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Therapeutic administration of Budesonide ameliorates allergen-induced airway remodelling

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Summary

Background—Airway inflammation and remodelling are important pathophysiologic features of chronic asthma. Although current steroid use demonstrates anti-inflammatory activity, there are limited effects on the structural changes in the lung tissue.

Objective—We have used a mouse model of prolonged allergen challenge that exhibits many of the salient features of airway remodelling in order to investigate the anti-remodelling effects of Budesonide.

Methods—Treatment was administered therapeutically, with dosing starting after the onset of established eosinophilic airway inflammation and hyper-reactivity.

Results—Budesonide administration reduced airway hyper-reactivity and leukocyte infiltration in association with a decrease in production of the Th2 mediators, IL-4, IL-13 and eotaxin-1. A reduction in peribronchiolar collagen deposition and mucus production was observed. Moreover, our data show for the first time that, Budesonide treatment regulated active transforming growth factor (TGF)- β signalling with a reduction in the expression of pSmad 2 and the concomitant up-regulation of Smad 7 in lung tissue sections.

Conclusions—Therefore, we have determined that administration of Budesonide modulates the progression of airway remodelling following prolonged allergen challenge via regulation of inflammation and active TGF- β signalling.

Keywords

allergy; asthma; remodelling; steroids; TGF- β signalling

Introduction

Asthma is characterized by chronic inflammation of the airways together with pathologic structural changes such as subepithelial fibrosis, an increase in mucus producing goblet cells

and an increase in smooth muscle mass, collectively known as airway remodelling {Bousquet, 2000 12927/id}. The cumulative chronic inflammation, increased deposition of extracellular matrix (ECM) proteins and smooth muscle cell proliferation increases airway wall thicknesses and may result in the decline in lung function characteristic of human chronic asthma [1].

Steroids are currently the most effective long-term treatment for asthma. Clinical studies have demonstrated that steroids inhibit pro-inflammatory cell recruitment, specifically activated eosinophils, mast cells and T-cells and down regulate the production of pro-inflammatory cytokines (reviewed by [2]). In addition, a decrease in airway hyper-reactivity (AHR) is observed following steroid treatment, attributed to the anti-inflammatory action in the lungs. However, some asthmatic patients still exhibit AHR following prolonged treatment with steroids, suggesting an irreversible component that is not effected by steroids [3]. Indeed, treatment of asthmatic patients with steroids demonstrated no significant effect on lamina reticular thickness [3–5]. In contrast, a significant reduction in ECM deposition was observed in other studies [6–8]. These differences appear to result from the severity of the asthma in the patient population studied, as well as from previous steroids use by the patients. Therefore, the effect of administration of steroids on established chronic pulmonary disease is less clear.

Transforming growth factor (TGF)- β is a profibrotic cytokine thought to play an important role in promoting the structural changes of airway remodelling. It has been shown to be essential for the transformation of fibroblasts into myofibroblasts, which are major producers of collagen. In asthmatics, increased TGF- β mRNA expression in bronchial biopsy sections is seen in comparison with normal subjects, and levels correlate with the depth of subepithelial fibrosis [9–12]. In addition, bronchoalveolar lavage (BAL) levels of TGF- β 1 are increased in asthmatic patients [13]. Hallmarks of TGF- β signal transduction pathways are the activation of TGF- β type I (ALK5) and type II receptors and the subsequent phosphorylation and translocation of the intracellular effectors Smad 2 and Smad 3 [14] to the nucleus where they regulate gene transcription [15]. Smad 7 is an intracellular inhibitor, which is rapidly induced by TGF- β family members and provides a negative feedback loop [16]. Recent studies on a mouse model of acute allergic inflammation have demonstrated *in situ* activation of these TGF- β signalling pathways [17]. However, the effect of steroid administration on active TGF- β signalling in airway lesions has not been determined.

We have recently established and characterized a murine model of prolonged allergen challenge featuring many characteristics similar to human chronic asthma, namely eosinophilic inflammation, subepithelial matrix deposition, mucus overproduction and expression of pro-fibrotic mediators [18]. The aim of this study was to determine the effects of therapeutic administration of Budesonide on the development of airway remodelling in order to identify potential mechanisms of action of steroids. Moreover, we investigated the effect of Budesonide treatment on active TGF- β signalling in airway lesions. Importantly, we administered Budesonide during the chronic phase of allergen-induced airway inflammation, after eosinophilic inflammation was established as this mirrors the therapeutic protocols usually employed by asthmatic patients.

Materials and methods

Allergen-induced airway inflammation and remodelling

Airway inflammation and remodelling was induced in female Balb/c mice sensitized with ovalbumin (OVA) in alum as previously described [18]. Prolonged inflammation was induced by subsequent exposure to aerosolized OVA (5%) three times a week for 20 min until groups of mice were sacrificed on days 35 and 55. A therapeutic regimen was instigated by initiating Budesonide treatment (Pulmicort, AstraZeneca, UK, 1mg/kg intra peritoneal (i.p.)) on day 26, after the establishment of considerable lung eosinophilia and airway hyper-reactivity. Control mice received the same dose of vehicle (phosphate-buffered saline (PBS)). Mice were sacrificed on day 35 (after four doses of Budesonide and OVA challenges) or on day 55 (after 13 Budesonide doses and OVA challenges). For comparison in some figures, we have included two more groups of mice: mice which have been sensitized and challenged through the airways with OVA but sacrificed on day 24 of the acute phase of allergic inflammation (day 24 group) and mice which have been given a sham immunization with alum alone (alum group) and challenged with OVA before sacrifice on day 55. The challenge and treatment protocol is shown in Fig. 1.

Measurement of airway hyper-reactivity

Airway responsiveness was measured indirectly by whole body plethysmography to calculate enhanced pause (Penh: Buxco Technologies, Winchester, UK). Response to inhaled methacholine (Sigma, Poole, UK) at concentrations of 3–100mg/mL were measured for 1 min, as described previously [19]. Results are shown for Penh on day 55 during prolonged allergen challenge in mice-treated with Budesonide or vehicle (PBS) in comparison with sham immunized alum group.

Quantification of pulmonary inflammation

Cell recovery from airway lumen—BAL was performed as described [20]. Briefly, the airways of the mice were lavaged three times with 0.4mL of PBS via a tracheal cannula. BAL fluid was centrifuged (1200 rpm, 5 min, 4 °C); cells were counted then pelleted onto glass slides by cytocentrifugation (5×10^4 cells/slide). Differential cell counts were performed on Giemsa (Shandon, Runcorn, UK) stained cytopins. Percentages of eosinophils, lymphocytes/monocytes, neutrophils and macrophages were determined by counting their number in eight high-power fields ($\times 40$ magnification; total area 0.5mm²/area) randomly selected and then dividing this number by the total number of cells per high-power field. To obtain absolute numbers of each leucocyte subtype these percentages were multiplied by the total number of cells obtained in the lavage fluid. All differential counts were performed blind and in a randomized order at the end of the study by the same observer.

Lung tissue histopathology

Paraffin-embedded sections (4 µm) were stained with hematoxylin/eosin (H&E) to evaluate general morphology. A semi-quantitative scoring system was used to grade the size of lung infiltrates, whereby +5 signified a large (>3 cells deep) widespread infiltrate around the

majority of vessels and bronchioles, and +1 signifies a small number of inflammatory foci [21].

Quantification of mucus producing goblet cells

Goblet cells were counted using an arbitrary scoring system, as previously described [22, 23]. Briefly, periodic acid Schiff (PAS)-stained goblet cells in airway epithelium were measured double-blind using a numerical scoring system (0 = <5% goblet cells; 1 = 5–25%; 2 = 25–50%; 3 = 50–75%; 4 = 75–100%). The sum of the airway scores from each lung was divided by the number of airways examined (20–30/mouse), and expressed as mucus score in arbitrary units.

Measurement of airway remodelling

Airway remodelling was assessed by determining the amount of ECM deposition in the lungs, by two different methods. Total peribronchiolar matrix deposition was assessed on Martius Scarlet Blue (MSB) stained sections. In addition a biochemical assay was used to determine total lung tissue levels of collagen.

Image analysis of peribronchiolar matrix deposition

Image analysis was performed on MSB stained lung sections using Scion Image Analysis software package (Scion Corporation, Frederick, MD, USA) as described previously [18, 24]. Digital photographs of four bronchioles per tissue section were taken at $\times 40$ magnification and these images were converted into monochrome. Ten measurements of 20 μm lines from each of the four bronchioles were drawn at a right angle from the basement membrane into the submucosa and the mean density of staining intensity along the 20 μm was calculated and expressed as pixels per μm^2 .

Lung collagen Assay

Total lung collagen was measured using a collagen assay kit that uses a dye reagent which selectively binds to the [Gly-X-Y]_n tripeptide sequence found within the triple helix sequence of mammalian collagens (Biocolor, Belfast, Ireland), a method described previously for the quantification of lung collagen [18, 25, 26].

Assessment of active transforming growth factor - β signalling

Paraffin-embedded lung tissue sections (4–5 μm) were cut from Budesonide or vehicle-treated mice and fixed in 2% paraformaldehyde (Sigma) for 20 min. Endogenous peroxidase activity was blocked by incubation with 1.5% H_2O_2 in methanol for 30 min. Thereafter, endogenous avidin and biotin were blocked using a blocking kit (Vector Laboratories, Peterborough, UK). All incubations were carried out in humidified chambers and slides were washed between steps for 15 min each in PBS containing 0.1% saponin (Sigma). Non-specific binding was blocked using 10% donkey serum in PBS for 20 min. Sections were subsequently incubated with the primary antibodies for 90 min. Polyclonal rabbit antibodies against phosphorylated Smad 2, (pSmad 2), Smad 7 and the TGF- β type I receptor, also known as ALK-5, were raised and used as previously described [17]. Normal rabbit IgG (Sigma) was used as a negative control. Sections were then incubated with biotinylated goat

anti-rabbit secondary antibody (Strattech Scientific, Soham, UK) for 30min, followed by streptavidin complex (DAKO, Ely, UK) for another 30 min. Slides were developed for approximately 5 min in diaminobenzidine (DAB, Vector Laboratories) and counterstained in Gills haematoxylin. Sections were subsequently dehydrated, mounted and studied by light microscopy.

Cytokine analysis

Cytokines were analysed in supernatants generated from homogenising isolated lobes of lung. Paired antibodies for murine IL-4, IFN- γ and active TGF- β 1 (all Pharmingen, Oxford, UK), IL-5 (Perbio, Tattenhall, UK), eotaxin-1 (CCL11) (R&D systems, Abingdon UK) were used in standardized sandwich ELISAs according to the manufacturer's protocol. Kits to measure IL-13 were purchased from R&D Systems.

Statistical analysis

Results were expressed as mean $\pm$ SEM with a group size of 6–8 from four different experiments. Data were analysed by ANOVA (Kruskal–Wallis) or Mann–Whitney *U*-test where appropriate and statistical significance was accepted when $P < 0.05$.

Results

Administration of Budesonide decreased allergen-induced airway hyper-reactivity

Airway hyper-responsiveness is a characteristic feature of the pulmonary allergic response. We determined the extent of AHR to methacholine during prolonged allergen challenge and assessed the effects of a therapeutic treatment regimen with Budesonide. Figure 2 shows dose response curves measured for Budesonide or vehicle treated mice 24 h after allergen challenge on day 55. Therapeutic administration of Budesonide significantly reduced AHR over a dose range of 10–100mg/mL inhaled methacholine.

Budesonide treatment reduced established pulmonary inflammation

Airway lumen

Inflammatory cell recruitment to the airways is thought to be a major factor in the development of allergen-induced pathophysiology. We determined the total number of cells within the airway lumen by BAL from mice treated with Budesonide or vehicle during prolonged allergen challenge. Therapeutic treatment with Budesonide reduced the total numbers of inflammatory cells within BAL (Fig. 3a). Differential counts of Wright–Giemsa stained cytopins revealed that Budesonide treatment significantly reduced the numbers of eosinophils, neutrophils and lymphomononuclear cells in airway infiltrates in comparison with vehicle-treated mice (Fig. 3a).

Lung tissue

We determined the extent of leukocyte recruitment to the lung tissue by examination of H&E stained histological sections (Figs 3b and c). The degree and extent of inflammation was assessed blind and assigned a semi-quantitative score (Fig. 3d). Prolonged OVA challenge of vehicle treated mice-induced widespread peribronchiolar inflammation. The

beneficial effect of Budesonide treatment on the extent of inflammation was reflected in a significantly reduced score, compared with vehicle controls.

Administration of Budesonide reduced established goblet cell hyper-plasia

Excessive mucus secretion from hyper-plastic goblet cells is a characteristic feature of chronic asthma and contributes to morbidity and mortality. Indeed, prolonged allergen challenge results in increased numbers of mucus producing goblet cells [18]. Mucus secreting cells were quantified in PAS stained sections from mice treated with vehicle or Budesonide. Therapeutic administration of Budesonide significantly reduced the number of mucus producing goblet cells within the respiratory epithelium on day 55 during prolonged allergen challenge in comparison with vehicle-treated mice (mean $\pm$ SEM: vehicle, 1.54 ± 0.35 ; Budesonide, 0.22 ± 0.11).

Therapeutic administration of Budesonide decreased peribronchiolar extracellular matrix deposition

Dysregulation of ECM proteins during tissue remodelling results in increased deposition within tissues. Subepithelial collagen deposition is characteristic of the remodelled airway. We determined the extent of ECM deposition in lung tissue from allergen challenged mice histologically, by staining lung sections with MSB and performing quantitative image analysis of collagen density in the airways. In addition, we determined levels of collagen within the lung tissue by biochemical assay. Prolonged allergen challenge of sensitized mice results in increased collagen deposition in the airways (Fig. 4a; [18]). Collagen deposition was substantially reduced in lungs from mice treated with Budesonide in comparison with vehicle-treated mice (Fig. 4b). In order to quantitate these differences in matrix deposition we performed image analysis to determine the relative density of matrix in 20 μ m lengths of the basement membrane in lung sections from each group of mice. Figure 4c shows that treatment with Budesonide significantly reduced the collagen density in the airways of mice on days 35 and 55 during prolonged allergen challenge. Further quantitation of collagen was made by biochemical analysis of lung collagen content. Again, there was a significant reduction in total lung collagen content after treatment with Budesonide at both time points in comparison with vehicle-treated mice (Fig. 4d).

Therapeutic Budesonide treatment reduced inflammatory mediator production in the lung

In order to gain some insight as to how treatment with Budesonide modulates matrix deposition, we determined the concentration of the Th2 cytokines, IL-4, IL-5 and IL-13 and eotaxin-1/CCL11 in the lung. Therapeutic treatment with Budesonide significantly reduced lung tissue levels of IL-4, IL-13 and eotaxin-1 on day 35 in comparison with vehicle-treated mice (Figs 5a, b and d, respectively). In contrast, Budesonide had no effect on lung tissue levels of IL-5 (Fig. 5c), or on any mediator at day 55. Interestingly, therapeutic administration of Budesonide significantly increased lung tissue levels of IFN- γ on day 35 in comparison with vehicle-treated mice (mean (pg/mL) $\pm$ SEM: vehicle, 389.7 ± 11.19 ; Budesonide, 468.8 ± 23.34). We also looked at the effect of Budesonide on levels of the pro-

fibrotic mediator TGF- β 1 and found that levels of active TGF- β 1 were not significantly reduced by treatment (mean (pg/mL) $\pm$ SEM: vehicle, 213.3 $\pm$ 65.63; Budesonide, 227 $\pm$ 29.0).

Therapeutic Budesonide treatment modulated transforming growth factor - β signalling pathways

Although we demonstrated that Budesonide treatment reduced collagen deposition in the airways we did not observe any reduction in lung TGF- β 1 levels by ELISA. Recent reports have demonstrated an improved method for investigating the expression of active TGF- β signalling *in-situ* [17, 27], which involved examination of the expression of the intracellular effectors, Smads. Therefore, we next investigated the expression patterns of pSmad2, ALK-5 and Smad 7 in lung following Budesonide administration in order to investigate any effect on active TGF- β signalling in airway lesions. An increase in expression of pSmad2 was observed during prolonged allergen challenge. Strong expression of pSmad2 was detected in bronchial epithelial cells, inflammatory infiltrates, fibroblast-like cells and alveolar macrophages in vehicle-treated mice (Fig. 6a). A number of the positive cells showed nuclear translocation of pSmad 2, indicating increased TGF- β signalling (Fig. 6a inset). Administration of Budesonide considerably decreased pSmad2 expression on day 55 during prolonged allergen challenge. Only a few bronchiolar epithelial cells and alveolar macrophages were positive whereas none of the infiltrating leukocytes expressed pSmad2 (Fig. 6b). ALK-5 was readily expressed in bronchial epithelial, inflammatory cells and in airway smooth muscle cells in vehicle treated mice during prolonged allergen challenge (Fig. 6c). Administration of Budesonide decreased ALK-5 expression in all cell populations on day 55 (Fig. 6d). Only weak Smad 7 immunostaining was observed in some bronchiolar epithelial cells and alveolar macrophages in vehicle-treated mice on day 35 during prolonged allergen challenge (Fig. 6e). Interestingly, marked up-regulation of Smad7 expression was detected in bronchiolar epithelium, infiltrating leukocytes and alveolar macrophages upon administration of Budesonide (Fig. 6f).

Discussion

Airway remodelling is increasingly recognized to occur as a consequence of chronic asthma. However, current therapeutic strategies of long-term steroid treatment do not always ameliorate the structural changes observed in the airways. Therefore, we have used a mouse model of prolonged allergen challenge that incorporates many of the features of the human disease in order to investigate the pathophysiological mechanisms underlying the effect of Budesonide on the remodelling process. We have determined that therapeutic administration of Budesonide is able to reverse established inflammation and AHR, and to ameliorate airway remodelling through a decrease in matrix deposition and mucus production. Moreover, we have shown that Budesonide modulates airway remodelling via a molecular mechanism involving regulation of active TGF- β signalling in the lung.

Airway inflammation is a characteristic feature of allergic airway disease and infiltrating inflammatory cells to the airways are thought to contribute to the remodelling response [28]. Administration of Budesonide after the onset of acute eosinophilic airway inflammation was

found to decrease BAL cellular inflammation during chronic allergen challenge. In addition, therapeutic treatment with Budesonide significantly reduced the size and extent of leukocyte infiltration to the lung. Moreover, therapeutic administration of Budesonide significantly reduced the number of mucus producing goblet cells within the bronchiolar epithelium. In support of our data, steroid treatment demonstrated an anti-inflammatory effect and decreased cell numbers, specifically eosinophils in the airways of human asthmatics [2, 29]. The observed reduction in pulmonary inflammation and mucus production might result from the concomitant reduction in lung tissue levels of Th2 type cytokines, IL-4 and IL-13 on day 35 following treatment with Budesonide [22, 30, 31]. In addition, the decreased levels of eotaxin-1 observed in the lungs of Budesonide treated mice, possibly resulted in a concomitant decrease of eosinophil recruitment to allergic airways. We have recently shown that eosinophils are critical for the development of airway remodelling [32]. It is conceivable that Budesonide mediates its anti-remodelling effect at least partly through modulation of eosinophilic inflammation. However, it is interesting to note that Budesonide had no effect on the levels of IL-5 in the lung.

Airway inflammation and structural changes in the airways of asthmatics are associated with an increase in airway hyper-reactivity to stimulants such as methacholine. Administration of Budesonide significantly inhibited development of established AHR during prolonged allergen challenge. In agreement with this data, administration of steroids to asthmatics generally results in a beneficial effect on lung function [33]. Similarly other studies using acute allergen challenge in the mouse have shown that Budesonide successfully reduces both inflammation and AHR [34–36]. However, we have shown for the first time that Budesonide reduces AHR and inflammation in a prolonged allergen challenge model using a therapeutic regimen. In our study, Budesonide treatment resulted in an early reduction in lung IL-13, which is thought to be critical in the establishment of AHR [22, 31]. Thus, the beneficial effect of Budesonide in AHR could also be derived from the observed reduction in pulmonary inflammation.

Administration of Budesonide after inflammation was established resulted in an abrogation of airway remodelling during prolonged allergen challenge. Budesonide treatment significantly decreased the density of the subepithelial region of bronchioles in MSB stained lung tissue sections. Moreover, administration of Budesonide significantly reduced lung tissue levels of total collagen during our chronic allergen challenge model. Similarly, a significant reduction in ECM deposition was observed following steroid treatment in asthmatic patients [6–8]. Steroids may be able to influence matrix production directly, via transcriptional inhibition of promoter activity, as has been shown for dexamethasone-induced down-regulation of type VII collagen activity [37]. Alternatively, the anti-fibrotic activity of steroids may be indirect in that they may reduce cytokines and/or growth factors which influence the fibrotic process. The anti-inflammatory effect of steroids such as Budesonide is mediated by inhibition of inflammatory cytokine-induced gene expression, via regulation of transcription factors such as AP-1 and nuclear factor (NF)- κ B (reviewed by Barnes [38]). In support of this Budesonide caused a reduction in the airway remodelling in our model concomitant with reduced IL-4 and IL-13 levels at day 35 during the induction phase of lung remodelling. IL-4 and IL-13 are known to be able to influence matrix production and proliferation of fibroblasts mainly through the production of TGF- β 2 from

bronchial epithelial cells [39–41]. Experiments *in vitro* have shown that addition of the steroid, fluticasone propionate, to cultures of myofibroblasts rapidly inhibited IL-4 and IL-13-induced JAK-STAT signal transduction in lung myofibroblasts [40]. Moreover, Budesonide was found to inhibit IL-13 or IL-4 mediated production of VEGF from *in vitro* cultured airway smooth muscle cells [42]. Budesonide treatment also upregulated IFN- γ production in the lung on day 35 of our model. IFN- γ not only blocks TGF- β signaling but directly inhibits collagen production from fibroblasts *in vivo* and *in vitro* [43]. Collectively, our data suggest that Budesonide might influence the remodelling process *in vivo* by modulation of mediator secretion.

Many reports demonstrate that steroid treatment of asthmatics had no effect on the expression of TGF- β [44–46]. Moreover, it has been suggested that the failure of steroids to decrease collagen deposition might be because of persistently elevated TGF- β expression [46]. Although we demonstrated that Budesonide treatment reduced collagen deposition in the airways we did not observe any reduction in lung active TGF- β 1 levels by ELISA. Recent reports have demonstrated an improved method for investigating the expression of active TGF- β signalling *in-situ* [17, 27], which involved examination of the expression of the intracellular effectors, Smads. Indeed, an up-regulation of phosphoSmad2 expression in lungs was observed after acute allergen challenge in both animal and human studies which also correlated with increased basement membrane thickening of the asthmatic airways [17, 27]. In order to investigate the effect of Budesonide treatment on active TGF- β signalling we investigated the expression of these molecules in the lung tissue. We show here for the first time that therapeutic administration of Budesonide regulated active TGF- β signalling in the airways as demonstrated by a decrease in pSmad2 and ALK5 (TGF- β type I receptor expression concomitant with an increase in the inhibitory molecule, Smad 7. In agreement with our findings, recent studies have shown that inflammatory molecules such as IFN- γ inhibit TGF- β function by regulating its signaling pathways (through a decrease in the intracellular effector pSmad2 and an induction of its inhibitor, Smad 7) [27, 47]. Moreover, it has been proposed that steroids also inhibit TGF- β functions through transcriptional inhibition of promoter activity, such as collagen genes in dermal fibroblasts, however the exact mechanism has not yet been shown [37]. Previously, *in vitro* studies have shown that steroids modulate active TGF- β signaling through a down-regulation of the constitutive TGF- β type II receptor [48] and an up-regulation of Smad 7 expression [49]. Here, we show induction of Smad 7 expression by Budesonide in the mouse lung. Previously a clinical study observed that expression of Smad7 in bronchial epithelial cells inversely correlated with basement membrane thickness and airway hyper-responsiveness in patients with asthma [49]. Moreover, adenoviral delivery of Smad7 has been shown to inhibit the fibrotic effect of TGF- β on renal tubular epithelial cells by blocking Smad2 activation [50]. Our data indicate that the anti-fibrotic effects of Budesonide are not mediated by down-regulation of TGF- β at the protein level but interestingly through inhibition of its signalling pathway.

In conclusion, our results suggest that Budesonide treatment reduces airway remodelling through mechanisms involving decrease in pulmonary inflammation and the production of cytokines as well as modulation of active TGF- β signalling in the lung.

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Fig. 1.

Schematic overview of study protocol for induction of prolonged allergen challenge and therapeutic administration of budesonide. Airway inflammation and remodelling was induced in female Balb/c mice sensitized with ovalbumin (OVA) in alum, and subjected to serial aerosolized OVA challenge. Prolonged allergic inflammation (chronic phase) was induced by subsequent exposure to aerosolized OVA three times a week until groups of mice were sacrificed on days 35 and 55. A therapeutic regimen of Budesonide was instigated on day 26, after acute eosinophilia had been established, and mice were given 1 mg/kg before each airway challenge in the chronic phase. A group of mice received vehicle (phosphate-buffered saline) instead of Budesonide and served as the control. Analysis was also performed on a group of OVA-treated mice on day 24 (acute phase of allergic inflammation) and on alum-treated mice for comparisons.

Fig. 2.

Therapeutic Budesonide treatment decreased established airway hyper-reactivity (AHR). AHR was measured 24 h after the final ovalbumin challenge on day 55 (chronic phase) using a Buxco system where mice were exposed to increasing concentrations of methacholine (3–100 mg/mL). Results are shown for Penh after allergen challenge in mice treated with Budesonide in comparison with vehicle-treated mice and alum control mice. Values are expressed as mean $\pm$ SEM, $n = 6$ –8/group/time point, * $p < 0.05$ in comparison with vehicle-treated mice.

Fig. 3. Therapeutic Budesonide treatment decreased established pulmonary inflammation. (a) Differential cell counts in BAL from mice treated with Budesonide or vehicle on days 35 and 55 during prolonged allergen challenge. Mice were sacrificed 24 h after the final ovalbumin (OVA) challenge and BAL cells were isolated as described in Materials and methods. Values are expressed as mean $\pm$ SEM, $n = 6-8$ /group, $*p < 0.05$ in comparison with vehicle-treated mice. (b-c) Representative photomicrographs of hematoxylin and eosin (H&E) stained lung sections from OVA challenged mice treated with either vehicle (b) or Budesonide (c) on day 55 are shown (original magnification $\times 40$). (d) Semi-quantitative analysis of H&E stained lung tissue sections from mice treated with Budesonide or vehicle during prolonged allergen challenge according to the methods described in Materials and methods section. Data are expressed as mean $\pm$ SEM, $*p < 0.05$ in comparison with vehicle-treated mice.

Fig. 4.

Therapeutic Budesonide treatment decreased the development of airway remodelling. (a, b). Representative photomicrographs of paraffin embedded (4 µm) Martius Scarlet Blue (MSB)-stained lung sections from mice treated with vehicle (a) or Budesonide (b) on day 55 are shown (original magnification $\times 40$). (c). Image analysis of MSB stained lung sections from mice treated with Budesonide during prolonged allergen challenge. Random measurements from the basement membrane into the submucosa (10 measurements of 20 µm in length) were taken and the mean density calculated from four bronchioles per mouse, $n = 6-8$ mice per group, $*p < 0.05$ in comparison with vehicle-treated mice. (d). Levels of total collagen were measured in lung homogenate samples taken from mice treated with Budesonide or vehicle during prolonged allergen challenge. Data are expressed as mean $\pm$ SEM, $n = 6-8$ /group. $*p < 0.05$ in comparison with vehicle-treated mice.

Fig. 5.

Therapeutic Budesonide treatment decreased lung tissue levels of inflammatory mediators during prolonged allergen challenge. Levels of IL-4 (a), IL-13 (b), IL-5 (c) and eotaxin-1 (d) were measured in lung tissue homogenate samples by ELISA in mice treated with Budesonide or vehicle during prolonged allergen challenge. Data are expressed as mean $\pm$ SEM, $n = 6-8$ /group/time point; * $p < 0.05$ in comparison with vehicle-treated mice.

Fig. 6. Modulation of active transforming growth factor- β signalling by Budesonide treatment during prolonged allergen challenge. Paraffin-embedded lung tissue sections from mice treated with vehicle (a, c, e) or Budesonide (b, d, f) were stained with specific antibodies to pSmad 2, ALK5 and Smad 7 (original magnification $\times 40$, except for 6a inset $\times 100$). Positive staining is depicted in brown and nuclei are stained blue. White arrows in a and inset show translocation of pSmad2 to the nucleus, compared with cytoplasmic staining (black arrow). Data are representative of $n = 4$ per group and Ig controls were negative.