/No ISS) induced a significant accumulation of mast cells in large airways at 1 mo ($p = 0.0001$, vs control), 2 mo ($p = 0.0001$, vs control), 3 mo ($p = 0.0002$, vs control), and 6 mo ($p = 0.0001$, vs control) ($n = 12$ mice/group). ISS treatment (OVA/ISS) significantly reduced the number of mast cells in large airways at 1 mo ($p = 0.006$, vs OVA/No ISS), 2 mo ($p = 0.006$, vs OVA/No ISS), 3 mo ($p = 0.02$, vs OVA/No ISS), and 6 mo ($p < 0.0001$, vs OVA/No ISS) ($n = 12$ mice/group). *B*, Medium airways. Repetitive OVA Ag challenge (OVA/No ISS) induced a significant accumulation of mast cells in medium-sized airways at 1 mo ($p = 0.0001$, vs control), 2 mo ($p = 0.0005$, vs control), 3 mo ($p = 0.0003$, vs control), and 6 mo ($p = 0.0001$, vs control) ($n = 12$ mice/group). ISS treatment (OVA/ISS) significantly reduced the number of mast cells in medium-sized airways at 3 mo ($p = 0.007$, vs OVA/No ISS) and 6 mo ($p < 0.0001$, vs OVA/No ISS) ($n = 12$ mice/group). *C*, Small airways. Repetitive OVA Ag challenge (OVA/No ISS) induced a significant accumulation of mast cells in small airways at 1 mo ($p = 0.0001$, vs control), 2 mo ($p = 0.0001$, vs control), 3 mo ($p = 0.0002$, vs control), and 6 mo ($p = 0.0004$, vs control) ($n = 12$ mice/group). ISS treatment (OVA/ISS) significantly reduced the number of mast cells in small airways at 2 mo ($p = 0.001$, vs OVA/No ISS), 3 mo ($p = 0.03$, vs OVA/No ISS), and 6 mo ($p = 0.001$, vs OVA/No ISS) ($n = 12$ mice/group).

0.02), and after 6 mo of OVA challenge (2.4 ± 0.6 vs 7.3 ± 0.6 mast cells per airway; ISS + OVA vs OVA; $p < 0.0001$) (Fig. 2A). ISS also significantly reduced the number of peribronchial mast cells in the medium-sized airways (Fig. 2B) after repetitive OVA challenge for 3 mo (ISS + OVA vs OVA; $p = 0.007$) and 6 mo (ISS + OVA vs OVA; $p = 0.0001$), as well as in the small airways at 2 mo (ISS + OVA vs OVA; $p = 0.001$), 3 mo (ISS + OVA vs OVA; $p = 0.03$), and 6 mo (ISS + OVA vs OVA; $p = 0.001$) (Fig. 2C).

Effect of chronic Ag challenge on expression of mast cell growth factors (IL-4, IL-9, stem cell factor)

To investigate the potential mechanism(s) by which repetitive OVA challenge induced an increase in the number of peribronchial mast cells, we measured levels of cytokines in the lung, which are known to stimulate mast cell proliferation (IL-4, IL-9, stem cell factor). Increased levels of lung IL-4 were detected after repetitive OVA challenge (141.2 ± 17.7 pg/ml vs 21.9 ± 7.1 pg/ml; 3 mo OVA vs no OVA; $n = 12$; $p < 0.0001$; Fig. 3A). Similarly, increased levels of lung IL-9 were detected after repetitive OVA challenge (229.5 ± 75.1 pg/ml vs 7.9 ± 7.9 pg/ml; 3 mo OVA vs no OVA; $n = 10$; $p < 0.0001$; Fig. 3B). Levels of lung stem cell factor were not increased after repetitive OVA challenge (521.0 ± 31.1 pg/ml vs 538.7 ± 20.5 pg/ml; 3 mo OVA vs no OVA; $n = 12$; $p = \text{NS}$).

ISS pretreatment decreased lung IL-4 (63.4 ± 8.7 pg/ml vs 141.2 ± 17.7 pg/ml; 3 mo OVA + ISS vs 3 mo OVA; $n = 12$; $p =$

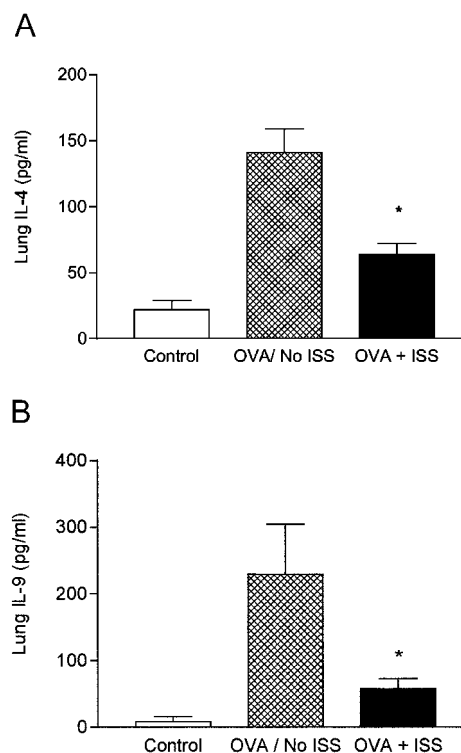


FIGURE 3. Chronic airway OVA exposure induces expression of IL-4 and IL-9: modulation by ISS treatment. *A*, Levels of IL-4 were measured in the lungs of mice by ELISA. Mice challenged with OVA for 3 mo had significantly higher levels of lung IL-4 compared with control mice ($p < 0.0001$, OVA/No ISS vs Control). ISS treatment for 3 mo significantly reduced lung IL-4 levels ($p = 0.0002$, OVA + ISS vs OVA/No ISS) ($n = 12$ mice/group). *B*, Levels of IL-9 were measured in the lungs of mice by ELISA. Mice challenged with OVA for 3 mo had significantly higher levels of lung IL-9 compared with control mice ($p < 0.0001$, OVA/No ISS vs Control). ISS treatment for 3 mo significantly reduced lung IL-9 levels ($p = 0.02$, OVA + ISS vs OVA/No ISS) ($n = 10$ mice/group).

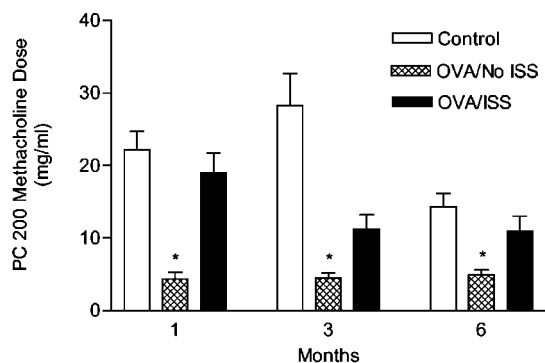


FIGURE 4. Chronic airway OVA exposure induces airway hyperreactivity: modulation by ISS treatment. The PC200 concentration of methacholine was calculated in mice repetitively challenged with OVA for up to 6 mo ($n = 12$ mice/group). Mice repetitively challenged with OVA (OVA/No ISS) had a significantly lower PC200 compared with age-matched control mice at 1 mo ($p = 0.0005$), 3 mo ($p < 0.0001$), and 6 mo ($p < 0.0001$) (Control vs OVA/No ISS). ISS-treated mice repetitively challenged with OVA (OVA/ISS) had a significantly higher PC200 at 1 mo ($p < 0.0001$), 3 mo ($p = 0.004$), and 6 mo ($p = 0.001$) (OVA/No ISS vs OVA/ISS), indicating that ISS treatment reduced the increased airway hyperreactivity to methacholine induced by chronic airway Ag challenge.

0.0002; Fig. 3A) as well as lung IL-9 (229.5 ± 75.1 pg/ml vs 57.6 ± 15.0 pg/ml; 3 mo OVA + ISS vs 3 mo OVA; $n = 10$; $p = 0.02$; Fig. 3B). ISS pretreatment did not change levels of lung stem cell factor (data not shown).

ISS inhibits airway hyperreactivity to methacholine induced by repetitive OVA challenge

In the absence of ISS therapy, mice developed significant airway hyperreactivity to methacholine after repetitive OVA exposure for up to 6 mo. Repetitively OVA-challenged mice had lower PC 200 values compared with non-OVA-challenged mice at 1 mo (4.3 ± 0.9 mg/ml vs 22.2 ± 2.5 mg/ml; $p = 0.0005$), 2 mo (3.5 ± 0.4 mg/ml vs 11.3 ± 0.8 mg/ml; $p = 0.0007$), 3 mo (4.5 ± 0.7 mg/ml vs 28.2 ± 4.5 mg/ml; $p < 0.0001$), and 6 mo (4.9 ± 0.7 mg/ml vs 14.3 ± 1.8 mg/ml; $p < 0.0001$) (Fig. 4). Pretreatment of repetitively OVA-challenged mice with ISS significantly reduced airway hyperreactivity, as evidenced by the higher PC200 values in ISS-treated mice at 1 mo (ISS + OVA vs OVA; $p < 0.0001$), 3 mo (ISS + OVA vs OVA; $p = 0.004$), and 6 mo (ISS + OVA vs OVA; $p = 0.0009$; Fig. 4). Overall, these results suggest that ISS significantly reduced the increase in airway hyperreactivity to methacholine induced by repetitive airway OVA exposure.

MBMMCs express TLR-9

RT-PCR studies demonstrated that MBMMCs strongly expressed the TLR-9 receptor (Fig. 5).

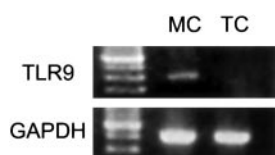


FIGURE 5. MBMMCs express the TLR-9 receptor by RT-PCR. RT-PCR demonstrated that MBMMCs express the TLR-9 receptor. Lane 1 is the m.w. ladder. Lane 2 shows MBMMCs (MC) expressing the 600-bp TLR-9 PCR product band. Lane 3 shows T cells (TC) that do not express TLR-9. Equivalent loading of the lanes is demonstrated by GAPDH.

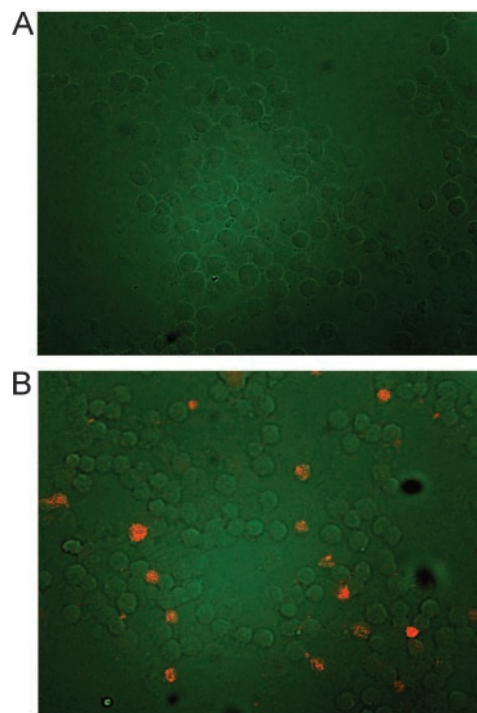


FIGURE 6. Confocal microscopy of MBMMCs incubated with rhodamine-labeled ISS. A, MBMMC incubation with rhodamine. As a negative control, MBMMCs were incubated with rhodamine alone for 6 h. No immunofluorescent MBMMCs were visualized by confocal microscopy. B, MBMMC incubation with rhodamine conjugated to ISS. MBMMCs (>99% purity) were incubated with rhodamine conjugated to ISS for 6 h. Approximately 10% of the MBMMCs stained positive on this confocal microscopy field.

MBMMCs bind rhodamine-labeled ISS

Having demonstrated that MBMMCs express TLR-9, the receptor for ISS, studies were performed to determine whether fluorescently labeled ISS bound to MBMMCs. Confocal microscopy studies demonstrated that MBMMCs bound rhodamine-labeled ISS but not rhodamine alone (Fig. 6). The MFI of MBMMCs incubated with control rhodamine alone (93.2 ± 2 ; $n = 29$) was similar to the background MFI of MBMMCs, which ranged from 80 to 100 (Fig. 7). The MFI of MBMMCs incubated with rhodamine-labeled ISS was significantly greater than that of MBMMCs incubated

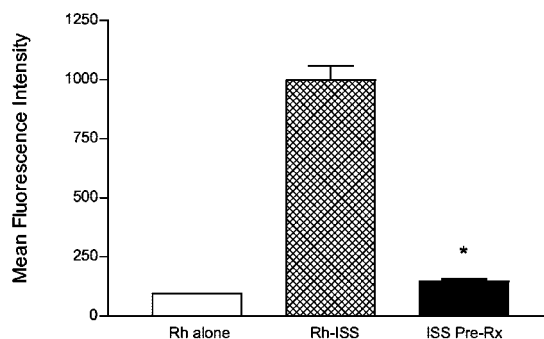


FIGURE 7. MFI of MBMMCs incubated with rhodamine-labeled ISS. MBMMCs were incubated in triplicate for 6 h with $2 \mu\text{M}$ rhodamine (Rh alone) or $2 \mu\text{M}$ rhodamine conjugated to ISS (Rh-ISS). The MFI of MBMMCs incubated with Rh-ISS was significantly greater than that of MBMMCs incubated with Rh alone ($p < 0.0001$). Pretreatment of MBMMCs with unlabeled ISS ($10 \mu\text{M}$; ISS Pre-Rx) 30 min before the addition of Rh-ISS significantly reduced the MFI ($p < 0.0001$).

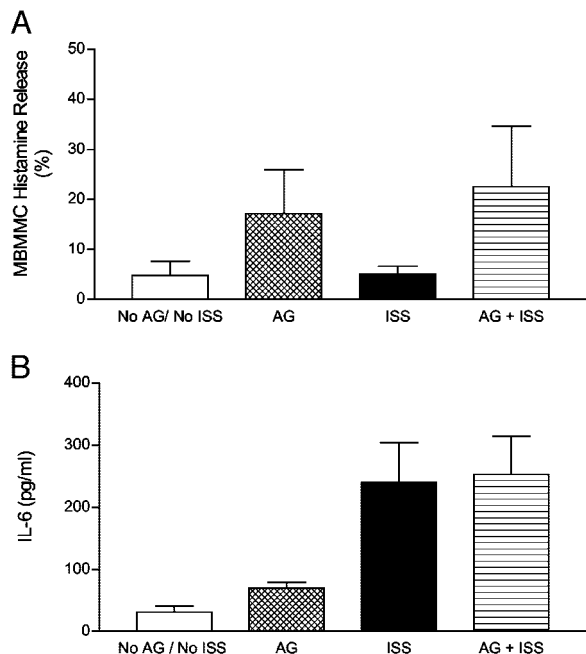


FIGURE 8. Effect of ISS on Ag/IgE-stimulated MBMMC mediator release in vitro. **A**, Effect of ISS on Ag/IgE-mediated histamine release from MBMMCs. MBMMCs were incubated with ISS for 4–6 h, sensitized with DNP-BSA IgE, and challenged with DNP-BSA Ag for 20 min. Ag induced significant histamine release from IgE-sensitized MBMMCs (Ag vs No Ag + No ISS) ($p = 0.05$). ISS did not inhibit Ag-induced histamine release from MBMMCs (Ag vs Ag + ISS) ($p = \text{NS}$). **B**, Effect of ISS on Ag/IgE-mediated IL-6 release from MBMMCs. MBMMCs were incubated with ISS for 4–6 h, sensitized with DNP-BSA IgE, and challenged with DNP-BSA Ag for 12 h. Ag induced significant IL-6 release from MBMMCs compared with control MBMMCs (Ag vs No Ag/No ISS) ($p = 0.05$). ISS induced significantly greater levels of IL-6 release from MBMMCs compared with Ag (ISS vs Ag) ($p = 0.001$).

with rhodamine alone (997.7 ± 58.6 vs 93.2 ± 2.1 ; $n = 29$; $p < 0.0001$). Pretreatment of MBMMCs with $10 \mu\text{M}$ ISS for 30 min significantly decreased the MFI of MBMMCs incubated with rhodamine-labeled ISS (148.1 ± 8.0 ; $n = 29$; $p < 0.0001$ compared with no pretreatment with ISS), suggesting that the uptake of rhodamine-labeled ISS was inhibited by the unlabeled ISS (Fig. 7). A rhodamine-labeled M-ODN (which only differs in not having the CpG motif present in rhodamine-labeled ISS) did not bind to MBMMCs (data not shown).

ISS does not inhibit mast cell proliferation in vitro

To determine whether the binding of ISS by MBMMCs induced any functional changes in MBMMCs, MBMMC proliferation assays were performed in the presence or absence of ISS in vitro for 1 or 7 days. In the 1 day experiments, the number of MBMMCs in the cultures containing $1 \mu\text{g/ml}$ ISS ($1.00 \pm 0.04 \times 10^5$ cells) and $10 \mu\text{g/ml}$ of ISS ($1.06 \pm 0.07 \times 10^5$) were not significantly different from the number of MBMMCs in cultures without ISS ($1.02 \pm 0.03 \times 10^5$) ($n = 9$) ($p = \text{NS}$).

There was a significant increase in the number of MBMMCs after 7 days of in vitro culture of MBMMCs without ISS (1.0×10^5 vs $1.48 \pm 0.10 \times 10^5$; MBMMC time, 0 h, vs MBMMC No ISS for 7 days; $p = 0.007$). After 7 days of incubation of MBMMCs with ISS, there was no significant difference in the number of MBMMCs in cultures containing $1 \mu\text{g/ml}$ ISS ($1.65 \pm 0.08 \times 10^5$ cells) compared with MBMMC cultures without ISS ($1.48 \pm 0.10 \times 10^5$ cells; $n = 9$; $p = \text{NS}$).

Effect of ISS on Ag/IgE-mediated mast cell activation in vitro

To determine whether ISS inhibits mast cell activation in vitro, MBMMCs were cultured in the presence or absence of ISS ($1 \mu\text{g/ml}$) and then activated by IgE receptor cross linking with Ag. MBMMC release of preformed granule mediators (histamine) was not inhibited by ISS (Fig. 8A). Although ISS did not induce histamine release from unstimulated MBMMCs, it did induce significant release of IL-6 from unstimulated MBMMCs (Fig. 8B). The levels of IL-6 released by MBMMCs in response to ISS stimulation were significantly greater than the levels of IL-6 induced by Ag/IgE stimulation of MBMMCs ($240.7 \pm 63.6 \text{ pg/ml}$ vs $70.2 \pm 18.7 \text{ pg/ml}$; ISS vs Ag; $p = 0.001$; Fig. 8B). There was no synergy in IL-6 release when MBMMCs were stimulated with Ag/IgE in combination with ISS (Fig. 8B).

Discussion

In this study, we have used a mouse model of repetitive OVA challenge to demonstrate that repetitive OVA challenge induces an accumulation of peribronchial mast cells in large, medium, and small airways. The accumulation of peribronchial mast cells could be due to recruitment and/or local proliferation of mast cell precursors or mature mast cells. Because mature mast cells are not noted in the circulation and very few mast cells are detected in the lungs of naive mice, we favor the mast cell precursor explanation for the peribronchial accumulation of mast cells. The lack of accumulation of peribronchial mast cells after an acute OVA challenge and the peak accumulation of peribronchial mast cells after 1 mo of repetitive OVA challenge are consistent with a model in which time is needed for mast cell precursors in the lung (resident or recruited) to differentiate under local cytokine stimulation into mast cells. Because there is no currently available marker to identify mast cell precursors in tissues, we were unable to demonstrate whether there was an increase in mast cell precursors in the lung after repetitive OVA challenge. Limiting dilution analysis has been used to determine the frequency of mast cell precursors in bone marrow, blood, or lung (39, 40). Characterization of mast cell precursors derived from limiting dilution analysis reveal that they are CD34^+ , IgE receptor $^-$, Kit^+ (39, 40). The number of mast cell precursors in bone marrow is $\sim 600/10,000$ mononuclear cells, with fewer detected in lung ($\sim 150/10,000$ mononuclear cells) (40). Additional evidence against circulating mast cells being recruited to the airway is derived from the histologic analysis of the lung distribution of mast cells in repetitively OVA-challenged mice in which there is an absence of a gradient of mast cells being detected in lung sections from pulmonary blood vessels to the airway. Whereas eosinophils can readily be detected in lung sections in and around blood vessels, in the interstitial space, and in peribronchial regions, this pattern of distribution was not noted for mast cells. We did not note mast cells adherent to pulmonary blood vessels, diapedesing between endothelial cells, or accumulating outside of blood vessels.

Because mast cells proliferate in response to a variety of cytokines and/or growth factors, we have investigated whether mast cell growth factors are expressed in the lung after repetitive OVA challenge. Initial studies focused on detecting mast cell growth factors released by Th2 cells because the repetitive OVA challenge model we have developed is associated with chronic expression of Th2 cytokines (IL-5, IL-13) and eosinophilic inflammation.⁴ Th2 cytokines known to induce mast cell proliferation include IL-4 (21, 22) and IL-9 (23–26). In vitro IL-4 enhances the growth of bone marrow-derived mast cells (21, 22), and IL-9 promotes mast cell

proliferation and differentiation (22–26). IL-9 expression in transgenic mice causes mast cell hyperplasia and bronchial hyperresponsiveness (24, 25). Repetitive OVA challenge was associated with increased expression of these Th2 cytokines (IL-4, IL-9), which can both affect mast cell proliferation. We also measured levels of stem cell factor (SCF), whose predominant cellular source is not Th2 cells, to determine whether repetitive OVA challenge induced expression of this mast cell growth factor (41). Repetitive OVA challenge did not induce increased levels of SCF, suggesting that SCF was not responsible for the increased numbers of peribronchial mast cells noted.

Because we have previously demonstrated that ISS inhibits Th2 cell-dependent accumulation of eosinophils in the lung (27), we performed experiments to determine whether ISS inhibited Th2 cytokines (IL-4 and IL-9) with mast cell growth factor activity, as well as mast cell accumulation in the lung. Although previous studies in an acute OVA challenge model have demonstrated that ISS inhibits IL-4 expression (28), this study is the first to demonstrate that ISS inhibits allergen-induced IL-9 expression. IL-9 expression is increased in asthmatic airways compared with normal subjects, and IL-9 mRNA expression has been correlated with FEV₁ and AHR to methacholine, identifying it as an important cytokine in allergic asthma (26). Although this study demonstrates an association between the ability of ISS to inhibit the expression of mast cell growth factors in the lung and the inhibition of mast cell lung accumulation, this study is not able to demonstrate whether this is a cause and effect relationship or only an association.

To determine whether ISS could be having any direct effect on mast cell proliferation, we initially determined whether mast cells express TLR-9 the receptor for ISS (42). RT-PCR studies demonstrated that MBMMCs strongly express TLR-9. Additional in vitro experiments demonstrated that MBMMCs were able to bind rhodamine-labeled ISS, and this binding was inhibited by pretreatment with unlabeled ISS. Although mast cells express TLR-9, ISS does not directly inhibit mast cell proliferation in vitro. ISS did not inhibit Ag/IgE-mediated MBMMC degranulation, but did it induce IL-6 generation. Previous studies using unstimulated MBMMCs have demonstrated that ISS induces MBMMC expression of TNF and IL-6 (43). The generation of IL-6 in response to ISS stimulation is likely to be a beneficial effect of ISS in allergic inflammation. Studies with IL-6 transgenic mice have demonstrated an important anti-inflammatory role for IL-6 in mouse models of asthma (44).

Overall, these studies demonstrate that mice exposed to repetitive OVA challenge, but not acute OVA challenge, have an accumulation of peribronchial mast cells and express mast cell growth factors including IL-4 and IL-9 in the lung. Although mast cells express TLR-9, ISS does not directly inhibit mast cell proliferation in vitro, suggesting that ISS inhibits accumulation of peribronchial mast cells in vivo by indirect mechanism(s) that include inhibiting the lung expression of Th2 cell-derived mast cell growth factors.

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