Supplementary data

Materials and Methods

Animals

Mice were lightly anaesthatised by inhalational isofluorane prior to receiving either intranasal HDM (15µg) or vehicle control (PBS 15µl volume) 3 days a week for up to 3 weeks.

Measurement of AHR

Direct measurements of dynamic lung resistance (R_L) and pulmonary compliance (C_{dyn}) were measured in anaesthetised and trachestomised mice. Mice were ventilated using a small animal ventilator (Harvard Apparatus, Kent, UK) and measurements were taken in response to inhalational doses of the cholinergic agonist methacholine (MCh - Sigma, Poole, UK) at increasing concentrations (3mg/ml - 100mg/ml) in an EMMS system (Electro-Medical Measurement Systems, Bordon, Hants, UK) in a modified version of previously described methods[1]. The trachea was exposed and cannulated then mice were ventilated at 150 breaths/minute with a stroke volume of 7ml/kg, comparable with normal breathing. Baseline measurements were obtained for 2 mins followed by a PBS dose. Each subsequent MCh dose lasted 20 seconds, and the response to each dose was measured for 4 minutes with measurements taken every 25 breaths. Lung R_L and C_{dyn} values were averaged for each 4 minute period following MCh challenge and expressed as cmH_2O/mls/s and ml/cmH_2O respectively.

Collection and preparation of samples

Bronchoalveolar lavage (BAL) was collected by lavage of the lungs three times with 0.4 mL of PBS via a tracheal cannula. BAL fluid was centrifuged (700 *g*, 5 min, 4°C) and cells were recovered. BAL cell supernatants were removed and analyzed for cytokines by ELISA. To disaggregate the cells from the lung tissue, one lobe (~100 mg) of lung was incubated at 37°C for 1 h in digest reagent (0.15 mg/ml collagenase type D, 25 µg/ml DNase type I) in complete RPMI media. The recovered cells were filtered through a 70-µm nylon sieve, washed twice, resuspended in complete media, and counted in a hemocytometer prior to cytocentrifugation. Lung and BAL cells were applied to glass slides by centrifugation ($5x10^4$ cells/slide) and stained with Wright-Giemsa (Thermo Fisher Scientific Inc, Waltham, MA). Percentages of macrophages, lymphocytes/mononuclear cells, eosinophils and neutrophils were determined under 40x magnification by counting cells in 8 randomly selected fields and dividing this number by the total number of cells counted. To obtain absolute numbers, this percentage was multiplied by the total number of cells recovered in 1 ml of lavage fluid and lung digest suspension which were normalised for the weight of the lung.

Assessment of airway remodeling

Goblet cells were visualized on periodic acid-Schiff (PAS)-stained lung sections and scored with results expressed as a mucus score in arbitrary units.[2,3] PAS-stained goblet cells in airway epithelium were measured double-blind using a numerical scoring system (0: <5% goblet cells; 1: 5 to 25%; 2: 25 to 50%; 3: 50 to 75%; 4: >75%) The sum of airway scores from each lung was divided by the number of airways examined, 20–50 airways per mouse, and expressed as mucus cell score in arbitrary units.

Muscle thickness was assessed by measuring the thickness of the peribronchial smooth bundle. Twenty measurements were made per airway and an average calculated in μm. Peribronchial muscle thickness was calculated for four airways per section. Constricted airways as determined visually in sections by concertinaed, ruffled or folded epithelium were not included when making this calculation.

Smooth muscle cell proliferation was assessed in lung sections stained with an antibody against PCNA. The percentage of $PCNA^+$ airway mesenchymal cells (an index of smooth muscle hyperplasia) was calculated by counting the number of PCNA positive mesenchymal cells as a percentage of the total number of peribronchial mesenchymal cells.

Neovascularisation and the number of pulmonary blood vessels was determined by counting the number of von Willebrand factor positive (as a marker of endothelial cells) vessels/mm^2 in at least four airways per section.

Peribronchial collagen deposition was determined in Sirius red stained sections using Scion Image Analysis software package (Scion Corporation, Frederick, MD, USA) adapted from the literature.[4] Digital photographs of four bronchioles per tissue section were taken at ×40 magnification under polarised light 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.

References

1. Martin TR, Gerard NP, Galli SJ, et al. and J.M. Drazen. Pulmonary responses to bronchoconstrictor agonists in the mouse. *J Appl Physiol* 1988;**64**:2318–2323.

2. Grunig G, Warnock M, Wakil AE, et al. Requirement for IL-13 independently of IL-4 in experimental asthma. *Science* 1998;**282**:2261-2263.

3. Townsend JM, Fallon GP, Matthews JD, et al. IL-9-deficient mice establish fundamental roles for IL-9 in pulmonary mastocytosis and goblet cell hyperplasia but not T cell development. *Immunity* 2000;**13**:573-583.

4. Flood-Page P, Menzies-Gow A, Phipps S, et al. Anti-IL-5 treatment reduces deposition of ECM proteins in the bronchial subepithelial basement membrane of mild atopic asthmatics. *J Clin Invest* 2003;**112**:1029–36