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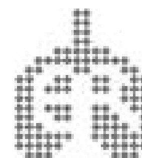

S-carboxymethylcysteine normalises airway responsiveness in sensitised and challenged mice

K. Takeda*, N. Miyahara*, T. Kodama*, C. Taube*, A. Balhorn*, A. Dakhama*, K. Kitamura#, A. Hirano#, M. Tanimoto# and E.W. Gelfand*

ABSTRACT: *S*-carboxymethylcysteine (*S*-CMC) has been used as a mucoregulator in respiratory diseases. However, the mechanism of action of *S*-CMC on allergic airway inflammation has not yet been defined.

In the present study, BALB/c mice were initially sensitised and challenged to ovalbumin (OVA) and, weeks later, re-challenged with OVA (secondary challenge). *S*-CMC (5–100 mg·kg^{-1}) was administered from 2 days before the secondary challenge through to the day of assay.

Mice developed airway hyperresponsiveness (AHR) 6 h after the secondary challenge and increased numbers of neutrophils were present in the bronchoalveolar lavage (BAL) fluid. At 72 h after secondary challenge, mice again developed AHR, but the BAL fluid contained large numbers of eosinophils. *S*-CMC treatment was found to reduce AHR and neutrophilia at 6 h, as well as eosinophilia and AHR at 72 h. These effects appeared to be dose dependent. Goblet cell hyperplasia, observed at 72 h, was reduced by *S*-CMC. In BAL fluid, increased levels of interferon-γ, interleukin (IL)-12 and IL-10 and decreased levels of IL-5 and IL-13 were detected.

In conclusion, the data indicate that *S*-carboxymethylcysteine is effective in reducing airway hyperresponsiveness and airway inflammation at two distinct phases of the response to the secondary allergen challenge in sensitised mice.

KEYWORDS: Airway hyperresponsiveness, eosinophils, neutrophils, *S*-carboxymethylcysteine

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Despite significant advances in asthma therapeutics since the 1990s, morbidity and mortality remain a worldwide concern [1]. In the pathogenesis of asthma, various types of inflammatory cells are thought to play an important role in the development of airway hyperresponsiveness (AHR) and allergic airway inflammation. A common theory is that the disease is the result of chronic airway inflammation, largely dependent upon eosinophils, leading to AHR and reversible airway obstruction [2, 3]. With the rising incidence and therapeutic insufficiencies, it is now more apparent that asthma is a heterogeneous syndrome, with numerous cell types and mediators contributing to the disease phenotype. Central to the pathogenesis of the airway disease are antigen-specific, memory T-cell responses and, perhaps to a lesser degree, antigen-specific immunoglobulin E responses [4, 5].

Conversely, mucus hypersecretion is one of the major symptoms in asthmatics and has been related to fatal asthma pathogenesis [6]. Although the contribution of mucus production on asthmatic airways is still unclear, MUC5AC and other mucin genes have been shown to be related to asthma status in clinical studies [7] and in animal models [8]. *S*-carboxymethylcysteine (*S*-CMC) has been used as a mucoregulator for treating chronic obstructive pulmonary disease (COPD), asthma and chronic nasal diseases, such as chronic sinusitis or otitis media, with some efficacy [9–11]. However, there have been few studies addressing potential efficacy or mechanism of action in allergen-induced asthma. Katayama *et al.* [12] showed that *S*-CMC decreased cough sensitivity, but not AHR in an antigen-induced guinea pig model, and Asti *et al.* [13] showed a suppressive effect on cigarette smoke-induced AHR. *S*-CMC is known to suppress neutrophil chemotaxis *in vitro* as well as *in vivo* [13, 14]. At early time points after allergen challenge, an increase in numbers of neutrophils in the airways has been shown to accompany changes in airway function [15]. However, it appears that depletion of neutrophils may have little effect on allergic airway eosinophilic inflammation or AHR [16].

In the present study, a murine model was utilised, which distinguishes a neutrophil-dominant phase early after antigen challenge and an eosinophil-dominant phase with few neutrophils at later time points. Both phases are associated with AHR. To determine if *S*-CMC can modulate these two phases of the inflammatory response, reagents were administrated prior to secondary challenge and changes in airway inflammation and AHR were monitored.

MATERIALS AND METHODS

Animals

Female BALB/c mice aged 6–8 weeks old were obtained from Jackson Laboratories (Bar Harbor, ME, USA). The animals were maintained on an ovalbumin (OVA)-free diet. Experiments were conducted under a protocol approved by the Institutional Animal Care and Use Committee of the National Jewish Medical and Research Center (Denver, CO, USA).

Experimental protocol

The experimental protocol for sensitisation and challenge to allergen was modified from previously described procedures [17]. Briefly, mice were sensitised by *i.p.* injection of 10 µg of OVA (Grade V; Sigma Chemical Co., St. Louis, MO, USA) emulsified in 2.25 mg aluminium hydroxide (AlumImuject; Pierce, Rockford, IL, USA) in a total volume of 100 µL on days 1 and 7. Mice were challenged (primary) *via* the airways with OVA (0.2% in saline) for 20 min on days 14, 15 and 16 by ultrasonic nebuliser (model NE-U07; Omron Healthcare, Vernon Hills, IL, USA). On day 30, mice received a single secondary challenge *via* the airways with 1% OVA for 20 min. Control mice received saline as the secondary challenge. Mice were studied 6 h and 72 h (fig. 1) after the secondary challenge.

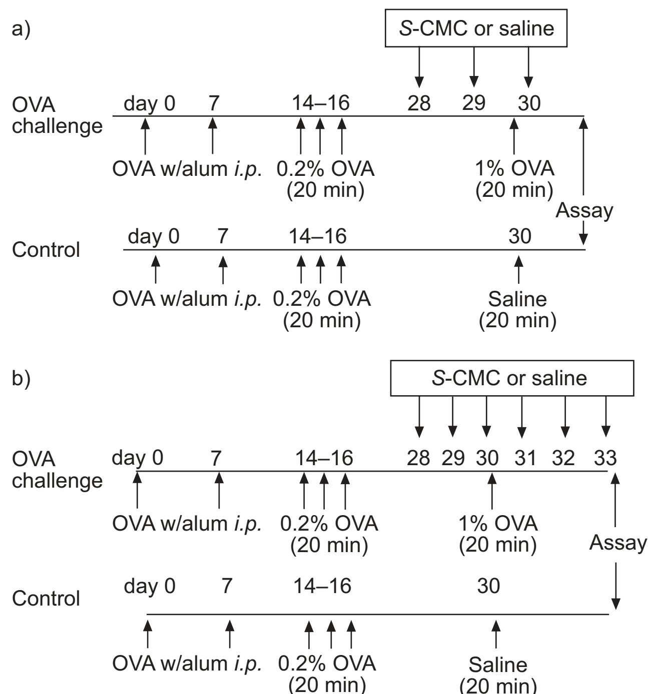

FIGURE 1. Diagram to show the experimental protocol a) 6 h and b) 72 h after the secondary challenge. *S*-CMC: *S*-carboxymethylcysteine; OVA: ovalbumin; w/alum: with aluminium hydroxide.

To determine the effects of *S*-CMC on airway allergic inflammation and AHR, 5, 10 or 100 $mg \cdot kg^{-1}$ of *S*-CMC was administered *b.i.d.* in 500 µL dH_2O by *i.p.*, from 2 days before the secondary challenge through to the day of study. Control groups of mice received saline in the same fashion. *S*-CMC was provided by Kyorin Pharmaceuticals (Tokyo, Japan).

Determination of airway responsiveness

Airway responsiveness was assessed as changes in airway function after challenge with aerosolised methacholine (MCh; Sigma). Mice were anaesthetised, tracheostomised and mechanically ventilated with lung function assessed as described previously [18]. Ventilation was achieved at 160 $breaths \cdot min^{-1}$ at a tidal volume of 0.16 mL with a positive end-expiratory pressure of 2–4 cmH_2O. Lung resistance (R_L) was continuously computed (Labview, National Instruments, TX, USA) by fitting flow, volume and pressure to an equation of motion using a recessive least squares algorithm.

Aerosolised MCh was administered through bypass tubing *via* an ultrasonic nebuliser (model 5500D; DeVilbiss, Somerset, PA, USA) placed between the expiratory port of the ventilator and the four-way connector. Aerosolised MCh was administered for 8 s with a tidal volume of 0.45 mL and frequency of 60 $breaths \cdot min^{-1}$ using another ventilator (model 683; Harvard Apparatus, South Natick, MA, USA). The data of R_L was continuously collected for up to 3 min and maximum values were taken. PC_{200} values (MCh concentration required to induce a 200% change in R_L relative to saline) were also calculated.

Bronchoalveolar lavage

Immediately after assessment of AHR, lungs were lavaged *via* the tracheal tube with Hank's balanced solution (HBSS; 1×1 mL, 37°C). The volume of collected bronchoalveolar lavage (BAL) fluid was measured in each sample and the number of leukocytes was counted (Coulter Counter; Coulter Corporation, Hialeah, FL, USA). Differential cell counts were performed by counting at least 200 cells on cytocentrifuged preparations (Cytospin 3; Shandon Ltd, Runcorn, UK). Slides were stained with modified Wright-Giemsa and white blood cells were differentiated by standard haematological procedures in a blinded fashion. BAL fluid supernatants were collected and stored at -70°C until measurement.

Cell preparation for in vitro cytokine production

To determine the effect of *S*-CMC on cytokine production at the single cell level, cells were isolated and cultured with different concentrations of OVA and *S*-CMC. Spleen, peribronchial lymph nodes and lungs were taken from mice 72 h after secondary challenge. Mononuclear cells from spleen and peribronchial lymph node (PBLN) were isolated. Lung mononuclear cells were obtained following collagenase digestion and gradient centrifugation (35% Percoll (Sigma)) to remove epithelial cells. Cells in BAL fluid were collected following saline challenge and instillation of 1 mL of HBSS four times. To purify airway macrophages, BAL cells were cultured in plastic dishes at 37°C for 1 h followed by washing three times with 37°C HBSS. Adhesive cells were collected and shown to be

>95% macrophages. Spleen, PBLN and lung cells (2×10^5) and alveolar macrophages (1×10^5) were cultured either with or without 10 μg·mL^{-1} of OVA. *S*-CMC was added to cultures at concentrations of 0, 1, 10 and 100 μg·mL^{-1}. After 24 h incubation at 37°C, supernatants were collected.

Measurement of cytokines

Cytokine levels in the BAL fluid or supernatants from cell culture were measured as previously described [19]. Briefly, measurements of interleukin (IL)-4, IL-5, IL-10 and IL-12 were performed by ELISA (BD PharMingen, San Diego, CA, USA) with 96-well plates (Immulon 2; Dynatech, Chantilly, VA, USA). IL-13 measurements were performed using an ELISA kit (QuantikineM; R&D Systems, Minneapolis, MN, USA), all following the manufacturers protocol. The limits of detection were: 1.5 pg·mL^{-1} for IL-13; 4 pg·mL^{-1} for IL-4 and IL-5; and 10 pg·ml^{-1} for IL-10 and interferon (IFN)-γ.

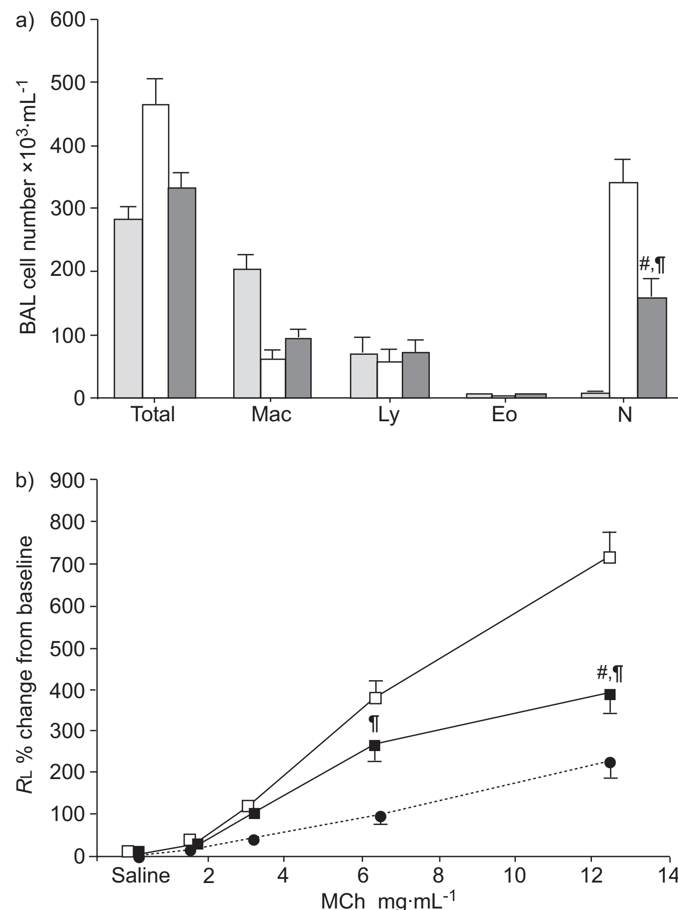

FIGURE 2. a) Cell composition in bronchoalveolar lavage (BAL) fluid obtained 6 h after secondary challenge. ■: control; □: saline; ■: *S*-carboxymethylcysteine (*S*-CMC). Mac: macrophage; Ly: lymphocyte; Eo: eosinophil; N: neutrophil numbers. b) Changes in lung resistance (*R*L) 6 h after secondary challenge. *R*L values were obtained in response to increasing concentrations of inhaled methacholine (MCh). □: saline challenge; ■: *S*-CMC treatment during secondary ovalbumin (OVA) challenge; ●: control (saline) treatment during secondary OVA challenge. n=8. #: $p<0.05$ significant difference between the saline treatment and *S*-CMC treatment group; ¶: $p<0.05$ significant difference between the saline challenge and *S*-CMC treatment groups.

Histological studies

Lungs were inflated through the trachea with 1 mL of 10% formalin and fixed in 10% formalin by immersion. Blocks of lung tissue were cut around the main bronchus and embedded in paraffin. Sections (6 μm) were cut and stained with haematoxylin-eosin (HE) to analyse inflammatory cell infiltration. For detection of mucus-containing cells in formalin-fixed airway tissue, sections were stained with periodic acid-Schiff (PAS) and counterstained with HE. Cells containing eosinophilic major basic protein (MBP) were identified by immunohistochemical staining as previously described using rabbit-anti-mouse MBP (provided by J.J. Lee, Mayo Clinic, Scottsdale, AZ, USA) [20]. The slides transferred to pictures using a Nikon microscope (Melville, NY, USA) equipped with a fluorescein filter system. PAS-positive cells and numbers of peribronchial eosinophils in the tissues were quantified using an NIH Image analysis system (version 1.63; NIH, Bethesda, MA, USA) and counting 6–8 different fields per slide in a blinded manner.

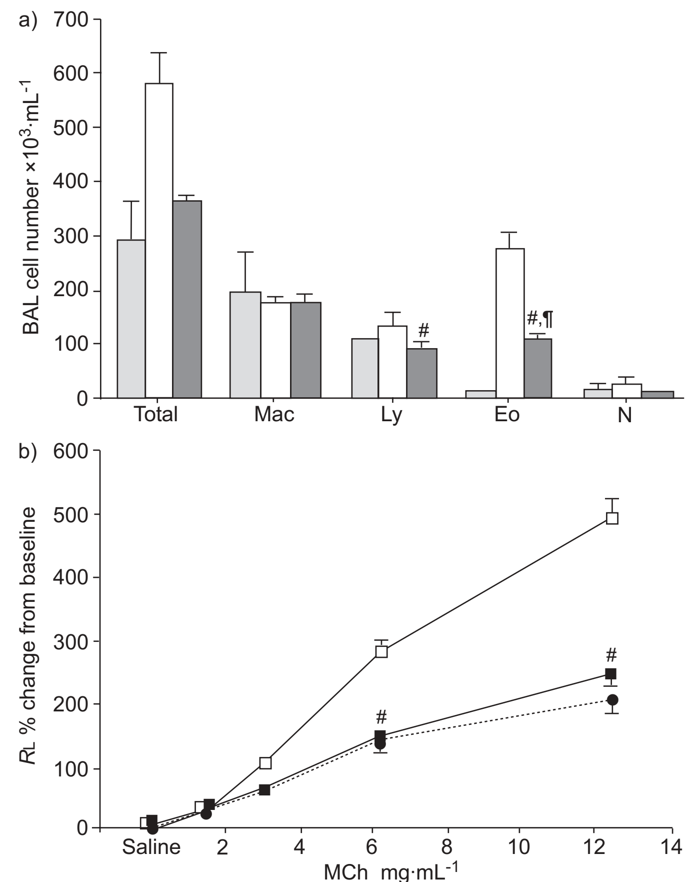

FIGURE 3. a) Cell composition in bronchoalveolar lavage (BAL) fluid obtained 72 h after secondary challenge. ■: control; □: saline; ■: *S*-carboxymethylcysteine (*S*-CMC). Mac: macrophage; Ly: lymphocyte; Eo: eosinophil; N: neutrophil numbers. b) Changes in lung resistance (*R*L) 72 h after secondary challenge. □: saline challenge; ■: *S*-CMC treatment during secondary ovalbumin (OVA) challenge; ●: control (saline) treatment during secondary OVA challenge. n=8. #: $p<0.05$ significant difference between the saline treatment and *S*-CMC treatment groups; ¶: $p<0.05$ significant difference between the saline challenge and *S*-CMC treatment groups.

Statistical analysis

All results were expressed as mean±SEM. ANOVA was used to determine the levels of difference between all groups. Groups were compared by unpaired two-tailed t-test. For analysis of IL-10 levels in alveolar macrophage cultures, the Mann-Whitney U-test was used. The p-value for significance was set at 0.05.

RESULTS

Neutrophilic airway inflammation and AHR 6 h after secondary challenge

Allergen-challenged, but not saline-challenged mice, showed a neutrophil-dominant inflammatory response in the BAL fluid (75% of total BAL fluid cells; fig. 2a) and AHR in response to inhaled MCh 6 h after secondary challenge (fig. 2b). When mice were treated with *S*-CMC, the numbers of neutrophils were decreased by ~50% and airway responsiveness to MCh was also decreased significantly compared with saline-treated mice.

Eosinophilic airway inflammation and AHR 72 h after secondary challenge

The mice developed an eosinophil-dominant inflammatory response 72 h after secondary challenge in the BAL fluid (45% of total BAL fluid cells), few neutrophils (fig. 3a) and AHR to inhaled MCh (fig. 3b). When *S*-CMC was administered, the numbers of eosinophils in BAL fluid were significantly decreased to ~37% of the control group, and airway responsiveness was reduced to the same levels as saline-challenged mice.

S-CMC dose-dependent effects on the airway

To determine if the effects of *S*-CMC were dose-dependent, 5, 10 and 100 $mg \cdot kg^{-1}$ of *S*-CMC were administered to mice. AHR and BAL cell composition were investigated at 6 or 72 h after secondary allergen challenge. Only 100 $mg \cdot kg^{-1}$ of *S*-CMC was effective in reducing neutrophil numbers in BAL fluid 6 h after secondary challenge (fig. 4a and b). In parallel, and only at 100 $mg \cdot kg^{-1}$, *S*-CMC was effective in reducing AHR resulting in a significant increase in PC200 values in airway resistance. Lower doses of *S*-CMC (5, 10, and 100 $mg \cdot kg^{-1}$) resulted in a dose-dependent decrease in eosinophil numbers in the BAL fluid as well as a dose-dependent increase in PC200 values for airway resistance 72 h after secondary challenge (fig. 4c and d).

Histological analysis

Histological studies monitoring HE, PAS and anti-MBP staining were performed on lungs removed from the same

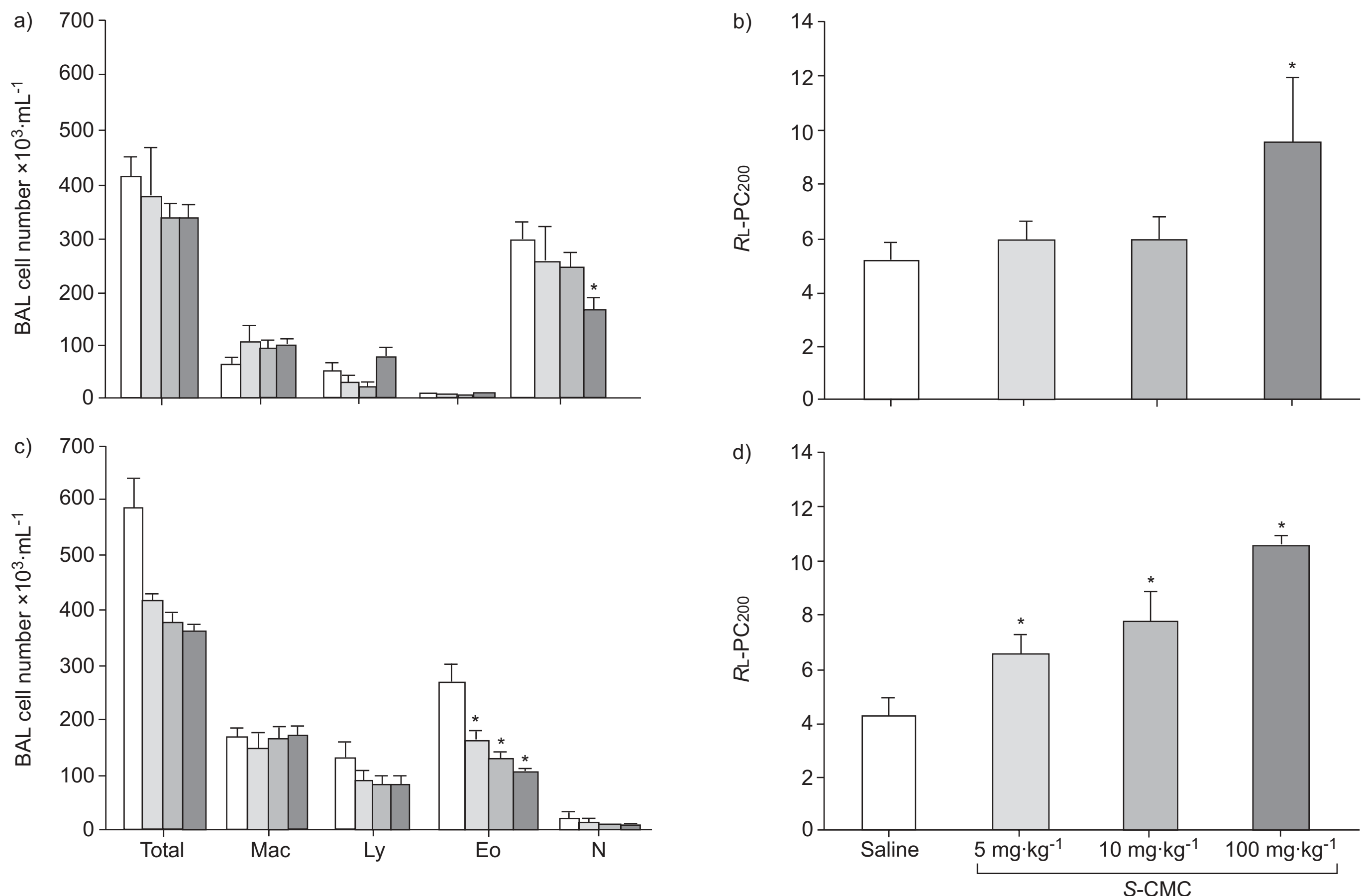

FIGURE 4. a, c) Cell composition in bronchoalveolar lavage (BAL) fluid, and b, d) PC200 changes (lung resistance (R_L)) following treatment with different doses of *S*-carboxymethylcysteine (*S*-CMC). Assessment was carried out at 6 h (a, b) and 72 h (c, d) after secondary allergen challenge. R_L changes are expressed as methacholine concentration required to induce a 200% change in R_L relative to saline challenged mice (PC200). n=8. *: $p<0.05$ significant difference between *S*-CMC-treated animals *versus* saline-treated animals.

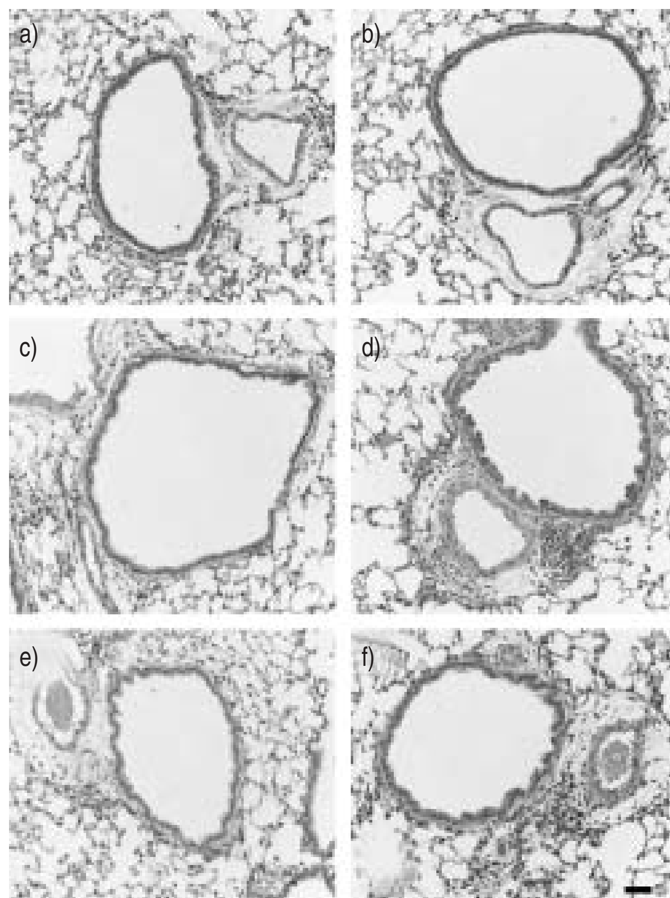

FIGURE 5. Sections of lung tissue were prepared 6 h after secondary challenge (a, c and e), and 72 h after secondary challenge (b, d and f), both with haemoxylin and eosin staining. The slides show saline challenge (a, b), secondary allergen challenge with saline treatment (c, d) and secondary allergen challenge following *S*-carboxymethylcysteine treatment (e, f). Scale bar=50 μm.

animals described previously. In OVA-sensitised mice, cell infiltration into the peribronchial regions was detected 72 h after secondary challenge (fig. 5). Few cells were observed in the peribronchial regions 6 h after secondary challenge, although increased numbers of neutrophils were observed in the alveolae. In the HE-stained sections, no obvious differences between *S*-CMC-treated and saline-treated mice were observed at any time point.

PAS-stained sections showed goblet cell hyperplasia and mucus hyperproduction 72 h after secondary challenge (fig. 6a–d), but not 6 h after challenge (data not shown). Following *S*-CMC treatment, PAS-positive cells were reduced significantly compared with saline-treated mice (fig. 6).

Anti-MBP staining of lung sections revealed the localisation of eosinophils in the tissue. At 72 h following secondary challenge, a significant accumulation of eosinophils in the peribronchial regions was observed (fig. 7a–f). Quantification of MBP-positive cells (eosinophils) revealed a small increase 6 h after secondary challenge, but a marked increase 72 h after the secondary challenge in the peribronchial regions (fig. 7g). This eosinophilic infiltration at 72 h was significantly reduced with *S*-CMC treatment.

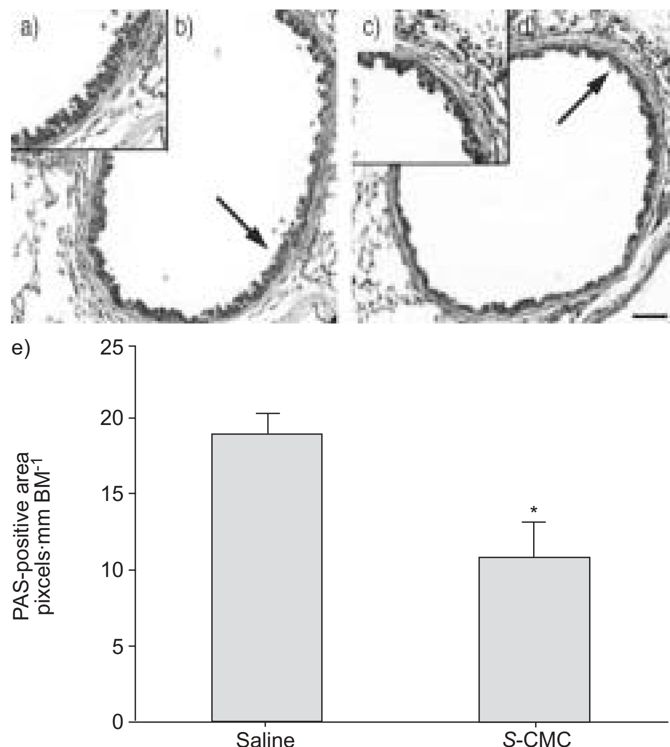

FIGURE 6. Assessment of goblet cell hyperplasia and mucin hyperproduction, using periodic acid-Schiff (PAS) and haemoxylin and eosin as a counter stain. Sections were prepared 72 h after secondary challenge following: a, b) saline, or c, d) *S*-carboxymethylcysteine (*S*-CMC) treatment (a and c are enlarged areas of b and d, respectively). e) Goblet cell hyperplasia 72 h after secondary allergen challenge was quantified in PAS-stained sections and expressed per mm of basement membrane (BM). n=6. *: $p<0.05$ significant difference between *S*-CMC treatment *versus* saline. Scale bar=50 μm.

Cytokine levels in BAL fluid

To address the mechanism of S-CMC on airway allergic inflammation, the levels of different cytokines in the BAL fluid were determined. As shown in figure 8, levels of the T-helper (Th)-2 cytokines, IL-4, IL-5, and IL-13, were significantly increased in the BAL fluid 6 h after secondary allergen challenge compared with saline challenge. These levels were much lower when assayed 72 h after secondary challenge. The levels of IL-10 were increased after secondary challenge and remained high at 72 h. *S*-CMC treatment induced further increases in IL-10 levels in BAL fluid at 72 h. The levels of IFN-γ and IL-12 were increased after secondary allergen challenge and *S*-CMC treatment further increased the levels of IL-12 at 72 h. Treatment with *S*-CMC reduced the levels of IL-5 and IL-13 at 72 h.

Cytokine levels in cultured cells

Lung, spleen and PBLN cells were collected and purified from mice after secondary OVA challenge. BAL alveolar macrophages were collected after saline challenge (as described in Material and methods) and cytokine levels in the supernates of cultured cells were determined. The levels of IL-10 were increased in lung cells when cultured with OVA (fig. 9a–c). Levels of IL-10 from spleen and PBLN cells, as well as levels of IL-5 and IL-13, also showed some increase (data not shown).

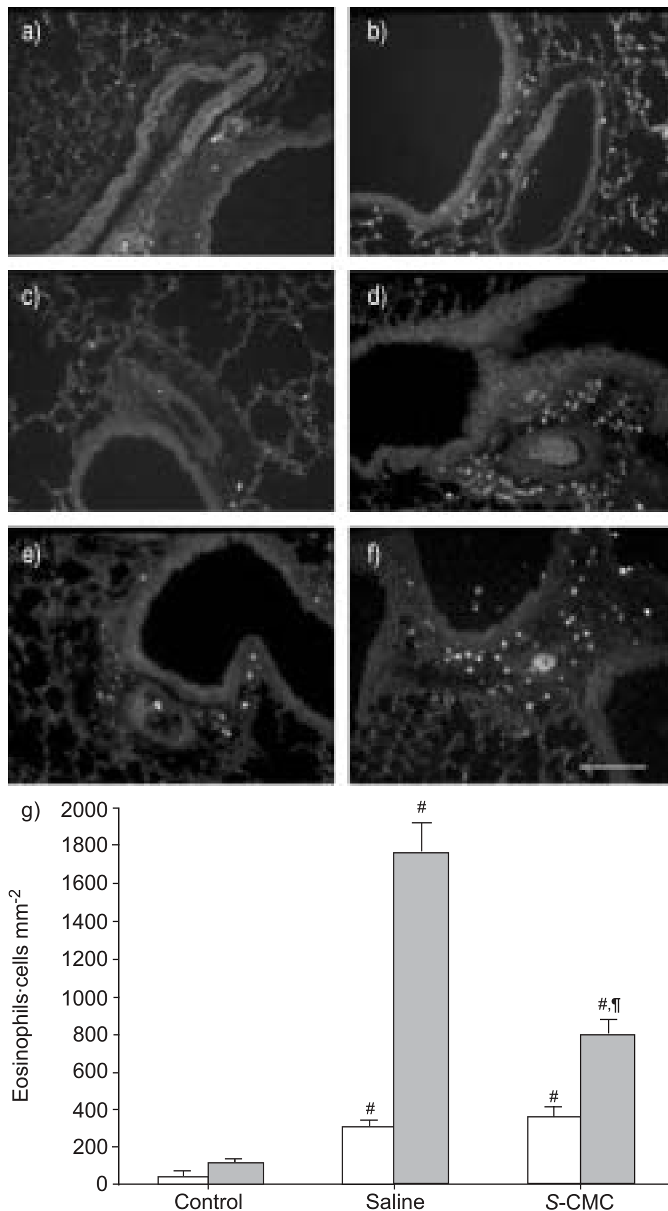

FIGURE 7. Anti-major basic protein (MBP) staining of airways prepared from lungs 6 h after secondary challenge (a, c and e) and 72 h after secondary challenge (b, d and f). Saline challenge (a and b), secondary allergen challenge following saline treatment (c and d) and secondary allergen challenge following *S*-carboxymethylcysteine (*S*-CMC) treatment (e and f). Scale bar=100 µm. g) The number of MBP-positive cells after secondary challenge was quantified. □: after 6 h; ■: after 72 h. n=6; *S*-CMC treatment *versus* vehicle. #: $p<0.001$ significant difference expressed to saline challenge; ¶: $p<0.001$ significant difference expressed to saline treatment.

However, these increased levels were not altered by inclusion of *S*-CMC in the cultures. Conversely, the levels of Th-1 cytokines IFN-γ and IL-12 in cultured lung cells were markedly increased with *S*-CMC treatment. These cytokines were not detected in cultures of spleen and PBLN cells (data not shown). In cultures of alveolar macrophage, IFN-γ, IL-12 and IL-10 were increased with *S*-CMC treatment (fig. 9d–f).

DISCUSSION

S-CMC has been widely used as a mucoregulator, since it is thought to improve mucus clearance by modifying its biochemical characteristics [21]. *S*-CMC has been used in the treatment of COPD. EDWARDS *et al.* [9] reported that *S*-CMC improved the symptoms of COPD patients. *S*-CMC has been reported to have other therapeutic effects in airway inflammation. For example, *S*-CMC has been shown to have an anti-oxidant effect [22] and improves SO_2 gas-induced lung inflammation. *S*-CMC normalised levels of fucose and sialic acid content in mucin glycoprotein and inhibited the increase in expression level of MUC5AC protein in the airway epithelium of rats [23]. *S*-CMC has been reported to inhibit neutrophil migration *in vivo* and *in vitro* [24]. ISHII *et al.* [14] showed that *S*-CMC may inhibit neutrophil activity through induction of phosphatidylinositol-specific phospholipase C *in vitro*. In asthma, *S*-CMC has been shown to have some efficacy with improvement of mucociliary transport or suppression of the cough reflex [25, 26]. However, controlled clinical studies have not been carried out to determine *S*-CMC efficacy on airway function or airway inflammation.

In the present study, mice developed a two phase airway inflammatory response after secondary allergen challenge, one neutrophilic and the other eosinophilic. AHR to inhaled MCh was detected at both phases of the response to secondary challenge. In the first phase, 6 h after last antigen challenge, mice developed AHR and a neutrophil-dominant airway inflammatory response with very few eosinophils in the BAL fluid. *S*-CMC administration improved both AHR and reduced the number of neutrophils in the airway. During this phase, only the higher dose of *S*-CMC appeared effective. In asthma patients [27, 28] and in animal models [15], neutrophils have been shown to be the first inflammatory cells in the airways after allergen challenge. Neutrophils are known to release several chemical mediators known to be toxic to the airways. The timing of the peak neutrophil influx coincided with development of AHR. The issue as to whether neutrophils directly or indirectly contribute to the development of AHR is not clear [16, 29]. Thus, although *S*-CMC treatment reduced both neutrophil accumulation and AHR, at the present time the two processes cannot be definitely linked.

The response to antigen challenge at 72 h was quite different and was characterised by a marked increase in numbers of eosinophils accompanied by development of AHR. *S*-CMC treatment reduced the numbers of eosinophils and virtually abolished AHR, suggesting greater efficacy at this later time point than at 6 h. At 72 h, the response to *S*-CMC showed a clear dose dependency. In part, this greater efficacy at 72 h compared with 6 h may reflect the longer duration of *S*-CMC treatment.

Mice developed a marked cellular infiltration in the peribronchial regions 72 h after secondary allergen challenge compared with saline-challenged mice. This infiltration was more marked than observed at 6 h. Staining of the lung sections with MBP antibody revealed some eosinophil accumulation (above controls) in the peribronchial regions at 6 h. Treatment with *S*-CMC had little effect on this response. When eosinophilic infiltration into the peribronchial regions and airway lumen was prominent 72 h after secondary antigen

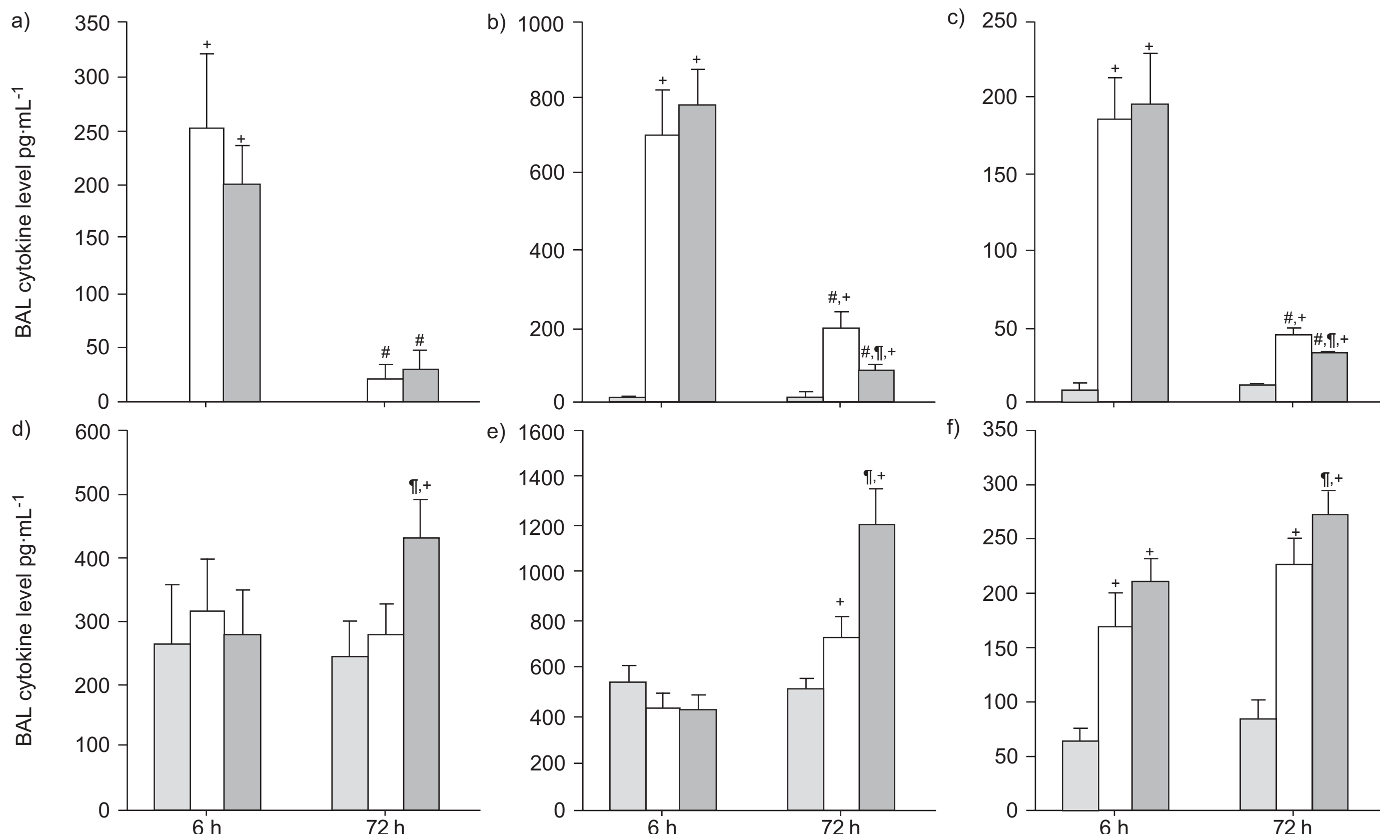

FIGURE 8. Cytokine levels in bronchoalveolar lavage (BAL) fluid in mice receiving saline challenge (■), secondary allergen challenge following *S*-carboxymethylcysteine (*S*-CMC; ■) or saline treatment (□). a) Interleukin (IL)-4, b) IL-5, c) IL-13, d) interferon-γ, e) IL-12 and f) IL-10. n=11–12 in each group. #: $p<0.05$ significant difference between the 6 h and 72 h time points after secondary allergen challenge; ¶: $p<0.05$ significant difference between saline-treated and *S*-CMC-treated groups; +: $p<0.05$ significant difference between secondary allergen challenge or saline challenge.

challenge, this inflammatory cell influx was significantly reduced by *S*-CMC treatment. Since eosinophils in the airway have been thought to contribute to the development of AHR [17, 30], this reduction by *S*-CMC may account for the normalisation of airway function. To date, no direct effects of *S*-CMC on eosinophils have been described.

Goblet cell hyperplasia was virtually undetectable 6 h after secondary antigen challenge, while goblet cell hyperplasia and mucus hyperproduction was markedly increased at 72 h. At 72 h, *S*-CMC treatment was effective in reducing this response which may have contributed to the decrease in AHR [6]. Since mucin-related gene MUC5AC expression has been shown to be upregulated by neutrophil elastase through reactive oxygen species [31], *S*-CMC may modulate mucus production through this mechanism as reported using SO_2 gas-induced lung inflammation [23].

Many of the responses leading to AHR, inflammation and goblet cell hyperplasia are the direct result of changes in specific cytokine levels. When cytokine levels in BAL fluid were determined, an increase in the Th-2 cytokines, IL-4, IL-5 and IL-13, was detected 6 h after secondary antigen challenge. In fact, by 72 h these levels were markedly reduced. *S*-CMC treatment was virtually ineffective in altering the levels of these cytokines at 6 h. *S*-CMC showed some efficacy in reducing cytokine levels at 72 h and at this time point the reduction in IL-13 may explain the decrease in goblet cell hyperplasia [32]. The levels of IL-5 were maximally increased when neutrophils were dominant at 6 h, but decreased by 72 h (from 700 to 200 pg·mL^{-1}) when eosinophils were dominant in the airways. It is known that IL-5 is a potent eosinophil inducer [33], but there may be a time lag for eosinophil induction by IL-5. When the temporal association of eosinophils in lung tissue and BAL was examined, eosinophil accumulation in the lung preceded that in the BAL by ⩾24 h [15], as was shown here.

The levels of IFN-γ and IL-12 remained stable at the 6 and 72 h time points after secondary challenge, and were significantly increased in *S*-CMC treated mice. The levels of IL-10 were increased at 6 and 72 h after secondary challenge, and were further increased with *S*-CMC treatment.

The Th-2 cytokines, IL-4, IL-5 and IL-13, have been associated with allergic eosinophilic airway inflammation and AHR in human asthma [29] as well as animal models [30]. However, IFN-γ may counteract Th-2 cytokine activities [34]. Administration of IL-12 has been shown to reduce AHR and eosinophilic inflammation [35]. The function of IL-10 is more complex. MAKELA *et al.* [36] reported that IL-10 is necessary for the development of AHR, while other reports showed that IL-10 can regulate allergen-induced airway inflammation [37].

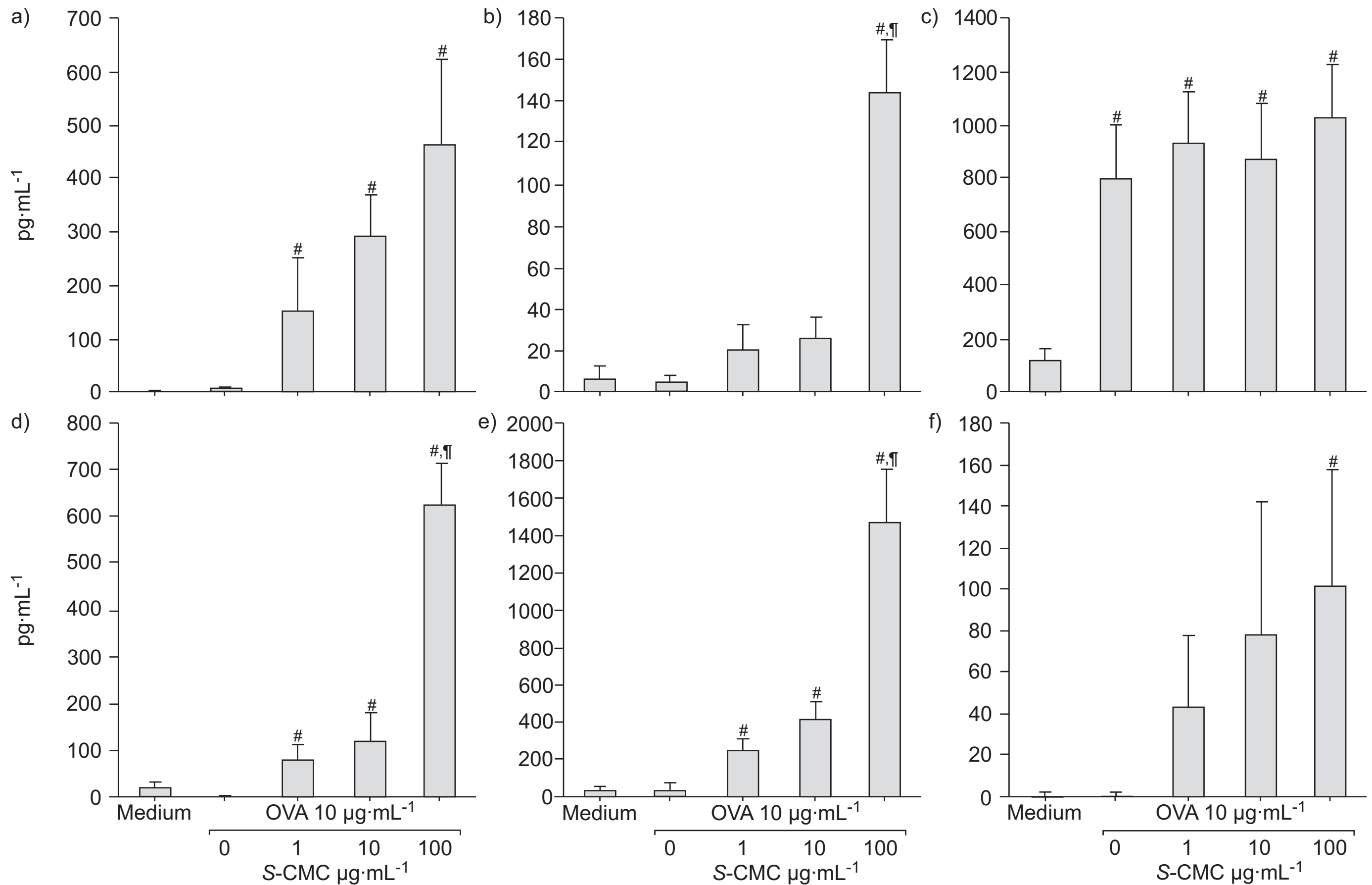

FIGURE 9. Cells were cultured for 24 h with or without 10 $\mu g \cdot mL^{-1}$ of ovalbumin (OVA) in the presence of various concentrations of *S*-carboxymethylcysteine (*S*-CMC; 0, 1, 10 and 100 $\mu g \cdot mL^{-1}$). a, b, c) Cytokine levels (interferon (IFN)-γ, interleukin (IL)-12 and IL-10, respectively) in supernates from cultures of isolated lung cells. d, e, f) Cytokine levels (IFN-γ, IL-12, IL-10, respectively) in supernates of cultured alveolar macrophages. n=6. #: $p<0.05$ significant difference *versus* medium; ¶: $p<0.05$ significant difference between *S*-CMC 100 $\mu g \cdot mL^{-1}$ and 10 $\mu g \cdot mL^{-1}$ treatment.

Both IL-10 and IL-12 are secreted mainly by monocytes or macrophages. As IL-10 and IL-12 were shown to be modulated by *S*-CMC treatment *in vitro*, *S*-CMC may regulate cytokine production from macrophages or monocytes *in vivo* as well [24]. The increased levels of IL-10 or IL-12 seen following *S*-CMC treatment may be advantageous for reducing AHR and normalising airway function, and the increases in IFN-γ may be more beneficial since IFN-γ is recognised as a negative regulator of allergic inflammation in the airways [38].

In summary, administration of the mucoregulator *S*-carboxymethylcysteine was shown to normalise airway hyperresponsiveness. This was accompanied by reducing neutrophils or eosinophils in the airways at the two distinct phases of the response to secondary allergen challenge. *S*-carboxymethylcysteine also modulated bronchoalveolar lavage fluid cytokine levels and goblet cell hyperplasia. Taken together, in both the neutrophil- and eosinophil-dominant phases of the response to secondary allergen challenge, *S*-carboxymethylcysteine reduced airway hyperresponsiveness to inhaled methacholine indicating the potential for its use as a modulator of the immune/inflammatory response in asthmatics repeatedly exposed to allergens.

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