

Chronic Asthma-induced Airway Remodeling Is Prevented by Toll-like Receptor-7/8 Ligand S28463

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Rationale: Allergic asthma is a heterogeneous disease, the pathology of which is a result of improper immune responses to innocuous antigens. We and others have previously shown that one of the Toll-like receptor (TLR)-7/8 ligands, the synthetic compound S28463 (resiquimod, R-848), is able to inhibit acute allergic asthma in mice. **Objectives:** Given that the efficiency of this pharmacologic compound against the smooth muscle mass increase and goblet cell hyperplasia that are characteristic of chronic allergic asthma has not been previously assessed, we investigated the ability of this compound to prevent these aspects of chronic airway remodeling. **Methods:** The impact of S28463 treatment was assessed in a Brown Norway rat model of chronic asthma by histologic, morphometric, and molecular techniques.

Measurements and Main Results: We demonstrate that treatment with S28463 is able to prevent the development of goblet cell hyperplasia and increases in airway smooth muscle mass, and that this effect is at least partially mediated by inhibiting proliferation of goblet and smooth muscle cells, respectively. Furthermore, we show that the abrogation of airway remodeling is preceded by inhibition of the inflammatory reaction normally occurring in response to allergen challenge in sensitized animals. This inhibition was associated with a reduction of both helper T cell type 1 and type 2 cytokine protein expression in the lungs, demonstrating the potent antiinflammatory effect of this pharmaceutical compound in the context of allergic reactions.

Conclusions: Taken together, our results indicate great potential for the use of S28463 as an antiinflammatory therapeutic agent for the management of chronic asthma.

Keywords: resiquimod; R-848; imidazoquinoline; airway remodeling; Toll-like receptor

Allergic asthma is a heterogeneous disease affecting the airways and is characterized by a chronic inflammatory response involving

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

Treatment with TLR7 ligand is effective in preventing the inflammatory reaction in an acute asthma model. The effectiveness of this compound in preventing chronic airway remodeling had not been assessed previously.

What This Study Adds to the Field

Treatment with S28463, a TLR 7/8 ligand, inhibits the increase in airway smooth muscle mass and the development of goblet cell hyperplasia that are associated with chronic asthma.

several cellular and humoral mediators. Prototypical asthmatic airway inflammation is believed to be triggered by IgE-dependent mast cell degranulation that results in the recruitment of monocytes, lymphocytes, and, particularly, large numbers of eosinophils as well as the induction of several helper T cell type 2 (Th2) cytokines. This chronic inflammatory response is believed to be the underlying cause of airway remodeling, characterized by subepithelial fibrosis, goblet cell hyperplasia, and increased smooth muscle mass (1), and has been associated with increased asthma severity (2).

Current asthma management strategies principally involve the use of bronchodilators, in the form of short- and long-acting β_2 agonists, as rescue medication during exacerbations and to prolong the time to exacerbation, respectively, and inhaled corticosteroids to minimize the underlying inflammation. Despite having been shown to be effective in preventing inflammation (3) by inhibiting cytokine transcription (4) and eosinophil survival (5, 6), inhaled corticosteroids have been relatively underprescribed because of concerns, on the part of primary care physicians and patients alike, over potential side effects, which may include oral candidiasis, dysphonia, growth retardation, skin thinning/bruising, and loss of bone mineral density (7). There is therefore a need for alternative approaches to the treatment of the inflammation underlying asthma pathology.

Considerable effort has been devoted to exploring the potential of immunomodulatory treatment of asthma by means of Toll-like receptor (TLR) activation (8). TLRs are a family of molecules that mediate pathogen-associated molecular pattern recognition and that play a crucial role in both innate and adaptive immunity (9, 10). These widely expressed proteins are some of the first molecules to be involved in generating immune responses to environmental stimuli, a process that is mediated by

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cellular activation and stimulation of cytokine production (11). Furthermore, several TLRs or TLR ligands have been associated with asthma. Elevated levels of lipopolysaccharides, which are TLR4 ligands, have been negatively correlated with the incidence of asthma in several studies (12, 13), and CpG oligodeoxynucleotides, which are TLR9 ligands, have been used, with various degrees of success, in the treatment of asthma in animal models (14–17).

The natural ligands of TLR7 are believed to be a subset of single-stranded RNA molecules of viral origin (18–20); however, this receptor, and TLR8, are also known to be ligated by members of the imidazoquinoline family of pharmaceutical compounds (21, 22). S28463 (also known as resiquimod and R-848) is a member of the imidazoquinoline family and has been shown to induce the expression of a wide range of cytokines in many species from rodents to primates (23). Our group (24, 25), and others (26, 27) have previously demonstrated that S28463 treatment can prevent the development of lung pathology, including airway hyperresponsiveness and eosinophilia, in mouse models of acute asthma. On the basis of studies using BALB/c mice, Quarcoo and colleagues suggested that treatment with S28463 causes a shift from an allergic Th2 to a nonallergic Th1-biased immune response (26). In two other strains of mice (C57BL/6, with low airway hyperresponsiveness, and A/J, with high airway hyperresponsiveness) treatment with S28463 resulted in a dramatic inhibition of the acute inflammatory reaction that results from allergen exposure in properly sensitized animals and this inhibition was accompanied by a concurrent inhibition of both Th1 and Th2 cytokines (24).

Given that S28463 treatment could prevent the acute inflammatory reaction associated with allergen challenge in several genetically diverse strains of mice, we postulated that this compound could also inhibit the acute inflammatory response in other species. Furthermore, we hypothesized that if treatment with S28463 was capable of preventing the acute inflammatory reaction caused by allergen exposure, it might also prevent the development of the chronic remodeling associated with repeated allergen exposure. The potential of Toll-like receptor 7 ligands in preventing changes associated with chronic asthma remains to be addressed.

The Brown Norway rat has been shown to develop airway remodeling consistent with asthma after only 2 weeks of allergen challenge (28, 29), making this species a convenient experimental model for the study of the chronic aspects of the disease. Using an acute ovalbumin (OVA)-sensitized and -challenged Brown Norway rat model of asthma, we demonstrate that S28463 treatment is able to prevent the development of allergen-induced inflammation and that this effect is associated with a reduction of both Th1 and Th2 cytokine levels and in a dramatic reduction of inflammatory cell infiltration into the lungs. Furthermore, using a chronic model of OVA-sensitized and -challenged rats, we demonstrate that continued treatment with S28463 is capable of preventing the increase in smooth muscle mass and goblet cell hyperplasia associated with airway remodeling in asthma. We also demonstrate that this effect is mediated, at least partially, by inhibiting the proliferation of epithelial and subepithelial airway cells.

METHODS

Animals

All animals used in this study were 8- to 10-week-old male Brown Norway rats (Harlan, Bicester, UK) and all procedures performed on the animals were in compliance with the Canadian Council of Animal Care (CCAC, Ottawa, ON, Canada) guidelines and were approved by the McGill University (Montreal, PQ, Canada) Animal Care Committee.

Sensitization and Challenge for Assessment of Acute Inflammatory Response

OVA sensitization was performed by concurrent subcutaneous injection of OVA adsorbed to aluminum hydroxide, and intraperitoneal injection of heat-killed *Bordetella pertussis* (provided by T. Issekutz, Dalhousie University, Halifax, NS, Canada). Fourteen days later, the animals were challenged by aerosol with an OVA solution (OVA-OVA and OVA/S28), or with phosphate-buffered saline (OVA-PBS and OVA-S28/PBS). Twenty-four hours before antigen challenge, two groups of rats (OVA-S28/OVA and OVA-S28/PBS) were administered an intraperitoneal injection of 1 mg of S28463 (S28463 was generously provided by R. Miller, 3M Pharmaceuticals, St. Paul, MN). All animals were killed 24 hours after antigen challenge. See the online supplement for additional details.

Sensitization and Challenge for Assessment of Chronic Airway Remodeling

OVA sensitization was induced as described above. Thereafter, three airway antigen challenges were performed on Days 14, 19, and 24. One group of rats received 1 mg of S28463 24 hours before each antigen challenge and all animals were killed 48 hours after the final antigen challenge. See the online supplement for additional details.

Bronchoalveolar Lavage Fluid and Cellular Analysis

On killing, lungs of the animals were rinsed with saline and total and differential cell counts were performed. Additional details can be found in the online data supplement.

Plasma IgE

On killing, plasma was recovered from the animals and total IgE levels were measured by ELISA. See the online supplement for additional details.

Cytokine Analysis

Cytokine analysis was performed on lung tissue homogenate samples, using a custom LINCOplex rat cytokine kit (LINCO Research/Millipore, St. Charles, MO). See the online supplement for additional details.

Preparation of Histologic Samples

Lung samples were prepared for histology by formalin fixation and paraffin embedding. See the online supplement for additional details.

Measurement of Chronic Inflammation

Chronic inflammation was assessed by histologic analysis of hematoxylin and eosin–stained lung sections. See the online supplement for additional details.

Measurement of Airway Smooth Muscle Mass

Airway smooth muscle (ASM) mass was estimated from measurements of the area of smooth muscle–specific α -actin (α -SMA) immunoreactivity as previously described (30). See the online supplement for additional details.

Goblet Cell Hyperplasia

Goblet cell hyperplasia was assessed by histologic analysis of periodic acid–Schiff (PAS)–stained lung sections. See the online supplement for additional details.

Proliferating Airway Epithelial Cell and Subepithelial Cell Quantification

Proliferation of airway epithelial and subepithelial cells was investigated by immunostaining for proliferating cell nuclear antigen (PCNA). See the online supplement for additional details.

Statistical Analysis

All data were analyzed with GraphPad Prism 4 (version 4.03; GraphPad Software Inc., San Diego, CA), using a parametric one-way analysis of variance method followed by a Bonferroni post-test procedure. Data were normalized before analysis by logarithmic or square root

transformation where appropriate. A *p* value of 0.05 or less was considered significant.

RESULTS

Treatment with S28463 Prevents the Influx of Inflammatory Cells into Bronchoalveolar Lavage Fluid

Having previously established that S28463 prevents acute airway inflammation in a mouse model of allergic asthma (24), in this study we investigated whether this compound had an effect on the development of airway remodeling in a rat model of chronic asthma. We first established the effectiveness of S28463 treatment in preventing the acute inflammatory reaction observed after a single allergen challenge. A prototypical finding in allergic asthma and its models is an influx of inflammatory cells, composed primarily of eosinophils but often also including neutrophils and macrophages, into the bronchoalveolar space. As expected (Figure 1A), there was a dramatic increase in the number of cells recovered in the bronchoalveolar lavage fluid (BALF) of OVA-sensitized and -challenged (OVA) rats when compared with rats that were challenged with vehicle only (PBS). Treatment with S28463 (S28/OVA) significantly reduced the number of inflammatory cells recovered in the BALF of OVA-challenged rats. Furthermore, treatment of sham-challenged animals (S28/PBS) did not lead to significant changes in BALF cell numbers. Although both macrophages and lymphocytes are increased in the BALF of OVA-challenged animals (Figure 1B), eosinophils, and to a lesser extent neutrophils, account for the majority of the increase. The dramatic increase in eosinophil numbers was significantly inhibited by treatment with S28463, and neutrophil

and lymphocyte numbers trended downward, but the observed decrease did not reach statistical significance.

Treatment with S28463 Prevents Airway Inflammation

It has been previously observed that, in human subjects treated with anti-IL-5 antibodies (31), it is possible to affect eosinophil levels in the BALF compartment to a greater extent than in the tissue compartment. We therefore evaluated inflammatory cell infiltration of the area surrounding the airways and blood vessels in the lung by histologic analysis. Sensitization with OVA followed by sham PBS challenge (Figure 2A), or followed by S28463 treatment and sham challenge (Figure 2B), did not cause any noticeable inflammation. The lungs of animals that were sensitized and challenged with OVA contained large numbers of inflammatory cells (Figure 2C) concentrated near the airways and in the perivascular areas between the airways and blood vessels. Treatment with S28463 before allergen challenge with OVA results in a marked decrease in inflammatory cell infiltration (Figure 2D).

Treatment with S28463 Causes a Decrease in Both Th1 and Th2 Cytokine Levels

Allergic asthma is an atopic disease, a class of diseases characterized by elevated blood IgE levels. OVA-sensitized and -challenged animals treated with S28463 (Figure 3) had significantly lower plasma IgE levels compared with animals that were not treated with the compound.

The predisposition toward an IgE-mediated immune response in individuals suffering from allergic asthma is closely related to an elevated number of Th2 cells and their associated cytokines (32, 33). Furthermore, TLR7 ligation has been associated with the induction of Th1 cytokines in several models (26, 34). We therefore used a cytokine protein multiplex assay to assess the production of both Th1 and Th2 cytokines elicited by OVA challenge of sensitized rats when treated with S28463.

Th2 cytokines IL-4 (Figure 4A), IL-5 (Figure 4B), and IL-13 (Figure 4D) and Th1 cytokine IFN- γ (Figure 4E) were strongly induced (16-, 85-, 10-, and 32-fold relative to PBS control, respectively)

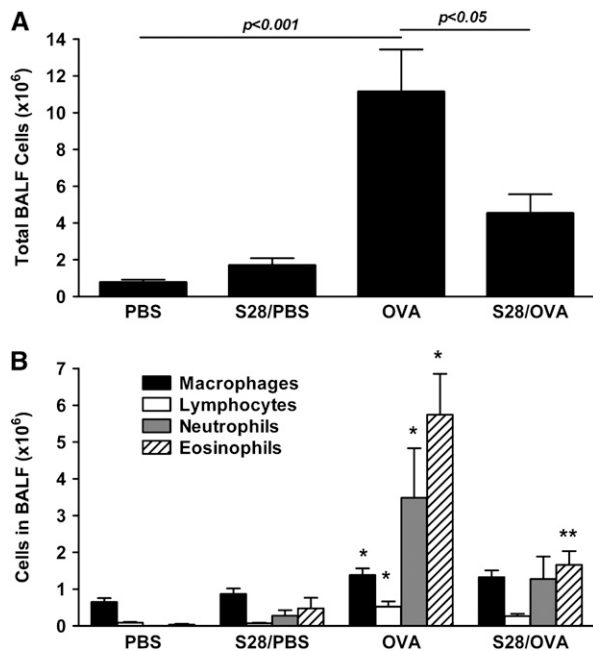


Figure 1. Analysis of inflammatory cells found in bronchoalveolar lavage fluid (BALF) after imidazoquinoline treatment (S28/OVA and S28/PBS) and OVA (OVA and S28/OVA) or PBS (PBS and S28/PBS) challenge. (A) Total number of inflammatory cells recovered in the BALF of animals. (B) Differential count of the inflammatory cells recovered. Results are represented as means $\pm$ SEM. *Significance between PBS and OVA groups; **significance between OVA and S28/OVA groups. *n* = 5 for S28/PBS group; *n* = 6 for all other groups. PBS = phosphate-buffered saline; OVA = ovalbumin; S28 = S28463.

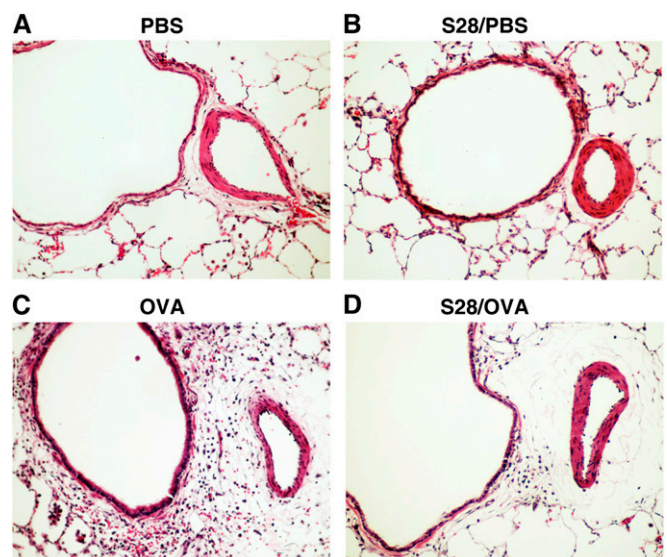


Figure 2. Representative hematoxylin-and-eosin-stained lung sections collected 24 hours after challenge from animals acutely sensitized with OVA and challenged with PBS (A and B) or OVA (C and D) with prior treatment with (B and D) or without (A and C) S28463.

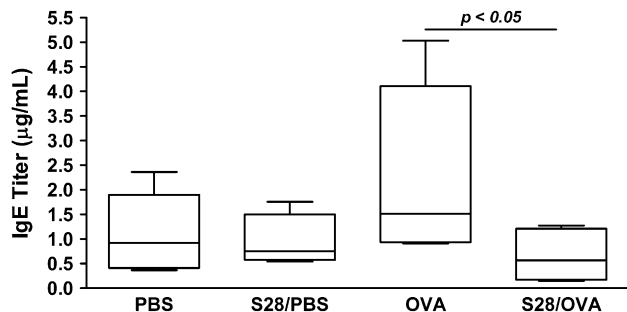


Figure 3. Total blood plasma IgE levels in rats after imidazoquinoline treatment (S28/OVA and S28/PBS) and OVA (OVA and S28/OVA) or PBS (PBS and OVA-S28/PBS) challenge. Data are represented as medians $\pm$ interquartile range ($n = 6$ for all groups).

48 hours after OVA challenge (OVA), compared with mock-challenged animals (PBS; Figures 4A, 4B, 4D, and 4E, respectively). The inflammatory cytokines IL-1 α (Figure 4F), IL-1 β (Figure 4G), and IL-6 (Figure 4C) were also found to be induced to a much greater extent in the OVA-challenged animals than in the PBS group (Figures 4C, 4F, and 4G). Furthermore, the chemokines CXCL-1 (Figure 4H) and macrophage inflammatory protein-1 α (Figure 4I) were also elevated in OVA-challenged animals, but to a lesser extent than the other cytokines.

Treatment with S28463 resulted in a dramatic decrease in cytokine production associated with OVA challenge. Production of the Th2 cytokines IL-4, IL-5, and IL-13 after OVA challenge was significantly lower (Figures 4A, 4B, and 4D, respectively) in the S28463-treated group (S28/OVA) than in the untreated controls. Similarly, production of the inflammatory cytokine IL-6 (Figure 4C) after antigen challenge was significantly lower in S28463-treated rats, whereas IL-1 α and IL-1 β (Figures 4F and 4G, respectively), as well as CXCL-1 (Figure 4H), levels trended downward but did not reach statistical significance. Importantly, the production of the Th1 cytokine IFN- γ (Figure 4E) induced by antigen challenge was also significantly reduced in animals treated with S28463.

The cytokine profile (Figure 4) of animals that received a mock challenge (PBS) was similar to the profile of animals that received both S28463 treatment and mock challenge (S28/PBS) as evidenced by the fact that none of the cytokine levels tested was significantly different. IL-9 and eotaxin were not found to be significantly modulated by any treatment (data not shown).

Treatment with S28463 Prevents the Airway Inflammation Associated with Repeated Allergen Challenge

After characterizing acute inflammatory responses to allergic stimulation with OVA, we assessed the ability of S28463 to prevent the development of airway remodeling and inflammation induced by repeated allergen challenge. The presence of inflammatory cells surrounding the airways of animals after repeated allergen challenge was evaluated by histologic analysis (Figure 5). Sensitization with OVA followed by repeated allergen (OVA) challenges resulted in significant inflammatory cell infiltration in the area surrounding airways (Figure 5B). Comparatively few inflammatory cells could be found surrounding the airways of animals that had been sensitized with OVA but sham challenged with PBS (PBS) (Figure 5A). Similarly, the airways of OVA-sensitized and -challenged animals that had received S28463 treatment (S28/OVA) displayed markedly lower numbers of inflammatory cells (Figure 5C).

The level of inflammation was quantified by counting the number of cells in the peribronchial area surrounding the airways of hematoxylin and eosin-stained lung sections and normalizing this count for airway size by dividing by the square of the perimeter of the basement membrane (Pbm²). Sensitization and challenge with OVA lead to a significant increase in the number of inflammatory cells, with an important eosinophil component accounting for 37% of all inflammatory cells surrounding the airways (Figure 5D). Treatment with S28463 leads to a complete abrogation of the increase in inflammatory cells (Figure 5D). In addition, treatment with S28463 leads to a dramatic decrease in eosinophil numbers when compared with OVA-sensitized and -challenged animals that did not receive treatment.

Treatment with S28463 Prevents the Increase in Airway Smooth Muscle-specific α -Actin-positive Cell Mass

One of the key structural changes observed in asthmatic lungs is an increase in ASM mass. Sensitization and repeated challenge

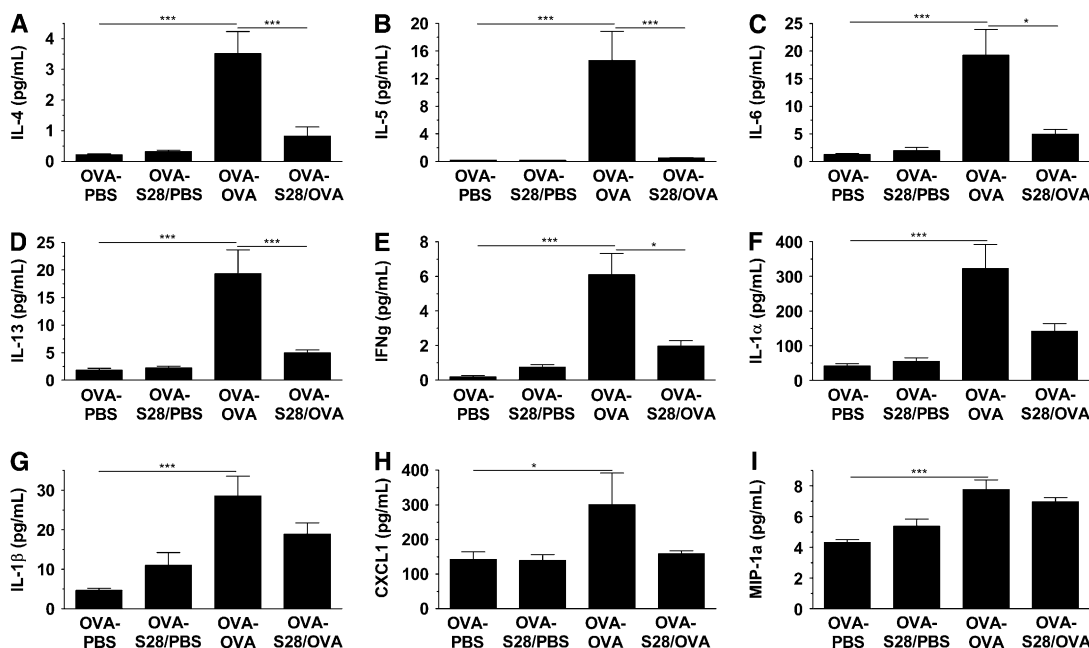


Figure 4. Cytokine levels in lung homogenates of OVA-sensitized animals after challenge with PBS (PBS and S28/PBS) or OVA (OVA and S28/OVA) with (S28/PBS and S28/OVA) or without (PBS and OVA) prior imidazoquinoline treatment. Data are presented as means $\pm$ SEM (* $p < 0.05$, *** $p < 0.001$; $n = 6$ for all groups). MIP-1 α = macrophage inflammatory protein-1 α .

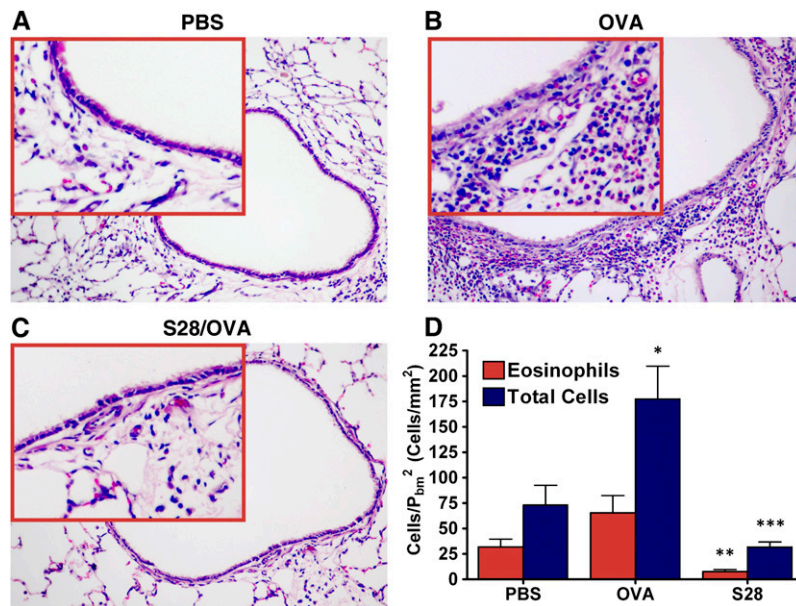


Figure 5. Representative hematoxylin and eosin-stained lung sections collected 48 hours after challenge from animals sensitized with OVA and chronically challenged with PBS (A) or chronically challenged with OVA (B and C) with prior S28463 treatment (C) or without prior S28463 treatment (A and B). Peribronchial inflammation was quantified (D) by counting the number of inflammatory cells, or eosinophils, surrounding airways and normalizing for airway size by dividing by the square of the perimeter of the basement membrane (Pbm²). Data are presented as means $\pm$ SEM (* p < 0.01 between PBS and OVA; ** p < 0.01 between OVA and S28; *** p < 0.001 between OVA and S28; n = 6 for all groups).

of an animal led to more ASM-specific α -actin staining (Figure 6B) in lung sections than could be observed in animals that were mock challenged (Figure 6A). The lungs of animals that were treated with S28463 (Figure 6C) were not visually distinguishable from the mock-challenged lung sections and showed less α -actin immunoreactivity than the OVA-sensitized and -challenged lung sections (Figure 6B).

ASM α -actin-positive cell mass was quantified as the area of ASM-specific α -actin-staining cells divided by the square of the perimeter of the basement membrane (Pbm²). Pbm² was used to correct measurements for airway size. Quantitation of ASM α -actin-positive cell mass confirmed our observations by visual inspection. OVA-sensitized and -challenged rats have almost threefold greater ASM α -actin-positive cell mass than do rats that were PBS challenged, and S28463 treatment leads to an

essentially complete abrogation of the ASM α -actin-positive cell mass increase (Figure 6D).

Goblet Cell Hyperplasia Is Prevented by S28463 Treatment

A second alteration of airway structure characteristic of the chronic remodeling changes in asthma is goblet cell hyperplasia. Goblet cells can be easily identified by the PAS method, which stains mucus glycoproteins. Only a relatively few and isolated PAS⁺ cells can be observed in the airway epithelium of OVA-sensitized and mock-challenged lungs (Figure 7A). OVA challenge leads to an easily observable increase in airway PAS⁺ cells (Figure 7B), which was inhibited in animals that were treated with S28463 (Figure 7C).

Goblet cell hyperplasia was quantified by counting the number of PAS⁺ airway epithelial cells and dividing this number by

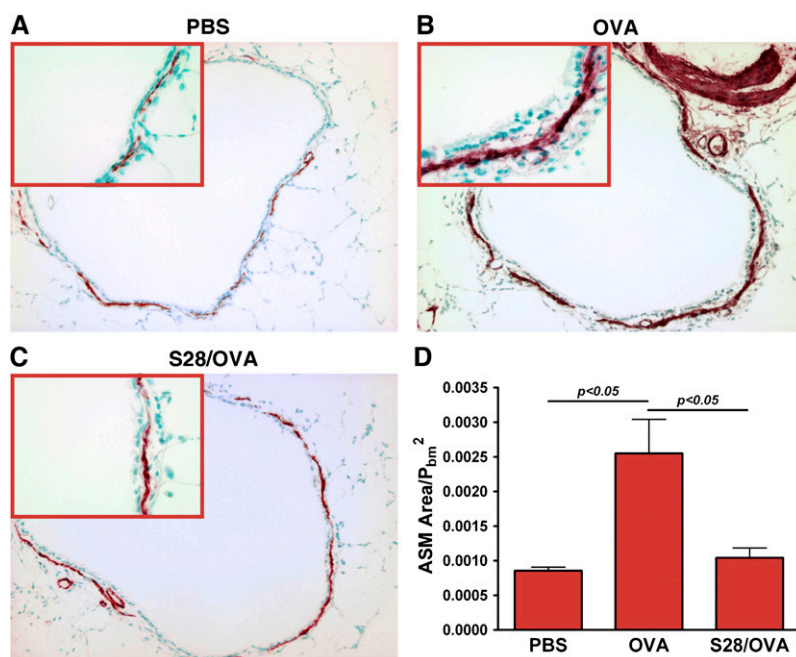


Figure 6. Representative airway smooth muscle-specific α -actin staining (red stain) of lung sections from OVA-sensitized animals chronically challenged with PBS (A), OVA (B), or OVA after S28463 treatment (C). Smooth muscle-specific α -actin-staining area was standardized for airway size by dividing by the square of the perimeter of the basement membrane of the airway (Pbm²). Data are presented as means $\pm$ SEM (n = 5 for OVA group; n = 4 for all other groups).

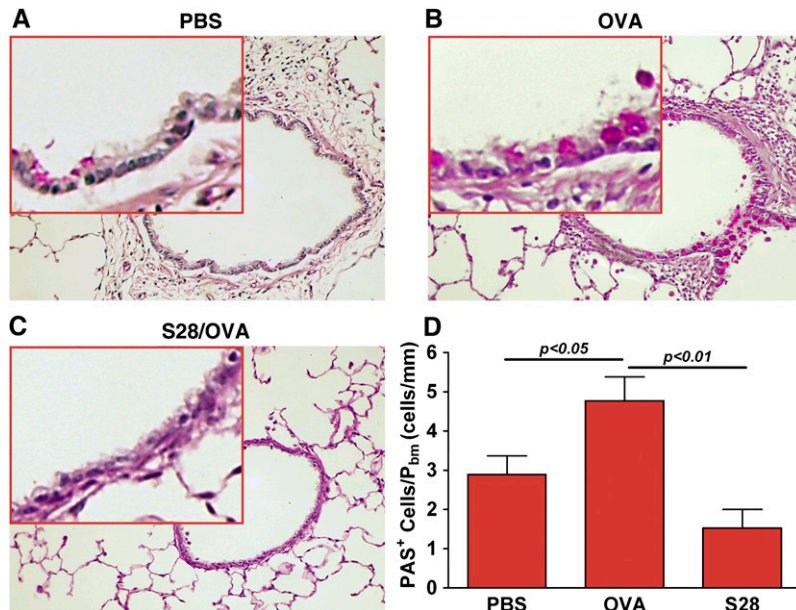


Figure 7. Representative periodic acid-Schiff (PAS) staining of lung sections from OVA-sensitized animals chronically challenged with PBS (A), OVA (B), or OVA after S28463 treatment (C). Goblet cells stain magenta in color. Goblet cell hyperplasia was determined by counting the number of PAS⁺ cells per airway and standardized for airway size by dividing by the perimeter of the basement membrane (Pbm). Data are presented as means $\pm$ SEM (n = 6 for all groups).

Pbm. Confirming the observations made by visual inspection of the slides, OVA-sensitized and -challenged animals had an elevated number of PAS⁺ airway epithelial cells compared with mock-challenged animals (Figure 7D). Again, S28463 treatment prevented the increase in PAS⁺ airway epithelial cell numbers induced by repeated allergen challenge (Figure 7D).

S28463 Treatment Prevents Proliferation of Airway Cells in Response to Repeated Allergen Challenge

One of the mechanisms believed to contribute to ASM mass increases in asthma is ASM cell proliferation (35, 36). Similarly, proliferation may account for the increase in the number of goblet cells found in the airways. We therefore evaluated the expression of PCNA in epithelial cells and in the subepithelial

cells corresponding to the smooth muscle zone to determine whether proliferation might be involved in the goblet cell hyperplasia or ASM mass increase, respectively.

The airways of PBS-challenged rats contain only a relatively low number of PCNA-positive epithelial (Figure 8A, red arrows) and subepithelial (Figure 8A) cells scattered along the airway wall. OVA challenge led to a clear increase in the number of PCNA-positive airway epithelial cells (Figure 8B, red arrows) visible in the histologic samples. Furthermore, OVA challenge led to a dramatic increase in the number of subepithelial PCNA⁺ cells that could be observed in the ASM zone (Figure 8B, blue arrow). Treatment with S28463 before allergen challenge prevents the increase in both epithelial and subepithelial PCNA⁺ cells, as can be observed by inspection of the lung sections, which

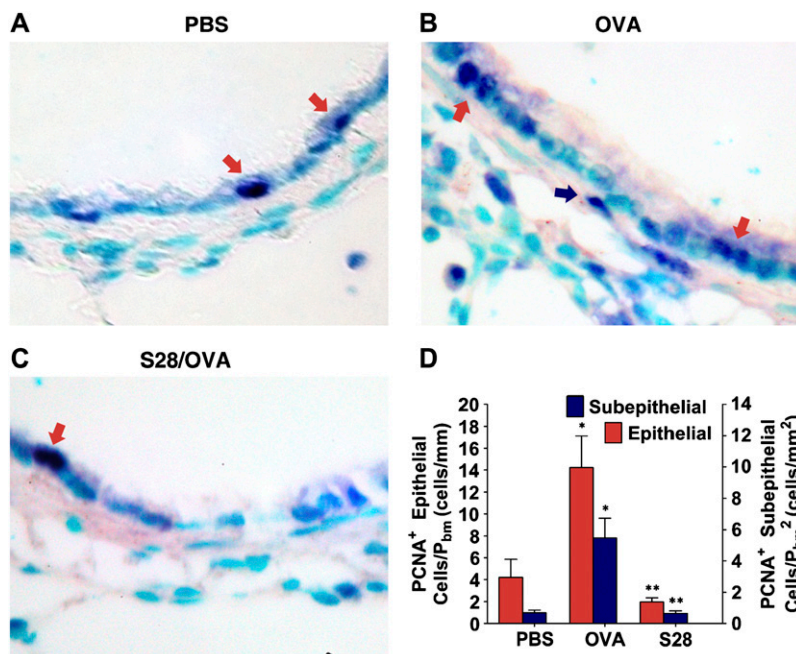


Figure 8. Representative proliferating cell nuclear antigen (PCNA)-specific stain from OVA-sensitized animals chronically challenged with PBS (A), OVA (B), or OVA after S28463 treatment (C). The nuclei of PCNA-positive cells stain dark blue. PCNA-positive epithelial cells are indicated by red arrows and PCNA-positive subepithelial cells by a blue arrow. (D) Quantification of PCNA⁺ cells in the airway epithelium and subepithelium (smooth muscle zone). PCNA⁺ cells were counted in each airway and standardized for airway size by dividing by the square of the perimeter of the basement membrane (Pbm², subepithelial cells) or the perimeter of the basement membrane (Pbm, epithelial cells). Data are presented as means $\pm$ SEM (*p < 0.01 between PBS and OVA; **p < 0.01 between OVA and S28/OVA; n = 6 for PBS, n = 4 for OVA, n = 5 for S28).

reveal relatively few isolated epithelial PCNA⁺ cells and even fewer PCNA⁺ subepithelial cells (Figure 8C).

Quantification of epithelial and subepithelial PCNA⁺ cells was accomplished by counting the number of epithelial or ASM zone subepithelial cells that were positively stained and dividing this number by Pbm and Pbm², respectively. Quantitative analysis of the histology indicates that repeated allergen challenge leads to an almost fourfold greater number of PCNA⁺ airway epithelial cells than that found in the mock-challenged control (Figure 8D). Furthermore, there was a significant eightfold increase in the number of PCNA⁺ subepithelial cells located in the ASM zone in OVA-challenged animals when compared with PBS-challenged animals (Figure 8D). Treatment with S28463 before allergen challenge completely abrogates the increase in both epithelial and subepithelial PCNA⁺ airway cells (Figure 8D).

Cytokine Levels in Lungs of Chronically Challenged Animals

As illustrated in Figure 4 and Table 1, we found a significant induction of both Th1 and Th2 cytokines in the lungs of OVA-sensitized and -challenged rats 24 hours after the first allergen challenge. Forty-eight hours after the final allergen challenge, however, we did not detect elevated levels of any cytokine (Table 1). Levels of the cytokines in rats that were chronically challenged with allergen (OVA) did not remain significantly elevated, when compared with mock-challenged animals (PBS), when assessed at the 48-hour time point. Both of these groups expressed the measured cytokines at levels comparable to those found in the lungs of mock-challenged animals 24 hours after the initial exposure.

DISCUSSION

This study was initiated to characterize the effect of treatment, with the TLR7 ligand S28463, on the early inflammatory responses to allergen challenge in a rat model of asthma. We found that, as in the mouse model, S28463 was able to effectively inhibit the inflammatory reaction in response to allergen. These encouraging findings prompted us to test whether S28463 could prevent the development of chronic airway remodeling. The results presented clearly demonstrate that continued treatment

with this TLR7 ligand could prevent the development of chronic airway remodeling.

Sensitization and challenge of Brown Norway rats resulted in significant recruitment of cells, composed mainly of eosinophils and to a lesser extent neutrophils, into the airway tissue and alveolar space. Animals that were treated with S28463 one day before allergen challenge in this acute model of allergic asthma showed greatly reduced eosinophilic inflammation, consistent with previous observations in murine models. Furthermore, we found that, during the initial phase of the allergic response to allergen exposure, total IgE levels were lowered by S28463 treatment, which is also consistent with the published results of experiments performed in murine models (24, 26). We also observed an increase in the Th2 cytokines IL-4, IL-5, and IL-13, the Th1 cytokine IFN- γ , as well as an increase in IL-6 after allergen challenge that was prevented by S28463 treatment.

Previous studies employing TLR7 ligands have demonstrated that signaling through this receptor can stimulate the production of the Th1 cytokines IL-12, IFN- α , and IFN- γ in a CD4⁺ lymphocyte and macrophage coculture (34). The cell type responsible for the bulk of the IL-12 production was determined to be the macrophage and, in addition, S28463 treatment of these cultures resulted in an inhibition of IL-5 production that was only partially mediated by IFN- α (34). More recently, studies conducted with human cells have shown that S28463 can directly induce proliferation of, and upregulate IFN- γ production in, effector memory CD4⁺ T cells (37). Neither of these mechanisms, however, would fully account for the available data.

The natural resistance-associated protein-1 (Nramp1, now SLC11A1) gene is macrophage restricted, involved in modulating macrophage responsiveness to TLR7 agonists (38), and has been shown to be necessary for the TLR7 ligand S28463-induced activation of macrophages in a murine model of *Mycobacterium bovis* BCG infection (39). In the BCG model, S28463 treatment allows wild-type Nramp1⁺ mice to clear infection with the intracellular parasite; on the other hand, Nramp1^{-/-} or Nramp1^s (susceptible) mice did not respond to S28463 treatment. Our previous results demonstrated that S28463 treatment in an acute model of allergic asthma was effective in both Nramp1⁺ and Nramp1^s mice (24). These findings suggest that prevention of

TABLE 1. CYTOKINE LEVELS IN LUNG HOMOGENATE OF SENSITIZED AND CHALLENGED RATS

	24 h after Challenge			48 h after Challenge		
	PBS	OVA	S28/OVA	PBS	OVA	S28/OVA
IL-4	0.22 $\pm$ 0.03	3.5 $\pm$ 0.7*	0.83 $\pm$ 0.30 [‡]	0.26 $\pm$ 0.09	0.20 $\pm$ 0.04	0.40 $\pm$ 0.15
IL-5	0.17 $\pm$ 0.02	14.6 $\pm$ 4.2*	0.51 $\pm$ 0.06 [‡]	0.29 $\pm$ 0.17	0.13 $\pm$ 0.05	0.09 $\pm$ 0.01
IL-6	1.3 $\pm$ 0.2	19.3 $\pm$ 4.6*	5.0 $\pm$ 0.9 [§]	2.8 $\pm$ 0.5	2.2 $\pm$ 0.6	1.79 $\pm$ 0.39
IL-13	1.8 $\pm$ 0.3	19.3 $\pm$ 4.3*	5.0 $\pm$ 0.5 [‡]	5.1 $\pm$ 0.7	3.7 $\pm$ 0.7	2.5 $\pm$ 1.0
IFN- γ	0.19 $\pm$ 0.07	6.1 $\pm$ 1.2*	2.0 $\pm$ 0.3 [‡]	0.67 $\pm$ 0.17	0.55 $\pm$ 0.09	0.61 $\pm$ 0.42
IL-1 α	41.8 $\pm$ 6.4	323 $\pm$ 70*	.141 $\pm$ 22	59.3 $\pm$ 10.8	59.4 $\pm$ 15.4	32.7 $\pm$ 4.9
IL-1 β	4.7 $\pm$ 0.6	28.6 $\pm$ 5.0*	19.0 $\pm$ 2.8	9.3 $\pm$ 1.6	9.5 $\pm$ 3.0	7.0 $\pm$ 1.6
CXCL-1	142 $\pm$ 22	301 $\pm$ 91 [†]	159 $\pm$ 9	144 $\pm$ 7	168 $\pm$ 22	128 $\pm$ 11
MIP-1 α	4.3 $\pm$ 0.2	7.8 $\pm$ 0.6*	7.0 $\pm$ 0.3	5.0 $\pm$ 0.3	5.2 $\pm$ 0.6	4.1 $\pm$ 0.3

Definition of abbreviations: MIP-1 α = macrophage inflammatory protein-1 α ; OVA = ovalbumin; PBS = phosphate-buffered saline; S28 = S28463.

Rats were OVA sensitized and challenged three times at 5-day intervals, with either OVA (OVA and S28/OVA) or PBS (PBS) with (S28/OVA) or without (PBS and OVA) prior treatment with S28463. The animals were killed 24 hours after the first challenge or 48 hours after the final challenge. Data, presented as means $\pm$ SEM, are expressed as picograms per milliliter (n = 6 for all groups).

* p < 0.001 for OVA group versus PBS group.

[†] p < 0.05 for OVA group versus PBS group.

[‡] p < 0.001 for OVA group versus S28/OVA group.

[§] p < 0.05 for OVA group versus S28/OVA group.

the acute allergic inflammation of the airways seen in asthma is not dependent on macrophage responsiveness to TLR7 ligands.

Whereas treatment with S28463 in a model of allergic asthma in the BALB/c mouse appears to result in an increase in Th1 cytokines, particularly IFN- γ (26), we have previously shown that, in both A/J and C57BL/6 mice, a similar treatment that successfully prevents the development of airway inflammation and hyperresponsiveness does not cause an increase in IFN- γ or IL-12 p70 (24). The present study indicates that treatment in the Brown Norway rat produces a similar result. In fact, in both the present study and in our previous work (24) we have found that IFN- γ is elevated after allergen challenge and that TLR7 ligand treatment prevents this induction. These findings suggest that it is unlikely that S28463 exerts its effect simply by redirecting the Th2 immune response typical of allergic asthma toward a Th1 response.

Th2 responses and activated CD4⁺ cells generated by the acute inflammatory reaction have both been implicated in the development of chronic airway remodeling (29, 40). It is therefore not surprising, but nonetheless important, that TLR7 ligand treatment can suppress the development of airway remodeling given the effectiveness with which it prevents the development of the initial inflammatory events.

Goblet cell hyperplasia may contribute to airway obstruction through excessive mucus production (41). We have demonstrated in this study that continued treatment with the TLR7 ligand S28463 prevents the development of goblet cell hyperplasia. Another important aspect of airway remodeling, which has been linked to asthma severity and is assumed to contribute to the development of airway hyperresponsiveness (2), is the increase in smooth muscle mass in asthmatic airways. Given that eosinophils have been shown to play a crucial role in the disease process leading to the marked increase in smooth muscle area (42), preventing the development of eosinophilic inflammation after allergen challenge would be expected to prevent the increase in smooth muscle mass observed in allergen-sensitized and -challenged animals. TLR7 ligand treatment behaved as expected in this respect, as we found no evidence of an increase in smooth muscle mass in S28463-treated animals.

The increase in ASM α -actin-positive cell mass can occur through hyperplasia or hypertrophy. We have observed an increase in PCNA⁺ cells in the subepithelial smooth muscle zone of OVA-sensitized and -challenged rats. We therefore believe that an increase in proliferation of ASM α -actin-positive cells is at least partially responsible for the ASM mass increase in our model. Similarly, we observed an increase in epithelial PCNA⁺ cells, which leads us to believe that the goblet cell hyperplasia we observed in chronically challenged animals is also at least partially caused by an increase in the proliferation of goblet cells or their precursors.

In summary, our data demonstrate that treatment with S28463, a TLR7/8 ligand, can prevent the development of the inflammatory reaction that results from allergen exposure in sensitized rats and that this was accomplished through inhibition of both Th1 and Th2 cytokines. Our data also demonstrated that continued treatment with S28463 can prevent the development of chronic airway remodeling, including goblet cell hyperplasia and airway smooth muscle hyperplasia. The ability of TLR7 signaling to modulate both acute and chronic asthma pathology makes this receptor a prime target for a new class of therapies aimed at minimizing the pathology associated with chronic inflammation in asthma. Furthermore, elucidation of the mechanism by which TLR7 modulates the immune response in the context of asthma should provide us with invaluable insight into the processes that lead to the development of the disease.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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Chronic Asthma Induced Airway Remodeling is Prevented by the Toll-like Receptor 7/8 Ligand S28463

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Online Data Supplement

Materials and Methods

Sensitization and Challenge for the Assessment of the Acute Inflammatory Response

Ovalbumin (OVA) sensitization was performed by concurrent subcutaneous injection of 1mg OVA (Grade V, Sigma, Oakville, Ontario, Canada) adsorbed to 100mg of aluminium hydroxide (EM Industries Inc., Hawthorne, NY) in 1mL of saline, and intra-peritoneal injection of 5×10^8 heat killed *Bordetella pertussis* bacilli (provided by Dr. Thomas Issekutz, Dalhousie University, Halifax, Nova Scotia, Canada) in 0.5mL of saline as adjuvant. Fourteen days following sensitization, the animals were challenged by aerosol exposure to either a 1% OVA solution, OVA-OVA and OVA/S28 groups, or to PBS vehicle only (OVA-PBS and OVA-S28/PBS groups), for 30 minutes. Twenty-four hours before the antigen challenge, two groups of rats, OVA-S28/OVA and OVA-S28/PBS, were treated by an intra-peritoneal injection of 1mg of S28463 (S28463 is generously provided by Dr. Richard Miller; 3M Pharmaceutical, Saint-Paul, MN). All animals were sacrificed 24 hours after antigen challenge, $n = 6$ for all groups, pooled from three separate experiments.

Sensitization and Challenge for the Assessment of Chronic Airway Remodeling

OVA sensitization was induced as described above. Thereafter, three antigen challenges were performed, at 5 day intervals and beginning on the 14th day following sensitization, by intra-tracheal instillation of a 5% solution of OVA ($n = 6$) in saline or with saline vehicle only ($n = 5$). One group of rats ($n = 6$) received 1mg of S28463 24

hours prior to each antigen challenge and all animals were sacrificed 48 hours after the final antigen challenge. Results are pooled from two separate experiments.

Bronchoalveolar Lavage (BAL) Fluid and Cellular Analysis

Upon sacrifice, 24 hours following the final allergen challenge, 25mL of saline was instilled into the lungs of the animals in 5 equal portions of 5mL. The total cell counts were determined by use of a hemacytometer. Three hundred cells were identified on each BAL cytopsin slide using the Diff-Quik Stain Set (Dade Behring Inc., Newark, DE).

Plasma IgE

Upon sacrifice of the animals, 24 hours following the final challenge, blood was collected by cardiac puncture and clotting was prevented by the addition of EDTA. Plasma was recovered after centrifugation of the blood samples and the total IgE levels were measured by ELISA. The ELISA was performed on Immulon 2 HB 96-well plates (Fisher, Ottawa, ON) coated with mouse anti-rat IgE monoclonal antibodies (BD Biosciences, Mississauga, ON). Rat IgE protein was detected with biotin-conjugated mouse anti-rat IgE monoclonal antibodies (BD Biosciences, Mississauga, ON) and streptavidin-HRP (R&D Systems, Minneapolis, MN). Rat IgE monoclonal isotype standard (BD Biosciences, Mississauga, ON) was used as a standard.

Cytokine Analysis

Upon sacrifice of the animals, 24 hours following the final challenge for the acute model and 48 hours following the final challenge for the chronic model, the right lung was dissected, frozen, and stored at -80°C. A single lobe from each sample was then

homogenized in 4mL of PBS containing a protease inhibitor cocktail (Complete Inhibitor, Roche Diagnostics, Laval, PQ, Canada) and the total protein concentrations were adjusted using the Bio-Rad protein assay dye (Bio-Rad Laboratories, Hercules, CA). Samples were concentrated 20-fold with Microcon YM-3 centrifugal filters (Millipore, Billerica, MA) before analysis using a custom Lincoplex rat cytokine kit (Linco Research, St. Charles, MO) on a Luminex 100 LS apparatus with software version 2.3 (Luminex Corporation, Austin, TX). The cytokine protein levels reported refer to the cytokine levels of the samples in the lung homogenates as they were originally prepared, and not the 20-fold concentrated homogenates as the samples were assayed.

Preparation of Histological Samples

For preparation of the histological sections, the left lung was dissected and fixed with 10% formalin overnight with a pressure of 25cmH₂O. The paraffin embedded tissues were cut into 5 µm sections for histological analysis, deparaffinized, hydrated, and then stained with a standard hematoxylin and eosin (H&E) procedure.

Measurement of Chronic Inflammation

Chronic inflammation was assessed by histological analysis of haematoxylin and eosin stained lung sections prepared as described above. Quantification of the inflammation present was accomplished by counting the number of cells in the peribronchial space surrounding each airway and standardizing for airway size by dividing the count by the square of the perimeter of the basement membrane (P_{bm}^2). Eosinophils were identified on the basis of nuclear morphology and the presence of eosin staining in the cytoplasm.

Measurement Airway Smooth Muscle (ASM) Mass

The mass of ASM was estimated from the measurement of the area of smooth muscle specific α -actin (α -SMA) immunoreactivity as previously described. Briefly, sections, as described above, were stained with a mouse monoclonal antibody to α -SMA (clone 1A4) (Sigma-Aldrich) and a biotinylated horse anti-mouse IgG (Vector Laboratories). The signal was detected with Vector Red (Vector Laboratories). The area of ASM was traced using a camera lucida side arm attachment to the microscope and digitized^{E1}. The mass of ASM was standardized for airway size by dividing the area of ASM by the square of the perimeter of the basement membrane (P_{bm}^2).

Goblet Cell Hyperplasia

Goblet cell hyperplasia was assessed by histological analysis of periodic acid-Schiff (PAS) stained sections of lungs prepared as described above. Goblet cell hyperplasia quantification was accomplished by counting the number of PAS⁺ cells, and standardizing for airway size by dividing the count by the perimeter of the basement membrane (P_{bm}), for at least five different airway cross-sections per animal.

Proliferating Airway Epithelial Cell and Sub epithelial Cell Quantification

Proliferation of airway epithelial and sub epithelial cells was investigated using immunostaining for proliferating cell nuclear antigen (PCNA) using a monoclonal antibody to PCNA (Calbiochem). Staining was preceded by high-temperature epitope unmasking in antigen retrieval solution (Vector Laboratories) and permeabilization in 0.2% Triton X-100 (Sigma-Aldrich). Sections were then blocked with 20% horse serum (Vector Laboratories) in universal blocking solution (Dako Cytomation), and a

monoclonal antibody to PCNA was detected with biotinylated horse anti-mouse IgG, avidin/biotin-alkaline phosphatase complex, and BCIP/NBT chromogen substrate (Vector Laboratories), followed by methyl green (Sigma-Aldrich) counterstain. The number of PCNA positive airway epithelial and sub epithelial cells was counted and corrected for airway size by dividing by P_{bm} and P_{bm}^2 respectively.

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