

Goblet Cell Rebound and Airway Dysfunction with Corticosteroid Withdrawal in a Mouse Model of Asthma

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Rationale: Although corticosteroids are highly effective at preventing allergen-induced increases in goblet cell numbers, we observed in unpublished experiments a rebound increase in goblet cell numbers in mice after the simultaneous withdrawal of corticosteroid and cessation of exposure to allergen that reached levels greater than those observed in mice exposed to allergen alone, without corticosteroid treatment.

Objectives: To formally explore the goblet cell hyperplasia rebound observed after corticosteroid withdrawal in allergen-exposed mice to determine the mechanism responsible for this previously undescribed pathology.

Methods: Mice airways were assessed for mucin-containing goblet cells after exposure to varying durations of allergen and corticosteroid.

Measurements and Main Results: We confirmed that the simultaneous withdrawal of corticosteroid and cessation of exposure to allergen resulted in a goblet cell hyperplasia rebound that reached levels greater than those observed in allergen-exposed corticosteroid naive mice. Importantly, the goblet cell rebound was associated with a significant airway dysfunction greater than that observed in allergen-exposed corticosteroid naive mice. The goblet cell hyperplasia rebound is independent of the type of corticosteroid or allergen and was associated with an increased level of bronchoalveolar lavage IL-13. Inhibition of IL-13, but not CD4⁺ T cells, completely inhibited the goblet cell hyperplasia rebound and, critically, the associated airway dysfunction.

Conclusions: These findings suggest that certain corticosteroid treatment regimes may actually potentiate airway remodeling and dysfunction in patients with asthma and lead to increased exacerbations and worsening of asthma symptoms.

Keywords: asthma; goblet cells; airway resistance; pathophysiology; glucocorticoids

Corticosteroids (CS) are the most effective treatment currently available for the management of asthma. In addition to reducing asthma symptoms (1, 2), CS reduce the risk of asthma exacerbations (3, 4), improve the quality of life (5), and reduce asthma-associated fatalities (6). Evidence suggests that the earlier inhaled CS are introduced in the course of asthma

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

Corticosteroid therapy is generally highly effective in human and experimental asthma, but treatment-related complications are incompletely understood.

What This Study Adds to the Field

We observed a goblet cell hyperplasia rebound pathology associated with airway dysfunction after the simultaneous withdrawal of prolonged corticosteroid therapy and chronic exposure to allergen in mice. Our findings suggest that certain corticosteroid treatment regimes may actually potentiate airway remodeling and dysfunction in patients with asthma and lead to increased exacerbations and worsening of asthma symptoms.

treatment, the greater the degree of improvement in lung function (7–9).

Airway goblet cell hyperplasia has been established as a pathologic characteristic of asthma, irrespective of disease severity. Mucus hypersecretion by hyperplastic goblet cells in airways contributes to the pathophysiology of asthma (10) and significantly contributes to patient morbidity and mortality (11). Indeed, the airways of patients with severe asthma may have greater than a 30-fold increase in mucus compared with people who do not have asthma (12). Although CS are highly effective at preventing allergen-induced increases in airway goblet cell numbers, we observed, in unpublished experiments, that the simultaneous withdrawal of CS and cessation of exposure to allergen in mice results in a rebound increase in goblet cell numbers, reaching levels greater than those observed in mice exposed to allergen alone without any CS treatment.

Although evidence suggests that the earlier inhaled CS are introduced in the course of asthma, the greater the degree of improvement in lung function, CS have been suggested to account for a major component of airway epithelial damage that is characteristically seen in patients with asthma (2, 13). Additionally, it has recently been reported that the withdrawal of CS in preschool children at high risk for asthma resulted in worsening of asthma-like symptoms and a more rapid decline in the proportion of episode-free days compared with untreated children (14).

Considering those findings, we hypothesized *a priori* that the withdrawal of CS therapy may actually potentiate airway disease and contribute to worsening of asthma control. We therefore sought to explore the goblet cell hyperplasia rebound observed after CS withdrawal in allergen-exposed mice to determine the mechanisms responsible for this previously undescribed pathology. We report here that the goblet cell hyperplasia rebound

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is associated with significant airway dysfunction. The goblet cell rebound is a delayed pathology, independent of the type of CS or allergen, and is associated with IL-13. Inhibition of IL-13 completely inhibits the goblet cell rebound, whereas inhibition of CD4⁺ T cells, thought to be a principal source of IL-13 in allergic airway disease, is only partially effective. These findings suggest that withdrawal of CS treatment during times of allergen avoidance may actually augment airway remodeling and airway dysfunction, contributing to worsened control of airway disease.

METHODS

Animals

Female BALB/c mice, 8 to 10 weeks of age, were purchased from Charles River Laboratories (Saint-Constant, PQ, Canada). Mice were housed using Biobubble technology in environmentally controlled specific pathogen-free conditions for the duration of the study. All procedures were reviewed and approved by the Animal Research Ethics Board at McMaster University (Hamilton, ON, Canada).

Study Design

Overall, the current study was made up of eight sequential studies, each exploring the goblet cell hyperplasia rebound, based on findings from the previous studies (Figure 1).

Allergen Exposure

Ovalbumin Exposure. Mice ($n = 8$ –12/group in studies 1, 5, 6, 7, and 8; $n = 5$ /group in study 2) were sensitized and challenged with ovalbumin (OVA) as described previously (see the online supplement for details) (15, 16). Control mice were subjected to the same sensitization protocol but received saline challenges. Naive mice ($n = 5$ /group in study 3) were not exposed to allergen. Mice were studied at 24 hours and 4 weeks after final exposure to OVA.

House Dust Mite Exposure. Mice ($n = 5$ /group in study 4) were sensitized to house dust mite (HDM) *Dermatophagoides pteronyssinus* extract (Greer Laboratories, Lenoir, NC) as described previously (see online supplement) (17). Chronic exposure to HDM was performed as with OVA. Briefly, mice were exposed to repeated intranasal HDM challenges 5 days per week for 2 weeks and then, beginning on Day 19/20, were subjected to 6 additional periods of HDM exposure, each lasting for 2 days and spread over a 10-week period. Mice were studied at 24 hours and again at 4 weeks after final exposure to HDM.

Corticosteroid Intervention

Mice were injected intraperitoneally with 200 μ l of CS (budesonide [3 mg/kg], fluticasone [2 mg/kg]) once a day at approximately 30 minutes before allergen challenge. CS treatment consisted of every other day intervention, concurrent with exposure to allergen, beginning on Day 19 for the duration of allergen exposure protocol, and was discontinued after the final exposure to allergen. CS was suspended in a vehicle of 1% carboxymethylcellulose solution after 10 minutes of water bath sonication (VWR International model 150D, Westchester, PA). Control mice were subjected to the same treatment protocol but received vehicle intervention.

Antibody Interventions

Immunodepletion studies were completed using a rat anti-mouse IL-13 mAb (CNTO 134, IgG2a isotype; Centocor Inc., Radnor, PA) and a rat anti-mouse CD4 mAb (GK1.5, IgG2b isotype; ATCC, Manassas, VA). CD4 mAb was generated using conventional immunization protocols and hybridoma technology as previously described (18, 19). Anti-IL-13 (500 μ l of 1 mg/ml) and anti-CD4 (500 μ l of 1 mg/ml) interventions were administered intraperitoneally on Days 1, 4, 7, 14, and 21 (Figure 1). Control mice were similarly treated with a control IgG mAb.

Airway Responsiveness

Airway responsiveness was measured using the forced oscillation technique (see online supplement). Briefly, animals were ventilated quasi-sinusoidally (150 breaths/min, 10 ml/kg, inflation/deflation ratio

of 2/3) with a peak-end expiratory pressure set at approximately 2 cm H₂O. Mice were intravenously injected sequentially with saline and 10, 33, 100, and 330 mg/kg of methacholine (MCh). During a 45-second period immediately after each injection, mice were subjected to 13 single-sinusoidal inflations and deflations of 0.4 second with a volume of 10 ml/kg (QuickSnap-150; SCIREQ Inc., Montreal, Canada) followed by a 6-second inflation to total lung capacity. Total respiratory system resistance (R_{RS}) was calculated using linear least squares regression analysis of simultaneous recordings of airway pressure, airflow, and lung volume change, based on a linear single-compartment model. Maximal R_{RS} was calculated as the maximal MCh-induced R_{RS} .

Bronchoalveolar Lavage

Bronchoalveolar lavage was performed as described previously (see the online supplement for details) (15).

Cytokine Analysis

Bronchoalveolar lavage fluid was assessed for IL-4, IL-5, IL-9, IL-13, IL-1 β , IFN- γ , and tumor necrosis factor (TNF)- α using a custom Bio-Plex mouse cytokine multiplex panel (Bio-Rad Laboratories Canada, Mississauga, ON, Canada), according to manufacturer instructions.

Lung Histology and Morphometry

Lungs were dissected and processed as described previously (see the online supplement) (15, 20). Lung tissue sections were stained with periodic acid-Schiff to quantify mucin containing goblet cells. Morphometric quantification of stained sections was performed as described previously (15, 20).

Flow Cytometry Analysis

Flow cytometry analysis on cells extracted from lung tissue by enzymatic digestion (21) was performed as described previously (see the online supplement for details) (18). Flow cytometry confirmed that the depletion of CD4⁺ T cells was greater than 99%, as previously described (see Figure E1 in the online supplement) (18).

Statistical Analysis

Reported values are expressed as means and SEMs. Data from saline control groups were combined as there were no significant differences between groups in any assessed measurement. Data analysis was performed for planned comparisons using two-tailed, unpaired Student's t test. To minimize the probability of type I errors, α was adjusted below 0.05 using Bonferroni correction, based on the number of comparisons. Data analysis was performed using *Statistica* software (StatSoft, Tulsa, OK).

RESULTS

Concurrent Cessation of Persistent Corticosteroid Intervention and Chronic Exposure to Allergen Results in a Goblet Cell Hyperplasia Rebound

To confirm that simultaneous cessation of both chronic CS treatment and allergen exposure results in a goblet cell hyperplasia, lung sections from mice chronically exposed to OVA and concurrently treated with budesonide were histochemically stained with periodic acid-Schiff to detect mucin containing goblet cells. When assessed immediately after final exposure to allergen in untreated mice (CS naive), there was a significant goblet cell hyperplasia compared with saline control mice (85.7 [7.9] vs. 0.0 [0.0]). This goblet cell hyperplasia persisted, albeit to a decreasing degree, for at least 4 weeks after final exposure to OVA (21.5 [6.2]; $P < 0.0001$) (Figure 2A, 2B, 2D). Treatment with budesonide throughout chronic exposure to OVA significantly attenuated the development of the allergen-induced goblet cell hyperplasia (18.1 [3.2] vs. 85.7 [7.9]) (Figure 1A, 1B, 1D). Although goblet cells were still elevated in budesonide-treated mice compared with unchallenged mice, prolonged CS

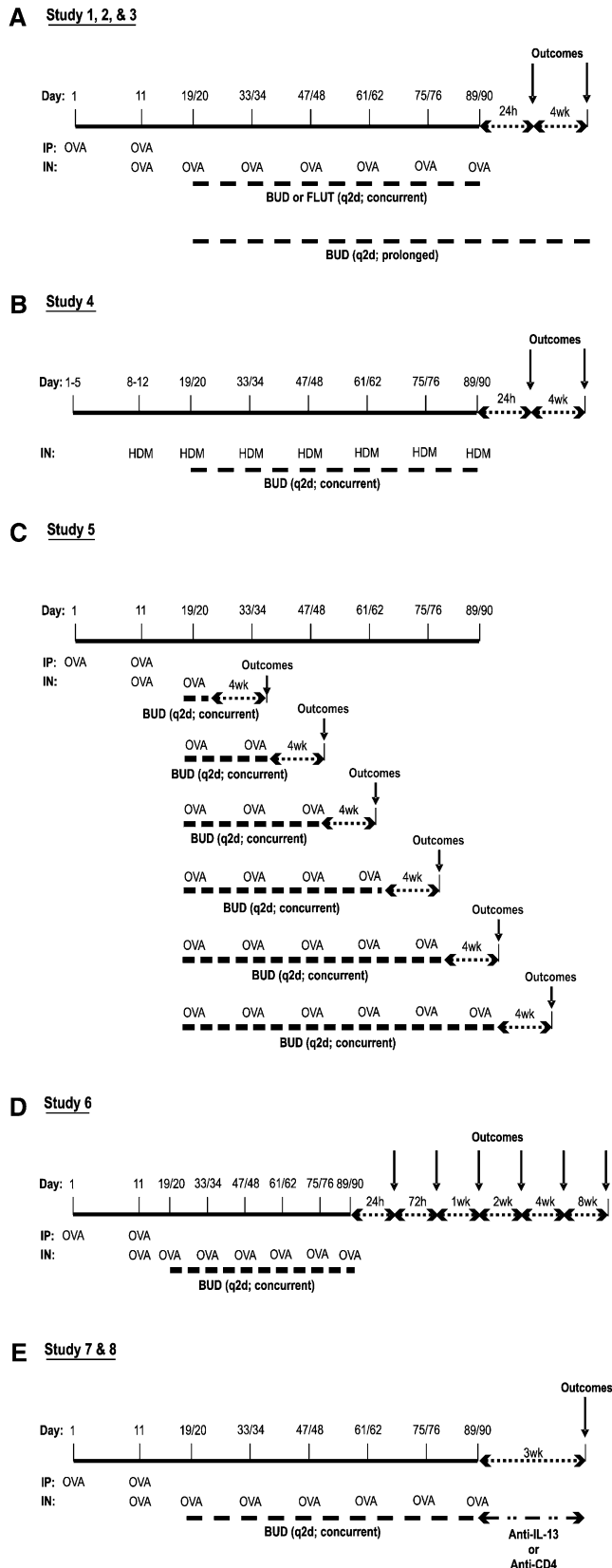


Figure 1. Study protocols for the eight separate studies performed. *Study 1.* According to protocol A, intraperitoneal (IP) sensitization with ovalbumin (OVA) was followed by chronic intranasal (IN) challenges with OVA for 13 weeks (11 d sensitization, 10 d rest + 10 wk challenge) as indicated, while negative control mice received saline. IP treatment with budesonide (BUD) (or vehicle for positive and negative control mice) occurred as indicated once every other day (q2d). Outcome measurements were made at either 24 hours or 4 weeks after final chronic exposure to OVA. *Study 2.* Was the same as protocol A, and identical to Study 1 but with fluticasone (FLUT). *Study 3.* Naive mice received the q2d BUD treatment for the durations shown in protocol A, but with no exposures. *Study 4.* According to protocol B, IN exposure to house dust mite (HDM) occurred as illustrated, while negative control mice received saline. IP treatment with BUD [or vehicle for positive and negative control mice] occurred as indicated q2d. Outcomes were assessed at either 24 hours or 4 weeks after final chronic exposure to HDM. *Study 5.* According to protocol C, sensitized mice received 2 days or 2, 4, 6, 8, or 10 weeks of IN OVA challenges and concurrent IP treatment with BUD. Outcomes were assessed 4 weeks after final exposure to OVA and treatment with BUD. *Study 6.* According to protocol D, outcome measurements were made at 24 and 72 hours and 1, 2, 3, 4, or 8 weeks after final exposure to OVA and treatment with BUD. *Study 7.* According to protocol E, mice having been exposed to OVA and simultaneously treated with BUD were treated with an anti-IL-13 monoclonal antibody on Days 1, 4, 7, 14, and 21 of a 3-week recovery period. Control mice were similarly treated with an IgG control antibody. *Study 8.* According to protocol E, mice were treated with an anti-CD4 antibody after exposure to OVA and treatment with BUD.

numbers (50.0 [4.9] vs. 18.1 [3.2]) (Figure 2A, 2B, 2D). This resulted in goblet cell numbers that were significantly greater than the level observed in never-treated mice assessed 4 weeks after chronic exposure to OVA alone (50.0 [4.9] vs. 21.5 [6.2]) (Figure 2A, 2B, 2D). If CS intervention offered only therapeutic benefits to the prevention of goblet cell hyperplasia, one would expect to see goblet cell numbers less than those observed with exposure to allergen alone. Therefore, this novel observation of rebound increases in goblet cells suggests that some CS treatment strategies may actually augment airway pathology.

Goblet Cell Hyperplasia Rebound Is Associated with Augmented Airway Dysfunction

Airway goblet cell hyperplasia and mucus overproduction are prominent features in respiratory disease that have important physiological consequences (10, 22). To investigate the potential functional consequences of the goblet cell rebound, we assessed airway responses to methacholine challenges. When assessed immediately after final exposure to allergen in untreated mice (CS naive), there was a significantly greater methacholine-induced maximum airway resistance compared with saline control (8.2 [1.7] vs. 3.7 [0.3]). This airway dysfunction persisted, albeit to a decreasing degree, for at least 4 weeks after final exposure to OVA (5.5 [0.8] vs. 3.7 [0.3]) (Figure 2A, 2B, 2D). Treatment with budesonide throughout chronic exposure to OVA significantly attenuated the development of the airway dysfunction (3.6 [0.4] vs. 8.2 [1.7]) (Figure 2A, 2B, 2D). Furthermore, we show that prolonged CS treatment beyond the cessation of exposure to allergen, prevents the development of airway dysfunction (3.4 [0.3]) (Figure 2D). Interestingly, however, when assessed 4 weeks after simultaneous cessation of both budesonide treatment and chronic exposure to OVA, in association with the goblet cell rebound, we observed an increased methacholine-induced maximum airway resistance when compared with mice assessed immediately after cessation of both budesonide intervention and chronic exposure to OVA (5.3 [0.8] vs. 3.7 [0.3]) (Figure

treatment beyond the cessation of exposure to allergen results in the complete resolution of goblet cell hyperplasia (Figure 2D). Interestingly, however, when assessed 4 weeks after the simultaneous cessation of both exposure to OVA and treatment with budesonide, there was a significant increase in goblet cell

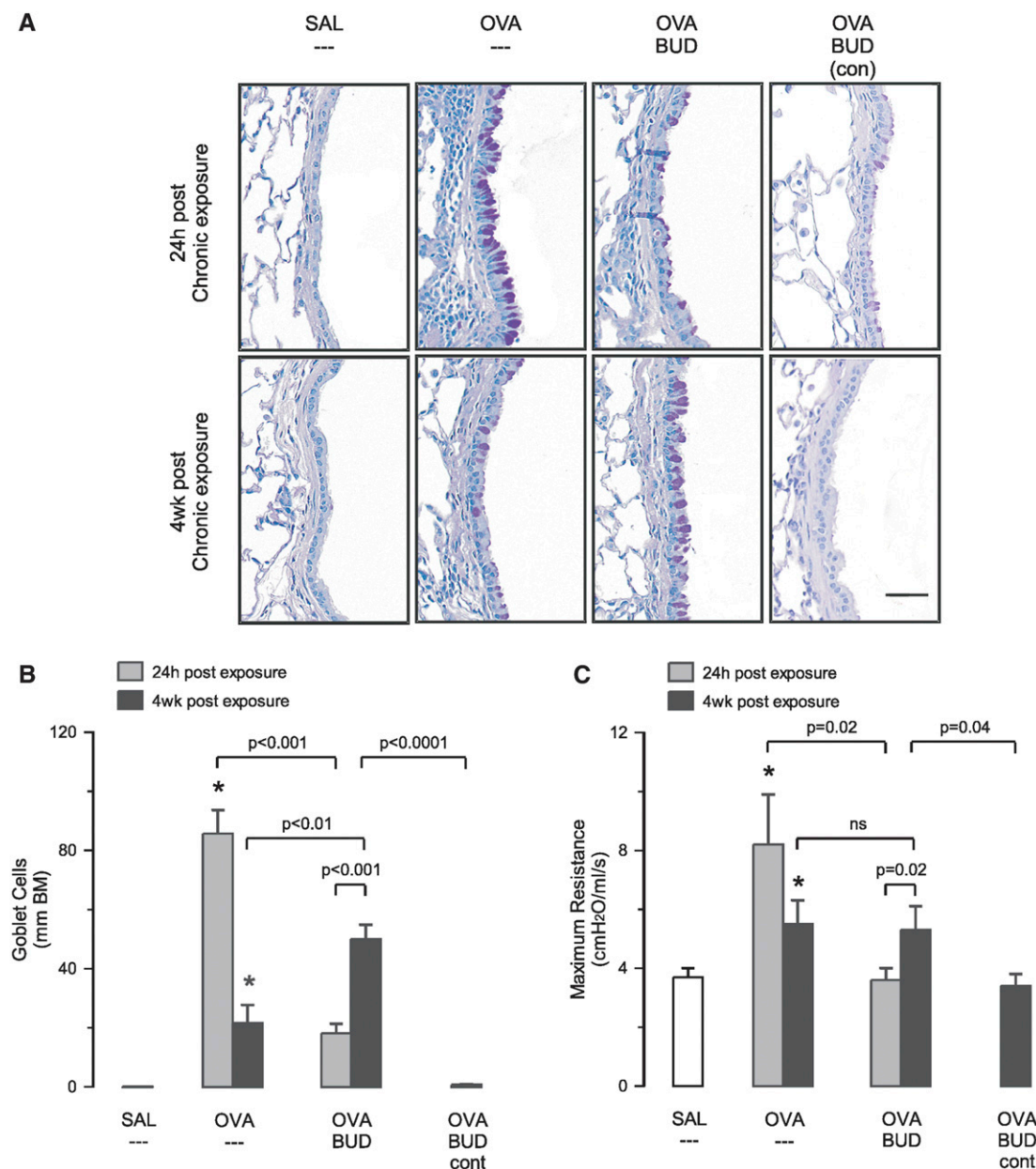


Figure 2. Simultaneous cessation of persistent glucocorticosteroid (CS) treatment and exposure to allergen contributes to a goblet cell hyperplasia rebound pathology associated with airway dysfunction. (A) Representative images of airway wall stained with periodic acid Schiff to quantify mucin containing goblet cells. Histological sections were assessed at 24 hours or 4 weeks after chronic saline (SAL), ovalbumin (OVA), OVA + budesonide (BUD), or OVA + BUD continuous (con) treatment. Bar = 50 μ m. Magnification 40 $\times$. (B) Morphometric quantification of airway mucin containing goblet cells expressed per millimeter of basement membrane (BM). Sections were assessed at 24 hours or 4 weeks after chronic SAL, OVA, OVA + BUD, or OVA + BUD con. * P < 0.05 compared with SAL. Values are means (SEM), n = 8–12/group. (C) Airway dysfunction represented by maximum methacholine-induced airway resistance assessed 24 hours or 4 weeks after chronic SAL, OVA, OVA + BUD, or OVA + BUD con. * P < 0.05 compared with SAL. Values are means (SEM), n = 8–12/group.

2C, 2D). These findings suggest that the goblet cell rebound contributes to airway dysfunction that clinically, may correlate with increased exacerbations and worsening of asthma symptoms.

Goblet Cell Hyperplasia Rebound Is Independent of the Type of Corticosteroid or Allergen

To determine if the goblet cell rebound pathology occurs after exposure to other CS, we repeated the study with fluticasone. As with budesonide intervention, treatment with fluticasone throughout chronic exposure to OVA significantly attenuated the development of allergen-induced goblet cell hyperplasia (45.3 [4.3] vs. 85.7 [7.9]) (Figure 3A). When assessed 4 weeks after simultaneous cessation of both exposure to OVA and treatment with fluticasone, however, there was a significant increase in goblet cell numbers compared with mice never exposed to OVA alone (62.2 [12.3] vs. 21.5 [6.2]) (Figure 3A).

Subsequently, to examine if a goblet cell rebound occurs with other allergens more closely associated with human asthma, we repeated the study with house dust mite (HDM) exposure. Chronic exposure to HDM resulted in a significant goblet cell

hyperplasia that persisted for at least 4 weeks after final exposure (79.6 [12.5] vs. 0.0 [0.0]) (Figure 3B). As expected, treatment with budesonide throughout chronic exposure to HDM significantly attenuated the development of the allergen-induced goblet cell hyperplasia (42.5 [18.2] vs. 79.6 [12.5]) (Figure 3B). When assessed 4 weeks after simultaneous cessation of both exposure to HDM and treatment with budesonide, however, there was a significantly greater number of goblet cells than in mice exposed to HDM alone (62.0 [13.6] vs. 25.8 [5.9]) (Figure 3B). These results suggest that the goblet cell rebound is a robust and consistent pathology that appears to be independent of the type of CS or allergen.

Goblet Cell Hyperplasia Rebound Is a Late Developing Transient Pathology Occurring in Response to Prolonged Corticosteroid Therapy

To address whether exposure to allergen was required for goblet cell rebound to occur, we exposed allergen-naïve mice to the same budesonide intervention protocol (Study 3 in Figure 1). These mice, however, did not develop goblet cell hyperplasia

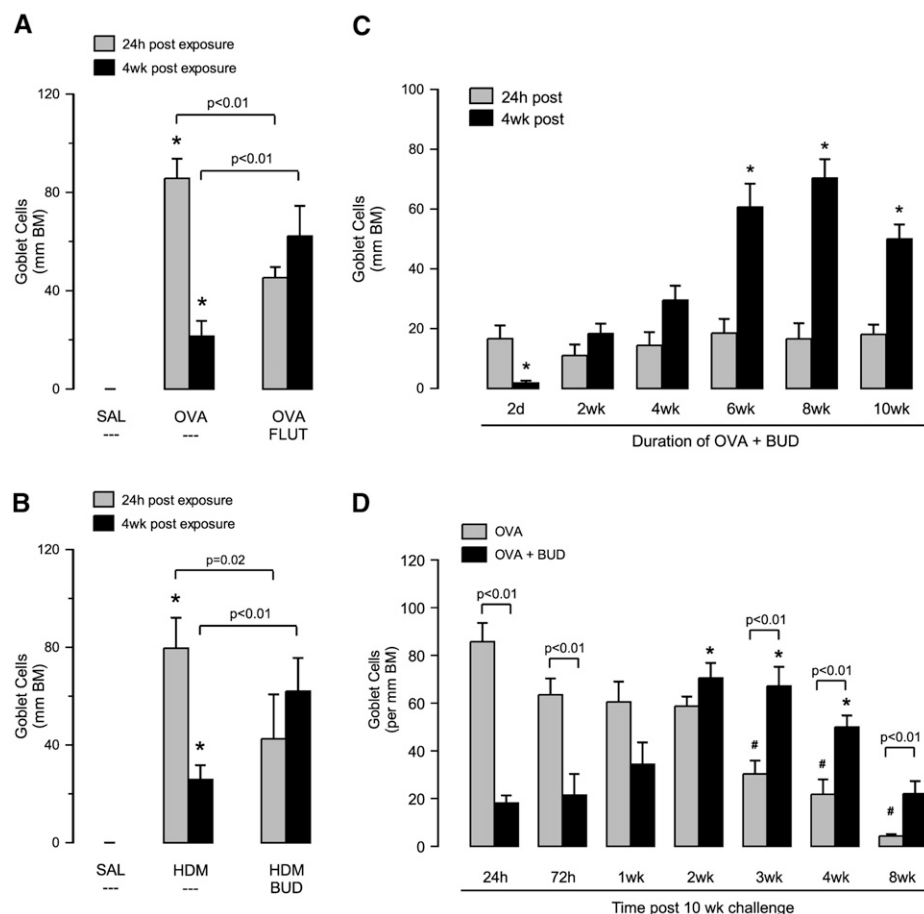


Figure 3. The goblet cell hyperplasia rebound is a late-developing pathology requiring prolonged glucocorticosteroid (CS) treatment and exposure to allergen, independent of CS or allergen type. (A) Morphometric quantification of airway mucin containing goblet cells expressed per millimeter of basement membrane (BM). Sections were assessed at 24 hours or 4 weeks after chronic saline (SAL), ovalbumin (OVA), or OVA + fluticasone (FLUT). * $P < 0.05$ compared with SAL. Values are means (SEM), $n = 5$ /group. (B) Morphometric quantification of airway mucin containing goblet cells expressed per mm basement membrane (BM). Sections were assessed at 24 hours or 4 weeks after chronic SAL, house dust mite (HDM), or HDM + budesonide (BUD). * $P < 0.05$ compared with SAL. Values are means (SEM), $n = 5$ /group. (C) Morphometric quantification of airway mucin containing goblet cells expressed per millimeter of BM. Sections were assessed at 24 hours or 4 weeks after 2 days and 2, 4, 6, 8, or 10 weeks after OVA + BUD. Values are means (SEM), $n = 8$ –12/group. (D) Morphometric quantification of airway mucin containing goblet cells expressed per millimeter of BM. Sections were assessed at 24, 72 hours, 1, 2, 3, 4, or 8 weeks after 10 weeks chronic OVA or OVA + BUD. * $P < 0.001$ compared with 24 hours post-OVA + BUD; # $P < 0.001$ compared with 24 hours post-OVA. Values are means (SEM), $n = 8$ –12/group.

(data not shown). Therefore, to determine if the goblet cell hyperplasia rebound pathology is attributed to prolonged exposure to allergen and CS treatment, mice were concurrently exposed to increasing durations of OVA and budesonide. Interestingly, only mice simultaneously exposed to allergen and CS treatment for greater than 4 weeks exhibited a goblet cell rebound (Figure 3C). We also investigated the time course of the goblet cell hyperplasia after cessation of both chronic exposure to OVA and budesonide treatment. Prolonged intervention with budesonide concurrent with chronic exposure to OVA resulted in a increase in goblet cell numbers (Figure 3D). The greatest rebound in goblet cell numbers occurred 3 weeks after cessation of chronic exposure to allergen (67.4 [8.1] vs. 30.3 [5.6]), after which time the numbers began to decline (Figure 3D). These findings indicate that the goblet cell rebound is a late-developing transient pathology that occurs in response to relatively prolonged CS therapy.

Goblet Cell Hyperplasia Rebound Is Associated with IL-13

To explore the potential mechanisms responsible for the goblet cell rebound, bronchoalveolar lavage was assessed for cytology and cytokine profiles. The goblet cell rebound was not associated with a distinctive inflammatory cell profile that differentiated CS treated from untreated mice (*see* Table E1 in the online supplement). IL-13, a classical T-helper type 2 (T_H2) cytokine, is known to regulate goblet cell hyperplasia and mucus production (23). When assessed immediately after final exposure to allergen, chronic exposure to OVA resulted in a significant increase in IL-13 (39.0 [13.2] vs. 6.3 [2.8]) (Figure 4A). Although the concentration of IL-13 declined, it was still elevated above baseline 4 weeks after final exposure to OVA. Intervention

with budesonide throughout chronic exposure to OVA significantly prevented the increase in bronchoalveolar lavage IL-13 levels, measured immediately after the exposure to allergen and CS treatment (6.8 [1.3] vs. 39.0 [13.2]) (Figure 4A). When assessed 4 weeks after simultaneous cessation of chronic exposure to OVA and treatment with budesonide, however, there was a significant increase in IL-13, occurring at the same time as the observed rebound increase in goblet cells compared with mice observed 4 weeks after chronic exposure to OVA alone (47.8 [6.7] vs. 26.7 [3.0]) (Figures 4A, 1A, 1B, and 1D). Additionally, we measured IL-4, 5, 9, and TNF- α , which have also been implicated in the development of goblet cell hyperplasia (10, 23, 24). The concentrations of these cytokines, however, were below levels of detection (IL-4, 5, IFN- γ , and TNF- α) or not different (IL-9) between CS treated and untreated mice (data not shown). These findings suggest a mechanistic relationship between IL-13 and the goblet cell rebound.

Inhibition of IL-13 Prevented the Development of the Goblet Cell Hyperplasia Rebound and Associated Airway Dysfunction

To explore a potential causal relationship between IL-13 and the goblet cell rebound, we treated mice with an anti-IL-13 mAb for 3 weeks after the simultaneous cessation of budesonide intervention and exposure to allergen. Inhibition of IL-13 completely prevented the development of the goblet cell rebound (1.1 [0.5] vs. 73.7 [5.6]) (Figures 4B and 4C) and, importantly, prevented the associated airway dysfunction (3.2 [0.3] vs. 5.4 [0.9]) (Figure 4D). These findings further strengthen the association between this pathology and the associated

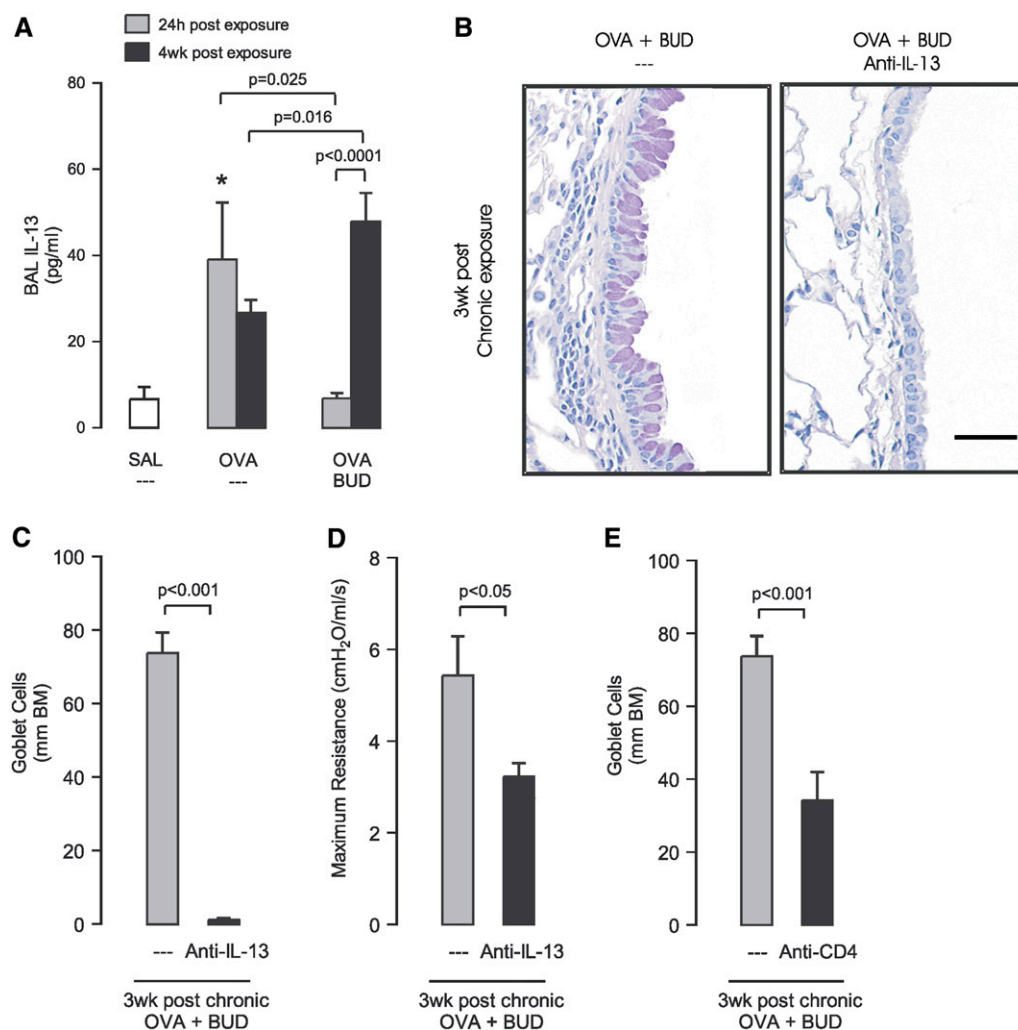


Figure 4. The goblet cell hyperplasia rebound and associated airway dysfunction after simultaneous cessation of persistent glucocorticosteroid intervention and exposure to allergen is IL-13 dependent. (A) Bronchoalveolar lavage (BAL) IL-13 levels assessed 24 hours or 4 weeks after chronic saline (SAL), ovalbumin (OVA), or OVA + budesonide (BUD). * $P < 0.05$ compared with SAL. Dashed line represents lower limit of detection. Values are means (SEM), $n = 5-6$ /group. (B) Representative images of airway wall stained with periodic acid-Schiff to quantify mucin containing goblet cells. Histological sections were assessed 3 weeks after chronic OVA + BUD. During the aforementioned 3 weeks of recovery, mice were treated with IgG control or anti-IL-13 intervention. Bar = 50 μ m. Magnification 40 $\times$. (C) Morphometric quantification of airway mucin containing goblet cells expressed per millimeter of basement membrane (BM). Sections were assessed 3 weeks after chronic OVA + BUD. During the aforementioned 3 weeks of recovery, mice were treated with IgG control or anti-IL-13 intervention. Values are means (SEM), $n = 8-12$ /group. (D) Airway dysfunction represented by maximum methacholine-induced airway resistance assessed 3 weeks after chronic OVA + BUD. During

the aforementioned 3 weeks of recovery, mice were treated with IgG control or anti-IL-13 interventions. Values are means (SEM), $n = 8-12$ /group. (E) Morphometric quantification of airway mucin containing goblet cells expressed per millimeter BM. Sections were assessed 3 weeks after chronic OVA + BUD. During the aforementioned 3 weeks of recovery, mice were treated with IgG control or anti-CD4⁺ intervention. Values are means (SEM), $n = 8-12$ /group.

airway dysfunction, and indicate that both are elicited through an IL-13 dependent mechanism(s).

IL-13 Independent of CD4⁺ T Cells Contributes to the Goblet Cell Hyperplasia Rebound

Given the well-described observation that the T_H2 subtype of CD4⁺ T cells are a principal source of IL-13 in allergic disease (24) and that these T_H2 allergic responses can be amplified after transient CS treatment (25), we sought to determine if the IL-13 responsible for the goblet cell hyperplasia rebound was derived from CD4⁺ T cells. Mice were treated with a CD4⁺ antibody as described previously (18), for 3 weeks after the simultaneous cessation of budesonide intervention and exposure to allergen, which resulted in ablation of CD4⁺ T cells. Interestingly, depletion of CD4⁺ T cells did not completely prevent the rebound in goblet cell hyperplasia (34.2 [7.7]) (Figure 4E). These findings indicate that although T_H2 cells are an important source of IL-13, additional sources of IL-13 contribute to the goblet cell rebound.

DISCUSSION

In this study, we report on a previously undescribed pathology of a goblet cell hyperplasia rebound associated with airway

dysfunction after the withdrawal of CS treatment (schematically illustrated in Figure 5). This rebound in goblet cells is a late-developing pathology, requiring the withdrawal of relatively prolonged CS intervention that is independent of the type of CS or allergen. IL-13, from sources in addition to T_H2 T cells, plays a critical role in the development of the goblet cell hyperplasia rebound. These findings suggest that certain CS treatment regimes may actually potentiate airway remodeling and dysfunction in patients with asthma and lead to increased exacerbations and worsening of asthma symptoms.

We have demonstrated that whereas CS treatment is highly effective at preventing allergen-induced increases in goblet cell numbers, the simultaneous withdrawal of CS and cessation of chronic exposure to allergen resulted in a rebound increase in goblet cell numbers assessed weeks after CS withdrawal. Intriguingly, the increase in goblet cell numbers reached levels greater than those observed at the same time point (>3 wk after allergen) in mice exposed to allergen alone and without CS treatment. Critically, the increase in goblet cell numbers is associated with marked airway dysfunction. Clearly, this is not evidence enough to conclude that the airway hyperresponsiveness (AHR) observed in these mice was a direct consequence of the goblet cell hyperplasia. Moreover, there was no morphological evidence that MCh challenge in these animals resulted in

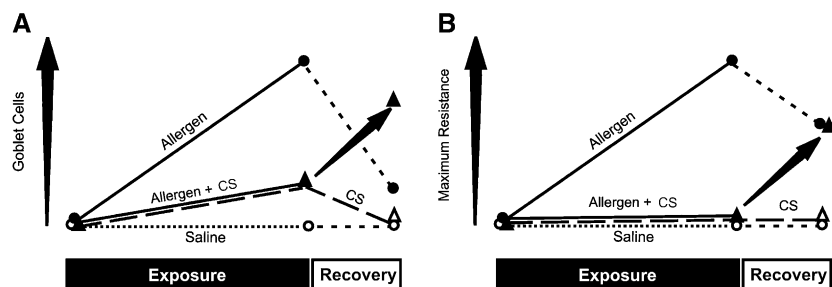


Figure 5. (A) Representative illustration of the goblet cell hyperplasia rebound after the simultaneous withdrawal of glucocorticosteroid (CS) treatment and chronic exposure to allergen. Goblet cell hyperplasia rebound is indicated by the arrow during the recovery component after cessation of both exposure to allergen and treatment with CS. (B) Representative illustration of the goblet cell rebound associated airway dysfunction after the simultaneous withdrawal of CS treatment and chronic exposure to allergen. Associated airway dysfunction is indicated by the arrow during the recovery component after cessation of both exposure to allergen and treatment with CS.

greater mucous secretion into the airway, which might have accounted for the greater increase in resistance. It has previously been reported that CS can induce apoptosis of airway epithelium, which may account for airway epithelial damage and remodeling in asthma (2, 13). Goblet cell hyperplasia has been established as a pathologic characteristic of asthma, irrespective of disease severity. Mucus hypersecretion by hyperplastic goblet cells in airways is believed to contribute to the pathophysiology of asthma (10) as well as patient morbidity and mortality (11). Whether this goblet cell hyperplasia rebound pathology augments airway inflammation, remodeling, or dysfunction of subsequent exposures to allergen, remains unknown. Furthermore, it would be of interest to determine if repeated bouts of intermittent corticosteroids contribute to an amplified goblet cell hyperplasia and associated AHR.

The goblet cell hyperplasia rebound pathology was associated with an increase in bronchoalveolar lavage IL-13, but not IL-4, 5, 9; IFN- γ ; IL-1 β ; or TNF- α . IL-13 is a classical T_H2 cytokine known to regulate goblet cell hyperplasia and mucus production (23). Inhibition of IL-13, after the withdrawal of CS, completely prevented the development of the goblet cell hyperplasia rebound and importantly the associated airway dysfunction. These findings further strengthen the association between the goblet cell hyperplasia rebound pathology after CS withdrawal and airway dysfunction, and indicate that both are elicited through an IL-13 dependent mechanism(s). The T_H2 subtype of CD4⁺ T cells are a principal source of IL-13 in allergic disease (24). Furthermore, T_H2 allergic responses can be amplified after transient CS treatment (25). Surprisingly, however, the depletion of CD4⁺ T cells did not completely prevent the rebound in goblet cell hyperplasia. Although T_H2 cells are an important source of IL-13, our findings indicated that additional sources of IL-13 contribute to the goblet cell hyperplasia rebound and airway dysfunction. Intriguingly, it remains unknown as to why the simultaneous withdrawal of CS treatment and chronic allergen exposure results in an apparent selective increase in IL-13. We and others have observed strong associations between IL-13 and several indices of airway remodeling (26, 27). We did not observe any other indices of airway remodeling in association with the goblet cell rebound, however, despite increases in IL-13. Whether such changes would occur with repeated episodes of CS withdrawal is unknown.

Interestingly, we observed that the goblet cell rebound was associated with the development of AHR that was not present during the period of allergen exposure. This may be interpreted as evidence that goblet cell hyperplasia has functional consequences. This may in fact be the case, however, our subsequent finding that the goblet cell rebound was associated with, and in fact dependent upon, an increase in IL-13 cautions against this conclusion. Evidence that IL-13 can directly affect smooth muscle function (28) may support the interpretation that the AHR occurred independently of the associated goblet cell hyperplasia.

An important question arising from our findings is whether the observed increase in goblet cells in mice, after the withdrawal of CS therapy, is of clinical relevance. Although this cannot be satisfactorily addressed here, the findings of this study may, in part, explain the surprising clinical findings in children with asthma. Although 2 years of CS intervention in preschool children at high risk for asthma unequivocally demonstrated that during CS treatment children experienced significantly less asthma-like symptoms and fewer exacerbations, CS did not alter the subsequent development of asthma symptoms or the eventual level of lung dysfunction during the after year without CS treatment (14). Intriguingly, however, those findings also illustrated that withdrawal of CS resulted in a greater proportion of previously treated children exhibiting worse asthma-like symptoms, and upon interpretation, indicate a more rapid decline in the proportion of episode-free days during the treatment-free year compared with children who were never treated (14). Furthermore, it has recently been reported that children with asthma who were prescribed intermittent CS treatment experienced an increased need for rescue medication compared with never-treated children (29). A plausible explanation for these results is that withdrawal of CS intervention may induce a goblet cell hyperplasia rebound or other pathology that actually potentiates disease progression and contribute to worsening of asthma control. In our studies, we have not addressed whether a time interval between the stopping of allergen and the stopping of CS will prevent the development of the rebound phenomena. Clearly, if such events are observed in patients with asthma, this will be a clinically important consideration.

The prospect of a goblet cell hyperplasia rebound pathology after CS withdrawal in human asthma provocatively raises questions regarding the current treatment strategy for pediatric asthma. Clinicians tend to prescribe intermittent CS therapy for children with wheeze or asthma (30, 31).

Whether animal models effectively undergo the same pathophysiologic changes as those that occur in human disease is unclear. Despite this potential limitation, the clinical plausibility and consequence of this goblet cell hyperplasia rebound highlight the importance of further exploring this observation to ensure that current treatment strategies are offering the best approaches for optimum disease management.

In summary, we report a previously unidentified goblet cell hyperplasia rebound pathology associated with airway dysfunction after the withdrawal of CS treatment. Whether similar events occur in human disease warrants further investigation.

Conflict of Interest Statement: D.S.S. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. R.E. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. J.W. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. W.G. was employed by Centocor from March 2005 to February 2008. M.D.I. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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Online Data Supplement

Goblet Cell Rebound and Airway Dysfunction with Corticosteroid Withdrawal in a Mouse Model of Asthma

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METHODS

Allergen Exposure

Ovalbumin Exposure

Mice ($n=8-12$ /group in study 1, 5-8; $n=5$ /group in study 2) were sensitized with intraperitoneal ovalbumin (OVA) (80 μ g in 200 μ l saline) conjugated to aluminum potassium sulfate and subsequently challenged intranasally with OVA (100 μ g in 25 μ l saline), as described previously (1;2). Control mice were subjected to the same sensitization protocol but received saline challenges. Naive mice ($n=5$ /group in study 3) were not exposed to allergen. Mice were studied 24 h and 4 wk following final exposure to OVA. We have previously reported that 25 μ l intranasal instillations results in a significant distribution to the lower respiratory tract (3).

House-dust-mite Exposure

Mice ($n=5$ /group in study 4) were sensitized to house-dust mite (HDM) *Dermatophagoides pteronyssinus* extract (Greer Laboratories, Lenoir, NC) intranasally (25 μ g in 35 μ l saline) on days 1 to 5 and 8 to 12, as described previously (4). Chronic exposure to HDM was performed as with OVA. Briefly, mice were exposed to repeated intranasal HDM challenges on two subsequent days every 2wk beginning on days 19/20 for 10wk. Mice were studied 24h and 4wk following final exposure to HDM.

Bronchoalveolar Lavage

Bronchoalveolar lavage was performed as described previously (1). Briefly, two aliquots of

250 μ l PBS were injected and withdrawn through a tracheal cannula. After centrifugation, the supernatant was stored at -20°C for subsequent measurements, and cytocentrifuge slides were prepared (Cytospin 3; Shandon Scientific, Sweickley, PA, USA) and stained with Diff-Quik (Dade Behring, Neward, DL, USA). Differential cell counts were performed on 400 cells by 1 investigator blinded to the experimental conditions. Cells were classified based on morphological criteria, as macrophages, neutrophils, lymphocytes, or eosinophils.

Airway Responsiveness

Airway responsiveness was measured as total respiratory system resistance (R_{RS}) to saline and increasing intravenous doses (10, 33, 100 and 330 μ g/kg) of MCh, using the forced oscillation technique. Briefly, mice were anaesthetized using 2,2,2-tribromoethanol (Avertin, 240 mg/kg intraperitoneally (Aldrich Chemical Co., Millwaukee, WI). When anaesthesia was established, the trachea was exposed and cannulated using a blunted 18-gauge needle. The needle was then attached to the *flexiVent* (SCIREQ, Montreal, PQ, Canada) computer-controlled small animal ventilator. Animals were ventilated quasi-sinusoidally (150 breaths/min, 10ml/kg, inflation/deflation ratio of b) with a peak-end-expiratory-pressure (PEEP) set at approximately 2 cmH₂O. Data was collected automatically based on a user defined script. The internal jugular vein was cannulated using a 25-gauge needle. Mice were paralysed using pancuronium (0.03mg/kg intravenously) to prevent respiratory effort. Data collection was preceded by a 6sec inflation to total lung capacity (25cmH₂O), in an effort to standardize the influence of the volume history of the lung, followed by 20sec of standard ventilation. Mice were intravenously injected sequentially with saline, 10, 33, 100, and 330 mg/kg of MCh. During a 45 sec period immediately following

each injection, mice were subjected to thirteen single-sinusoidal inflations/deflations of 0.4sec with a volume of 10 ml/kg (SCIREQ, QuickSnap-150) followed by a 6 sec inflation to total lung capacity. Each breath was preceded by 1 sec of relaxed expiration. Between each injection, mice were ventilated as described previously herein. Total respiratory system resistance (R_{RS}) was calculated using linear least squares regression analysis of simultaneous recordings of airway pressure, airflow, and lung volume change, based on a linear single-compartment model using the following equation:

$$R_{RS} = \frac{P - (E_{RS} \times V) - PEEP}{\dot{V}}$$

Where: P is airway pressure, R_{RS} is total respiratory system resistance, $\dot{V}$ is airflow, E_{RS} is respiratory system elastance, V is volume, and $PEEP$ is peak-end-expiratory-volume.

Maximal R_{RS} was calculated as the maximal MCh-induced R_{RS} .

Lung Histology and Morphometry

Lungs were dissected and processed as described previously (1;5). Briefly, lungs were removed and inflated with 10% formalin to a pressure of 20 cm H₂O and the trachea tied off. Lungs were fixed in formalin for 24h before the lower left lung was isolated, sectioned, and embedded in paraffin. Three- μ m thick transverse tissue sections were cut and stained with periodic acid-Schiff to quantify mucin containing goblet cells. Morphometric quantification of stained sections was performed as described previously (1;5). Briefly, quantification of stained sections was performed using a customized digital image analysis system (Northern Eclipse, Empix Imaging, Mississauga,

ON, Canada) in a double-blinded fashion. Goblet cell number was determined as the number of positively stained cells (PAS) within the airway epithelium and expressed per millimetre basement membrane.

Flow Cytometry Analysis

Flow cytometry analysis was performed as described previously (7), on cells extracted from lung tissue by enzymatic digestion as described previously (6). The viability of extracted cells was > 95%. A total of 1×10^6 cells pre-incubated with purified with anti-mouse CD16/CD32 mAb were stained with saturating amounts of phycoerythrin-anti-mouse CD4 mAb. Analysis was performed on a Becton Dickinson (Franklin Lakes, NJ) FACScan flow cytometry, using Cellquest and WinMDi software packages (BD Biosciences, Oakville, Ontario, CA). CD4⁺ stained cells were identified by detection of FL-2 (Phycoerythrin) and expressed as percentages of total lymphomononuclear cells gated. The number of positive cells was calculated by multiplying total cell count obtained after lung digestion. All stain anti-bodies; Pharmingen, Mississauga, Ontario, CA). Flow cytometry confirmed that the depletion of CD4⁺ T cells was > 99% as previously described (see Figure E1a,b). Although we have used naive mice to demonstrate the effectiveness of this antibody treatment, we have previously demonstrated that this treatment protocol was as effective at reducing lung CD4⁺ cells in allergen challenged animals (7).

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TABLES AND FIGURES

Table E1 Bronchoalveolar lavage fluid cellular contents following chronic exposure to allergen

	Eosinophils	Neutrophils	Lymphocytes
SAL	0.000 (0.000)	0.313 (0.080)	0.819 (0.176)
24 post			
OVA	9.585 (2.941)*	18.164 (4.863)*	1.637 (0.395)
OVA + BUD	1.663 (0.653)†	18.332 (2.679)	2.026 (0.486)
4wk post			
OVA	0.083 (0.034)*	2.663 (0.539)*	3.283 (0.592)*
OVA + BUD	0.329 (0.132)‡	0.384 (0.163)†‡	1.497 (0.336)†
OVA + BUD (cont)	0.000 (0.000)	0.760 (0.184)	2.554 (0.472)

Numbers in each column are means $\times 10^3$; (SEM) in parentheses

* $P < 0.01$ compared with SAL

† $P < 0.01$ compared with OVA 24h or 4wk post respectively

‡ $P < 0.05$ compared with OVA + BUD 24h post

Figure E1. Lung CD4⁺ T cells in allergen naive mice are abrogated following brief intervention with anti-CD4 monoclonal antibody. Representative plot illustrating comparison for CD4⁺ staining between control and anti-CD4 treated mice.

Supplementary Figure E1.

