Combination therapy with relaxin and methylprednisolone augments the effects of either treatment alone in inhibiting subepithelial fibrosis in an experimental model of allergic airways disease

Simon G. ROYCE*, Amelia SEDJAHTERA*, Chrishan S. SAMUEL†‡§ and Mimi L. K. TANG*∥¶

*Department of Allergy and Immune Disorders, Murdoch Children’s Research Institute, Parkville, Victoria 3052, Australia
†Howard Florey Institute, Parkville, Victoria 3052, Australia
‡Department of Biochemistry and Molecular Biology, University of Melbourne, Parkville, Victoria 3010, Australia
§Department of Pharmacology, Monash University, Clayton, Victoria 3800, Australia
∥Department of Allergy and Immunology, The Royal Children’s Hospital, Parkville, Victoria 3052, Australia
¶Department of Paediatrics, University of Melbourne, Parkville, Victoria 3010, Australia

Abstract
Although CSs (corticosteroids) demonstrate potent effects in the control of airway inflammation in asthma, many patients continue to experience symptoms and AHR (airway hyper-responsiveness) despite optimal treatment with these agents, probably due to progressive airway remodelling. Identifying novel therapies that can target airway remodelling and/or airway reactivity may improve symptom control in these patients. We have demonstrated previously that the anti-fibrotic hormone RLN (relaxin) can reverse airway remodelling (epithelial thickening and subepithelial fibrosis) and AHR in a murine model of AAD (allergic airways disease). In the present study, we compared the effects of RLN with a CS (methylprednisolone) on airway remodelling and AHR when administered independently or in combination in the mouse AAD model. Female mice at 6–8 weeks of age were sensitized and challenged to OVA (ovalbumin) over a 9-week period and treated with methylprednisolone, RLN, a combination of both treatments or vehicle controls. Methylprednisolone was administered intraperitoneally on the same day as nebulization for 6 weeks, whereas recombinant human RLN-2 was administered via subcutaneously implanted osmotic mini-pumps from weeks 9–11. RLN or methylprednisolone alone were both able to significantly decrease subepithelial thickness and total lung collagen deposition; whereas RLN but not methylprednisolone significantly decreased epithelial thickness and AHR. Additionally, combination therapy with CS and RLN more effectively reduced subepithelial collagen thickness than either therapy alone. These findings demonstrate that RLN can modulate a broader range of airway remodelling changes and AHR than methylprednisolone and the combination of both treatments offers enhanced control of subepithelial fibrosis.

Key words: airway remodelling, allergic airways disease, asthma, corticosteroid, relaxin

INTRODUCTION
Asthma is a chronic airway disease associated with bronchoconstriction that is characterized by three key features: AHR (airway hyper-responsiveness), airway inflammation [1] and airway remodelling (the structural changes that occur in the airways). New therapies should address all of these three aspects [2]. Persistent airway inflammation and excessive cellular infiltrate surrounding the lining of the airway is believed to contribute to AHR [3]. However, abrogation of inflammation by CS (corticosteroid) treatment fails to eliminate AHR or the progressive loss of lung function [4]. Structural changes in the airway wall, known
as airway remodelling, have been suggested to contribute to the manifestation of AHR [3].

Airway remodelling is characterized by abnormal deposition of collagen (fibrosis) in the subepithelial basement membrane region and deeper in the airway wall, damage of the epithelial cells (epithelial shedding), goblet cell metaplasia, angiogenesis and airway smooth muscle hyperplasia and hypertrophy. These changes contribute to thickening of the airways, increased secretion of mucins into the airway lumen and AHR. Airway remodelling also correlates with severity of asthma [5–7]. Of note, high levels of collagen deposition are observed before the development of chronic inflammation in young children [8]. This implies that thickening of the basement membrane could actually occur via mechanisms other than inflammation that have not yet been fully elucidated [1,2].

ICSs are potent anti-inflammatory drugs that are currently used to control airway inflammation in asthma. Binding of CS to the GR (glucocorticoid receptor) can reduce the expression of cytokines that are normally activated during chronic inflammation in asthma [9]. Many studies have suggested the potential of CS to reverse structural changes in the airway via increasing the rate of collagen degradation [10,11], but this action still remains controversial. A reduction in sub epithelial thickness was observed in acute asthma patients following ICS (inhaled CS) administration and this was correlated with improved lung function [11]. In contrast, prolonged administration of CS was unable to reduce several airway changes [4], including collagen thickness, in comparison with placebo treatment [12], suggesting the inability of CS to consistently prevent or reverse airway remodelling. Furthermore, treatment with ICS was unable to reduce the expression of TGF-β1 (transforming growth factor-β1), one of the main factors that drives airway fibrosis and remodelling [13]. Of concern, prolonged use of higher doses of CS can cause adverse effects such as adrenal suppression, abnormal growth, skin bruising, reduced bone density [14]; and although CS are effective in controlling symptoms in most patients with asthma, many sufferers fail to respond to ICS [15].

We have reported previously that the reproductively and lung-derived hormone RLN (relaxin) can improve AHR, through reversal of airway fibrosis and epithelial structural changes. The major circulating and stored form of RLN found in humans, H2 RLN (equivalent to RLN in rodents) [16] mediates its effects via the LGR7 [LRR (leucine-rich repeat) containing GPCR (G-protein-coupled receptor) 7] [17] that has been re-named RXFP-1 (RLN family peptide receptor-1) [18], to modulate a number of downstream signal transduction pathways. In asthma, it has been shown that the presence of RLN in the lung is important in controlling airway fibrosis [19] and smooth muscle thickening [20,21]. RLN- {-/−} and RXFP-1- {-/−} knockout mice (Rln−/- and RXFP-1−/-) underwent an increased deposition of ECM (extracellular matrix) in the airway and lung which correlated with increased total lung collagen content and decreased lung function [20,21]. Importantly, the treatment of RLN-deficient mice with recombinant RLN reversed the fibrotic lung phenotype [22]. Furthermore, the administration of exogenous RLN to wild-type mice sensitized and challenged with OVA (ovalbumin) reversed airway remodelling changes (airway fibrosis and epithelial thickening) and improved AHR [24]. In addition to its well-characterized interaction with RXFP-1, RLN has also been observed to act as a GR agonist and to activate glucocorticoid downstream pathways [25].

On the basis of these findings, the aim of the present study was to compare the airway remodelling effects of RLN with that of a synthetic CS in clinical use (methylprednisolone) and to evaluate the efficacy of combination treatment with RLN and methylprednisolone in a well-established murine model of chronic AAD (allergic airway disease) that mimics several features of human asthma.

**MATERIALS AND METHODS**

**Animals**

Female wild-type BALB/c mice at 6–8 weeks of age (18–22 g in weight) were maintained under specific pathogen-free conditions in the animal housing facility at the MCRI (Murdoch Children’s Research Institute) and were maintained under a fixed lighting schedule with access to food and water ad libitum. Female mice were used because they demonstrate better responses in OVA AAD models [26]. All experimental procedures were performed according to the regulations approved by the MCRI’s Animal Ethics Committee which follows the Australian Guidelines for the Care and Use of Laboratory Animals for Scientific Purposes.

**Chronic mouse model of AAD**

The experimental protocol for the AAD model in mice was performed as described previously [21,24,27]. Mice were sensitized with grade V OVA (Sigma) on days 1 and 14. Sensitization was achieved via intraperitoneal injection with 10 μg of OVA and 1 mg of alum (aluminium potassium sulfate adjuvant) in 500 μl of saline (Baxter). Mice were challenged with aerosol solution of 2.5% (w/v) OVA in saline solution for 30 min using an ultrasonic nebulizer (NE-U07; Omron) three times a week for 6 weeks (from days 21 to 63). Control animals were sensitized with 1 mg of alum in 500 μl of saline and nebulized with saline solution. The mice were assigned to seven different groups (n = 20 per group) according to their treatments: saline, OVA, OVA CS vehicle control (CS control), OVA CS, OVA RLN vehicle control (RLN control), OVA RLN and OVA CS and RLN (CS RLN).

**Methylprednisolone (CS) and RLN treatments**

Mice were treated with 0.3 mg of methylprednisolone/kg of body weight (Hospira) or vehicle (saline) control (intraperitoneal) in 500 μl of saline over the 6-week period that they received nebulized OVA (from days 21 to 63), i.e. the injections were given three times per week during the challenge period. At the end of the challenge period with OVA/saline, mice were treated with recombinant H2 human RLN (0.8 mg/ml; kindly provided by Corthera) or sodium acetate buffer (20 μmol/ml; vehicle) and delivered via ALZET mini osmotic pumps (model 2002; Durect) at 0.5 μl/h for 14 days (from weeks 9 to 11). The pumps were implanted subcutaneously and were left for 14 days, since rodents mount antibody responses to exogenous RLN after this time [22]. RLN has been shown previously to reverse established airway remodelling when administered at the end of the chronic AAD
model [24], without affecting airway inflammation, whereas CS are known to suppress airway inflammation and thereby may prevent airway remodelling if administered during the challenge period [29].

Measurement of AHR

Methacholine-induced AHR was measured by invasive plethysmography 14 days after the last nebulization treatment (Biosystem XA version 2.7.9; Buxco Electronics) [21,24,27]. Mice were anaesthetized, a tracheotomy was performed and the jugular vein cannulated to deliver acetyl-β-methacholine (Sigma). Baseline airway resistance was recorded for 5 min, then up to five increasing doses of methacholine were delivered, with airway resistance recorded. Tidal flow was derived from the differentiation of the volume signal. RL and Cdyn were then calculated by fitting an equation of motion to the measurements of pressure, flow and volume. In this equation, $\text{PTP} = V \times \text{RL} + \text{VT} / \text{Cdyn}$, PTP is the transpulmonary pressure, $V$ is the tidal airflow, RL is the pulmonary resistance, VT is the tidal volume and Cdyn is the dynamic pulmonary compliance [30]. Mice were killed via cardiac puncture. The blood was collected and the serum was stored for measurement of OVA-specific IgE levels. The largest lung lobe was fixed in 10% formalin overnight and the remaining lobes were frozen in liquid nitrogen and stored at −80°C for subsequent analyses.

Quantification of serum OVA-specific IgE levels

The OVA-specific IgE levels were determined by measuring the concentration based on the standard curve for each run (ELISA unit) [21,24,27]. The levels obtained were expressed as arbitrary units (AU) where 1 AU = $A$ (absorbance) of 1:50 dilution of a standard positive control.

BAL (bronchoalveolar lavage)

The BAL protocol involved for inflating and washing the mouse airways three times with 500 μl of PBS. Total viable cell counts were calculated using a haemocytometer and Trypan Blue exclusion (Sigma). Differential cell counts were determined by counting approximately 200 cells under a light microscope [21,24,27].

Processing of lungs for histology

The largest lung lobe from each mouse was fixed by instillation of 500 μl of 10% NBF (neutral-buffered formalin) and placed in ice-cold 10% NBF before being fixed overnight for routine processing. The lung tissue was cut transversely and processed in ice-cold 10% NBF before being fixed overnight for routine processing. The lung tissue was cut transversely and processed in ice-cold 10% NBF before being fixed overnight for routine processing.

Masson's trichrome stain

The slides were stained in water and incubated in pre-warmed Bouin's fluid for 1 h at 60°C. The slides were then stained with a mixture of Weigert's A and B (1:1) (Amber Scientific) for 10 min and Masson's Red for 1 h. After the incubation with 1% phosphotungstic acid for 7 min, the slides were transferred to Aniline Blue solution and stained for 20 min [21,24,27].

Image analysis

The analysis for each parameter tested in each different staining was performed as described previously [21,24,27,31]. It was performed by selecting four or five different airways that were 150–350 μm in diameter. The pictures were taken using Q-capture digital camera (SciTech). The mean thickness of epithelial and subepithelial regions stained was traced using a digitizer pad and the length determined by Image Pro-discovery software (Media Cybernetics). Epithelial thickness was measured from the luminal surface of the epithelium to the basement membrane. Subepithelial collagen thickness was a measure of the mean airway wall collagen thickness. The measurement included collagen-stained blue from the Masson’s trichrome-stained basement membrane (i.e. under the basal surface of bronchial epithelial cells), through the lamina propria and submucosal collagen.

Hydroxyproline analysis of lung collagen

Hydroxyproline is an amino acid that is most specific to collagen and used as a marker of collagen content. The protocol was used as described previously [32]. Briefly, frozen lung portions were freeze-dried for dry weight measurements before being hydrolysed in 6 M hydrochloric acid for 24 h at 110°C; for determination of hydroxyproline content in each sample. Hydroxyproline values were then multiplied by a factor of 6.94 to determine total collagen content, as hydroxyproline usually represents ∼14.4% of the amino acid composition of collagen in most mammalian tissues. Collagen content was expressed as a percentage of the tissue dry weight (collagen concentration).

Immunohistochemistry

A rabbit H-300 polyclonal antibody (sc-8992; 1:400 dilution; Santa Cruz Biotechnology), rabbit RXFP-1 (L7-2) [33], polyclonal antibody (1:10 000 dilution) and human RLN-2 monoclonal antibody (2F11; 1:200 dilution; Immunodiagnostics) were used as primary antibodies to detect GR, RXFP-1 and RLN in mouse tissues respectively. Citrate (GR) or EDTA (RXFP-1 and RLN) antigen retrieval was performed by incubating the slides in warmed citrate (pH 6.0) or EDTA (pH 9.0). An EnVision anti-rabbit secondary antibody was used to bind to the polyclonal antibody (GR and RXFP-1), whereas, for the monoclonal antibody, SHRP (streptavidin–horseradish peroxidase) was used. The slides were incubated with DAB (diaminobenzidine) (DakoCytomation). Staining was evaluated by two independent observers and was defined by a scale of 0–3 based on their staining intensity.

Statistical analysis

Results are expressed as means ± S.E.M., except for OVA-specific IgE, where the result is expressed as the median. The results were analysed with a non-parametric Mann–Whitney U test and, for each analysis, $P < 0.05$ was considered to be statistically significant. The data were analysed using the software package Graph Pad Prism 5.0.
RESULTS

Validation of the chronic model of AAD

To validate successful induction of the chronic AAD model in the OVA sensitized/challenged mice, we first confirmed that serum OVA-specific IgE was increased in all OVA sensitized/challenged mouse groups, as compared with respective measurements obtained from saline-treated controls (Table 1).

Effects of RLN and CS treatment on airway inflammation and body weight

Total and differential BAL cell counts

Total BAL cell counts were significantly higher in all six OVA treatment groups compared with saline controls (all $P < 0.001$ compared with control animals; Table 1). There was a significant increase in the percentage of eosinophils in all six OVA treatment groups as compared with saline controls ($P < 0.001$ compared with saline controls; Table 1), without any changes in neutrophils, monocytes and lymphocytes. There were no significant differences in BAL total or differential cell counts, however, in the active treatment groups (CS, RLN and CS + RLN) as compared with the vehicle control mice.

Mouse body weight

All treated mice had equivalent weights before and after treatment (Figure 1), but were increased in weight after the 11-week experimental period ($P < 0.05$ compared with starting body weight for each experimental group). Thus the treatments employed at the concentrations used did not significantly affect the weight of mice.

Effects of RLN and CS treatment on airway remodelling

Subepithelial collagen deposition

Subepithelial collagen thickness was significantly increased in the OVA-alone sensitized/challenged group and both OVA sensitized/vehicle-treated groups compared with that measured in saline controls ($P < 0.001$ compared with saline controls; Figures 2 and 3). This OVA-induced increase in collagen thickness, however, was significantly decreased by methylprednisolone or RLN treatment of OVA-sensitized animals ($35.2 \pm 0.99$ compared with $41.2 \pm 1.8$ μm in the CS and CS vehicle control groups ($P = 0.0094$); and $35.89 \pm 1.0$ compared with $44.6 \pm 1.5$ μm in the RLN and RLN control groups ($P < 0.0001$)) (Figures 2 and 3). Furthermore, combination treatment with methylprednisolone and RLN similarly decreased the collagen thickness ($30.98 \pm 0.95$ ($P < 0.0001$) compared with CS control and RLN control), which was statistically different from the effects of either treatment alone ($P = 0.0024$ and $P = 0.0007$ compared with CS and RLN respectively), but was still higher than that measured in saline control mice ($P = 0.0119$).

Total lung collagen

Total lung collagen content was also significantly increased in the OVA-alone sensitized/challenged group and both OVA sensitized/vehicle-treated control groups as compared with the saline control group (all $P < 0.05$ compared with the saline control group) (Figure 4). This OVA-induced increase in total collagen content was significantly decreased by methylprednisolone alone (by ~60%), but almost completely reversed by RLN alone (by ~93%) in comparison with that measured in the respective OVA-sensitized/vehicle-treated control groups (both $P < 0.05$ compared with respective vehicle-treated groups; Figure 3). A significant reduction in total lung collagen content was also observed in the combination treatment group compared with the OVA-alone and OVA-sensitized/vehicle-treated control groups (by ~85%) ($P < 0.05$ compared with OVA alone and OVA-sensitized/vehicle-treated control groups; Figure 3), which was not significantly different from the effects of H2 RLN alone.

<table>
<thead>
<tr>
<th>Group</th>
<th>Total cells ($\times 10^6$)</th>
<th>Eosinophils (%)</th>
<th>Neutrophils (%)</th>
<th>Monocytes (%)</th>
<th>Lymphocytes (%)</th>
<th>OVA-specific IgE level</th>
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<td>Saline</td>
<td>5.40 ± 0.66</td>
<td>0.36 ± 0.14</td>
<td>0.27 ± 0.12</td>
<td>92.6 ± 1.1</td>
<td>2.85 ± 0.75</td>
<td>0</td>
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<tr>
<td>OVA</td>
<td>15.7 ± 1.44***</td>
<td>2.60 ± 0.64***</td>
<td>0.25 ± 0.13</td>
<td>95.0 ± 0.95</td>
<td>2.1 ± 0.81</td>
<td>1753 ± 242.4***</td>
</tr>
<tr>
<td>CS control</td>
<td>13.7 ± 1.27***</td>
<td>3.7 ± 0.47***</td>
<td>0.36 ± 0.12</td>
<td>93.0 ± 1.1</td>
<td>3.0 ± 0.87</td>
<td>4656 ± 1317***</td>
</tr>
<tr>
<td>CS</td>
<td>12.5 ± 0.63***</td>
<td>3.0 ± 0.32***</td>
<td>0.33 ± 0.11</td>
<td>92.6 ± 1.1</td>
<td>2.89 ± 0.70</td>
<td>1803 ± 380.1***</td>
</tr>
<tr>
<td>RLN control</td>
<td>10.45 ± 0.85***</td>
<td>3.47 ± 0.47***</td>
<td>0.38 ± 0.21</td>
<td>90.7 ± 1.7</td>
<td>3.1 ± 0.78</td>
<td>7805 ± 1750***</td>
</tr>
<tr>
<td>RLN</td>
<td>10.5 ± 1.28***</td>
<td>2.50 ± 0.35***</td>
<td>0.23 ± 0.10</td>
<td>93.4 ± 1.0</td>
<td>2.4 ± 0.45</td>
<td>3468 ± 642.3***</td>
</tr>
<tr>
<td>CS RLN</td>
<td>14.9 ± 1.12***</td>
<td>2.38 ± 0.38***</td>
<td>0.31 ± 0.14</td>
<td>93.6 ± 0.9</td>
<td>3.1 ± 0.65</td>
<td>2403 ± 256.5***</td>
</tr>
</tbody>
</table>

Table 1 Differential cell analysis of lung lavage from different group in chronic AAD model following treatments

***$P < 0.001$ compared with the saline group.

Figure 1 Mouse body weights in the control and treated groups

All treated mice had equivalent weights before and after treatment, but were significantly ($*P < 0.05$ compared with respective starting body weight) increased in weight after the 11-week experimental period. Thus the treatments employed at the concentrations used did not significantly affect the weight of mice.
Treatment of airway remodelling using relaxin and corticosteroids

Figure 2 Representative Masson-trichrome-stained sections from saline (A), OVA (B), CS control (C), CS (D), RLN control (E), RLN (F) and CS RLN (G)

Epithelial thickness and subepithelial collagen deposition were increased in all of the OVA groups when compared with the saline control group. Treatment with methylprednisolone and/or RLN decreased the epithelial and collagen deposition compared with their control groups. Scale bar, 100 $\mu$m. (H) Inset of (G) showing an example of the measurement of the subepithelial collagen thickness (white caliper) and epithelial thickness (yellow caliper).

Epithelial thickening

In a similar trend to subepithelial collagen thickness and total lung collagen content, mean epithelia thickness was significantly increased in OVA alone and OVA-sensitized/vehicle-treated mice compared with that measured in saline control animals (all $P < 0.01$ compared with saline control group; Figure 5). There was no marked effect of CS treatment on the mean epithelial thickness of the airway compared with its vehicle-treated control group ($P = 0.2981$) (Figure 5), whereas RLN treatment significantly decreased epithelial thickness compared with its corresponding vehicle-treated control group (RLN, $34.1 \pm 1.24 \mu m; P = 0.0286$). Similarly the effect of combination treatment [$34.6 \pm 1.01 \mu m (P = 0.0384$ compared with CS control and $P = 0.0298$ compared with RLN control)] returned epithelial thickness back to that observed in saline-treated mice ($P = 0.2614$).

Figure 3 Morphometric analysis of subepithelial thickness in the chronic AAD model following treatments

The Masson-trichrome-stained lung tissue sections from saline and OVA-sensitized/challenged groups were examined by morphometric analysis to measure the collagen deposition in the subepithelial region. The result are the mean thickness of each airway examined, and there was a significant increase in mean subepithelial thickness following OVA sensitization ($*P < 0.05$ and $***P < 0.0001$). There was a significant decrease in the subepithelial thickness following treatment with methyl-prednisolone ($†††P < 0.001$) or RLN ($‡‡‡P < 0.0001$) in comparison with their control groups. Combination treatment with methyl-prednisolone and RLN further reduced the collagen thickness compared with the CS control and RLN control ($†††P < 0.001$ and $‡‡‡P < 0.0001$). Combination treatment effectively reduced the mean subepithelial thickness better than a single treatment alone ($§§P = 0.0024$ compared with the CS group and $¶¶¶P = 0.0007$ compared with RLN group).

Figure 4 Airway collagen content in the chronic AAD model following treatments

Lung samples ($n = 6–12$) were freeze-dried and analysed for the presence of hydroxyproline. There was a marked increase in total collagen content in the OVA-sensitized/challenged groups ($*P < 0.05$). Treatment with methylprednisolone and/or RLN significantly reduced the collagen content ($†P < 0.05$ and $‡P < 0.05$ compared with the control groups) back to the level in the saline group.

AHR

Consistent with the airway remodelling changes reported above, maximal resistance change from baseline AHR measurements were significantly increased in the OVA alone and OVA vehicle-treated groups compared with that measured in the saline control group (both $P < 0.001$ compared with saline group; Figure 6A). These dose–response curves were unaffected by CS vehicle control, CS, and RLN vehicle control treatment of mice, but were significantly reduced by RLN treatment alone ($P < 0.01$ compared with RLN vehicle control) and in combination with CS ($P < 0.05$ compared with CS vehicle control; $P < 0.05$ compared with RLN...
Airway epithelial thickness was observed by using Masson trichrome staining. Values are the mean thickness from each mouse in the groups. A significant increase in the epithelial thickness was observed in groups sensitized with OVA ($* P < 0.05$) compared with the saline control group. There was a significant decrease in the epithelial thickness following treatment with RLN ($‡ P < 0.05$) and combination treatment ($† P < 0.05$ compared with the OVA CS control and $‡ P < 0.05$ compared with the OVA RLN control).

There was no significant difference in PC200 (the methacholine concentration that produced a 2-fold increase in resistance above basal level; Figure 6B). However, at the maximal dose of AHR measured, OVA alone and OVA vehicle treatment of mice significantly increased AHR ($5.41 \pm 0.44$ cmH$_2$O/ml per s in the OVA group compared with $3.07 \pm 0.31$ cmH$_2$O/ml per s in the saline group; $P = 0.0007$) (Figure 6C). Treatment with CS alone did not significantly improve AHR compared with the CS vehicle-treated control group ($4.61 \pm 0.33$ and $5.17 \pm 0.44$ cm of H$_2$O/ml per s respectively; $P = 0.159$). However, AHR was significantly decreased by RLN treatment alone compared with that measured in the RLN vehicle-treated control group ($3.57 \pm 0.37$ and $5.23 \pm 0.43$ cm of H$_2$O/ml per s respectively; $P < 0.0042$). Additionally, combination treatment resulted in reduced AHR ($3.844 \pm 0.3287$ cmH$_2$O/ml per s in CS RLN) compared with the CS vehicle-treated control ($P = 0.0157$) and RLN vehicle-treated control ($P = 0.0251$) groups.

Expression of RLN, the RLN receptor and GR

**Expression of RLN in the airway**

Staining for H2 RLN using the mouse monoclonal antibody (2F1Fred) showed that strong expression of H2 RLN in the mouse lung was localized to the nuclei and cytoplasm of the airway epithelium (Figures 7A–7C). OVA sensitization/challenge resulted in a significant decrease in the expression of RLN (all OVA treatment groups) in comparison with the saline control group (OVA, $P = 0.0382$; CS control, $P = 0.0140$; CS, $P = 0.0089$; RLN control, $P = 0.0447$; CS RLN, $P = 0.0290$). However, there were no differences in RLN expression between the various OVA treatment groups (untreated, CS, RLN, CS + RLN, intraperitoneal vehicle control and subcutaneous vehicle control).

**Expression of the RLN receptor in the airway following treatments**

Staining for the RLN receptor with the L7-2 antibody showed that RXFP-1 was strongly expressed in airway epithelial cells, with moderate expression seen in fibroblasts and airway smooth muscle cells and only weak expression in the parenchyma of the lung (Figures 7D–7F). The saline control group had high mean intensity of staining for the RLN receptor. In the OVA group, there was a significant decrease in the expression of RXFP-1 compared with the saline group ($P < 0.001$). RXFP-1 expression in the CS-treated group was not significantly different from the intraperitoneal vehicle control group. In contrast, RLN-treated mice had significantly increased intensity of RXFP-1 expression compared with the respective subcutaneous vehicle-control group ($P = 0.0269$). Combined treatment with CS + RLN showed a trend towards increased RXFP-1 expression compared with the untreated OVA and vehicle-treated groups; however, these differences did not reach statistical significance.
Treatment of airway remodelling using relaxin and corticosteroids

Figure 7 Immunohistochemical staining for RLN, the RLN receptor and GR
Lung sections from the saline, untreated OVA and treated OVA groups were stained with anti-RLN, -RXFP-1 or -GR (sc-8992) antibodies. (A), (D) and (G) are the negative-control-stained images for each antibody used. (B), (E) and (H) show the expression of RLN, the RLN receptor and GR respectively. Scale bar, 100 μm. (G) Expression of the RLN receptor in the lumen of the airway from each saline and OVA-sensitized/challenged groups was detected by immunohistochemistry (n = 18–20). There was a significant decrease in RXFP-1 expression after sensitization and challenge with OVA in comparison with saline (†††P < 0.01 and ***P < 0.001). Treatment with RLN statistically increased RXFP-1 intensity compared with the RLN control group (#P < 0.05). Scale bar = 100 μm.

DISCUSSION
There is much evidence to suggest the importance of both airway inflammation and airway remodelling in asthma. An increase in the subepithelial collagen thickness correlates with damage of the epithelium and activation of pro-fibrotic and pro-inflammatory factors. Collectively, both airway inflammation and airway remodelling contribute to an increase in AHR. CSs are the current...
mainstay of asthma therapy and are effective in decreasing inflammation in asthma. However, studies suggest that CS have a minimal and limited effect on airway remodelling. Therefore novel therapies that target airway remodelling may offer added benefit for asthma management.

The chronic model of AAD used in the present study resembles the clinical manifestation of chronic allergic asthma and allows study of the pathophysiology of airway remodelling [34]. We observed significantly increased BAL inflammatory cell infiltration in mice sensitized and challenged with OVA as compared with saline sensitized and challenged controls. Airway remodelling changes of increased airway subepithelial collagen deposition, increased total lung collagen content, increased epithelial thickness and goblet cell metaplasia were also observed in the chronic model of AAD. These changes observed in the chronic model of AAD induced in this study are well-characterized and replicate previous findings from our laboratory and the laboratories of the originators of this model [13,39].

There have been many studies demonstrating the importance of both endogenous and exogenous RLN in the inhibition and reversal of fibrosis [21,24,31,35]. Our previous work has shown that exogenous RLN treatment can reverse several structural remodelling changes in a mouse model of chronic AAD, in particular epithelial thickening, subepithelial fibrosis and AHR [24]. In the present study, we examined whether combined treatment with CS and RLN may have additive effects on airway remodelling through the added inhibition of airway inflammation by CS. We have demonstrated that: (i) both methylprednisolone or RLN treatment alone could decrease OVA-induced subepithelial collagen thickness and total lung collagen content; (ii) however, only RLN was able to additionally decrease OVA-induced epithelial thickening and AHR in the mouse model of AAD, suggesting that RLN has more potent airway remodelling actions compared with methylprednisolone; and (iii) combined treatment with methylprednisolone and RLN resulted in enhanced effects on subepithelial collagen deposition compared with the effects of each individual therapy on its own, demonstrating that RLN has potential as an combination therapy to CS for the treatment of airway remodelling in asthma. Although these measures were performed by morphometry, which may be influenced by factors such as underinflation of the lung, our findings that changes in subepithelial collagen (by morphometry) resembled those of total collagen (by hydroxyproline analysis) confirms the significance of the results presented. In addition, we found that RLN and RXFP-1 expression were both significantly decreased in the OVA-induced AAD model although GR expression remained unchanged, and that RLN treatment led to increased RXFP-1 expression, while having no effects on GR expression.

Airway wall fibrosis is prominent in the RBM (reticular basement membrane) and may also occur in the deeper submucosal, around smooth muscle and arterioles. During airway remodelling, the true basement membrane becomes supported by a less ordered excess of ECM proteins constituting the RBM [36]. RBM thickness can be used as a surrogate marker for smooth muscle area, submucosal gland area and entire wall thickness. Thickening of the RBM layer has been found to correlate with airflow limitation and AHR in mild asthma [37] and to be negatively correlated with airway distensibility [38].

The airway epithelium has been found to be thicker in endobronchial biopsies from patients with severe asthma as compared with normal controls [39]. Furthermore, airway epithelial thickness increases with age and correlates with the thickness of the RBM as well as goblet cell number [40]. Goblet cell metaplasia will contribute to luminal narrowing. We have also shown a correlation between epithelial thickness and subepithelial collagen thickness in the mouse model of AAD used in the current investigation [41].

The ability of the airway wall to respond to bronchodilators may be considerably impaired by the remodelling sequence [42]. Mathematical models have predicted that an increase in the volume of the airway wall on the luminal side of the smooth muscle layer increases the effect of smooth muscle contraction in narrowing the lumen of an airway [43].

Our observation that RLN immunostaining was decreased in the OVA-induced 9-week chronic model of AAD is consistent with our previous findings [24] and confirms that endogenous RLN expression may inversely correlate to asthma pathogenesis. Our novel findings in this study that RXFP-1 expression was also decreased by OVA-induced AAD may suggest that a reduction in receptor levels may compromise the airway remodelling actions of RLN (which is also reduced), leading to the OVA-induced increase in subepithelial and total collagen (fibrosis), epithelial thickening and airway reactivity demonstrated. Furthermore, our additional findings that RLN was able to up-regulate RXFP-1 expression to levels measured in saline-treated controls, in the absence of any changes in GR immunostaining suggests that the hormone was mediating its airway remodelling actions in this AAD model via RXFP-1 and not through GR, which RLN can also bind and activate [25]. Surprisingly, GR expression was unaffected by any of the treatment groups compared with levels measured in saline-sensitized/-challenged controls. Additionally, CS treatment did not have any effects on RLN or RXFP-1 expression, suggesting that its mode of action was distinct to that of RLN; despite a recent report demonstrating that GREs (glucocorticoid response elements) are present on the H2 RLN gene [44]. Halls et al. [45] used HEK (human embryonic kidney)-293 cells, which transgenically express high levels of RXFP-1, to demonstrate that RLN binds to a GRE (in the presence of RXFP-1) [45]. The finding that we could not mimic that data with our in vivo studies is consistent with the fact that physiological levels of RXFP-1 are much lower in fibroblasts compared with that seen in HEK (human embryonic kidney)-293 cells.

In the present study, systemic treatment with methylprednisolone was used in an attempt to get maximal effect of CS in a mouse model of chronic AAD. There have been many studies that have evaluated the effects of CS in an acute (2–3-week OVA challenge period) model of AAD that is well documented to decrease airway inflammation via the reduction of eosinophils, whereas in the chronic model (>6-week OVA challenge period) there is limited airway inflammation at the termination of the experiment. Thus, effects of CS on airway inflammation in the chronic AAD model may be more difficult to demonstrate.
CS had a therapeutic effect on airway remodelling, as administration with methylprednisolone was found to reduce both collagen deposition around airways and total collagen deposition in the lung. The fact that there was some effect of CS on fibrosis supports the notion that there was also an effect on airway inflammation albeit a more subtle effect that was not identified by cell counts. There is some controversy as to whether CSs are able to directly prevent and reverse airway collagen deposition. In fibroblast cell culture models, some authors have demonstrated effects of CS in decreasing ECM content via inhibition of fibronectin expression [46,47]. On the other hand, others have reported that CSs were unable to change ECM thickness [48]. Prolonged systemic CS treatment regimes, similar to the one used in the current study have been shown to prevent airway collagen deposition in vivo [49–51]. In human studies, there was less evidence with appropriate and safe CS treatment protocols, showing the effect of CS in long-term remodelling (severe asthma), with conflicting reports regarding the effect of CS on fibrosis. This then would similarly be consistent with the finding that, in our model, CS only influenced one aspect of remodelling. The finding that RLN modified several parameters supports the benefit of adding RLN to CS treatment [52–54] in chronic disease.

Studies have shown that prolonged treatment with CS during and after OVA aerosol challenge in animal models of asthma decreased inflammation and were associated with an improvement in lung function [50,51,55,56]. Consistent with animal models, there is considerable evidence in the literature to suggest that CS can have a useful therapeutic effect on AHR [10,56–59]. Interestingly, in the current study, we did not detect an improvement in AHR following CS treatment at both low (Figure 6B) and maximal (Figure 6C) responses to methacholine. As fibrosis is shown to be important in remodelling, we would have expected a reduction in subepithelial collagen to reduce AHR similar to our previous findings [24,27]. However, the effects of CS on AHR might not be persistent enough to be detected 2 weeks after the cessation of treatment, as we and others have observed that symptoms returned once the CS treatment stopped [4]. Importantly the enhanced anti-remodelling effects of RLN were able to significantly reduce maximal responses to methacholine (Figure 6) without shifting the dose–response curve, confirming the stronger and more persistent effects of the hormone.

Combination treatment with CS and RLN revealed an additive effect on reduction of subepithelial collagen thickness compared with either treatment alone. Surprisingly though, the same trend was not observed in total collagen content measurements, as there was no additive effects of combination treatment compared with each treatment alone. This discrepancy may be explained by the fact that the hydroxyproline assay estimates all collagens in the lung including the airways, parenchyma, vascular regions and interstitium, whereas the morphometric analysis specifically measured subepithelial thickness of the airway (which mainly consists of type IV collagen). In addition, the treatments administered might only have been able to decrease collagen levels in the airway as most of the OVA-induced inflammation and structural changes are localized in the airways.

Furthermore, combination treatment did not influence the expression of GR in the airways. It is possible that high levels of exposure to GR with CS and RLN could interact with nuclear and cytoplasmic GR and reduce the number of receptors being expressed [60]. There was a trend towards an increase in RXFP-1 expression in the combination treatment group, but this was not as significant as that caused by RLN treatment alone. In addition, combination treatment did not significantly affect RLN expression in this model, confirming that the actions of RLN and CS are independent of regulating each other, but interact at a downstream level to reduce subepithelial collagen deposition rather than having any direct effects via cross-talk between RXFP-1 and GR (in the model studied), which warrants further investigation.

In conclusion, the findings from this project revealed that RLN has better airway remodelling effects compared with a clinically used CS, and enhances the airway remodelling actions of methylprednisolone on subepithelial collagen thickness (a hallmark of asthma). In addition, RLN modified other aspects of airway remodelling, including epithelial thickness as well as AHR. These findings suggest that RLN has real potential to supplement CS treatments, which are best known for their anti-inflammatory effects and only have modest airway remodelling effects (as further demonstrated in the present study). These findings further confirm that the RLN–RXFP-1 interaction controls the rate of airway fibrosis, epithelial thickening and airway reactivity in the lung exposed to AAD; with great promise as a treatment of the future.

**CLINICAL PERSPECTIVES**

- Although corticosteroids demonstrate potent effects in the control of airway inflammation in asthma, many patients continue to experience symptoms and AHR, probably due to progressive airway remodelling. Thus identifying novel therapies that can target airway remodelling and/or airway reactivity may improve symptom control in these patients. We have demonstrated previously that the anti-fibrotic hormone RLN can reverse airway remodelling (epithelial thickening and subepithelial fibrosis) and AHR in a murine model of AAD.
- In the present study, we compared the effects of RLN with a CS (methylprednisolone) on airway remodelling and AHR when administered independently or in combination in a well-established mouse AAD model.
- Our findings demonstrate that RLN can modulate a broader range of airway remodelling changes and AHR than methylprednisolone, and the combination of both treatments offers enhanced control of subepithelial fibrosis, highlighting the clinical potential of RLN/combination treatment as novel therapies for asthma.

**AUTHOR CONTRIBUTION**

Simon Royce designed and performed experiments, analysed data and wrote the paper; Amelia Sedjahthera performed experiments and analysed data; Chrihan Samuel performed experiments, analysed data and contributed to the paper; Mimi Tang designed experiments, analysed data and contributed to the paper.
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