OVEREXPRESSION OF SMAD2 DRIVES HDM MEDIATED AIRWAY REMODELLING AND AHR VIA ACTIVIN AND IL-25

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ONLINE DATA SUPPLEMENT

Methods

Animals. Female BALB/c mice (6-8 weeks old) (Charles River) were housed in filter cupboards and a 12-h light/dark cycle and received food and water *ad libitum.* Mice received either 15μg HDM extract (1mg/ml *D. pteronyssinus* in PBS) (Greer laboratories, Lenoir, NC) or 15μl PBS intranasally for 3 days a week (Wed, Fri and Mon) for up to 3 weeks.

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. All cell counts were performed blind by the same observer.

Western blotting. Briefly, lungs were weighed and tissue samples homogenised in 1:20 (w/v) of tissue in T-PER Tissue Protein Extraction reagent containing a protease inhibitor cocktail (Roche). Protein contents in clarified supernatants were determined by comparison with an OVA standard curve (ICN Biomedical), using the Bio-Rad protein assay.

The expression of total Smad2 and of phosphorylated Smad2 were assessed by Western blot after protein fractionation (15 µg) by 4-12% NuPAGE Novex Bis-Tris Mini Gels in MOPS SDS Running Buffer (Invitrogen), transfer to polyvinylidene difluoride membranes (Bio-Rad) and sequential reaction with a rabbit anti-mouse Smad2 Ab (1/150 dilution; Zymed Laboratories), or with a rabbit anti-mouse pSer465/467 Smad2 Ab (1/1000 dilution; Calbiochem) and with a mouse anti-alpha-tubulin mAb (1/5000 dilution; Sigma-Aldrich). Immunoblots were then incubated with peroxidase-conjugated goat anti-rabbit, or anti-mouse Abs at a 1/2000 dilution (DakoCytomation) and developed using the ECL Western blotting detection system (Amersham). Densitometry analysis was performed using ImageJ 1.41 software.

Pathology. Paraffin-embedded sections (4 µm) were stained with haematoxylin/eosin (H&E) to evaluate general morphology. Goblet cells were visualized on periodic acid-Schiff (PAS)-stained lung sections and scored with results expressed as a mucus score in arbitrary units (E1, E2). 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 (U).

Collagen deposition was assessed on Sirrius red stained sections. Image analysis was performed using Scion Image Analysis software package (Scion Corporation, Frederick, MD, USA). Digital photographs of four bronchioles per tissue section were taken at ×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.

ASM cells were counted in proliferating cell nuclear antigen (PCNA) immunostained lung tissue sections at ×40 magnification. Dark elongated smooth muscle cell nuclei and round nuclei in the muscle bundles were counted per bronchiole in four bronchioles per mouse. Cells positive for PCNA stained dark brown. Total cell counts and those positive for PCNA were counted, meaned and divided to obtain an index of hyperplasia. All scoring and measurements were performed blind by the same observer

Epithelial cell height and thickness of the airway smooth muscle layer around medium sized conducting airways were measured from paraffin sections. 10 measurements were made per airway and a minimum of 10 airways were measured per section. Data presented are an average.

Quantification of total lung collagen. Recently synthesised acid soluble collagens were extracted by overnight incubation of chopped lung tissue in 0.5M acetic acid containing protease inhibitor cocktail. Collagen content was measured in lung tissue supernatants by biochemical assay according to the manufacturer's instructions (Sircol collagen assay, Biocolor, Belfast, UK). All presented data have been normalised for the weight of the lung.

References

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E2. Townsend JM, Fallon GP, Matthews JD, Smith P, Jolin EH, McKenzie NA. 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.

Figure Legends

Figure E1. No effect of AdC on inflammatory profile in lung. (A). Differential counts of cells isolated from lung tissue, M macrophage, E eosinophil, N neutrophil, L/M lymphocyte monocyte. (B) Flow cytometric analysis of T cell subsets isolated from lung tissue. (C) Increased Penh measured in response to increasing doses of MCh. (D) Lung sections stained with H & E showing peribronchial and perivascular cellular infiltrate in HDM treated groups. Data shown represent means ± SEM (N = 6–12). * = $p < 0.05$ compared with PBS controls.

Figure E2. Cytokine and growth factor levels in the lung after 3 week exposure to HDM in the presence or absence of epithelial overexpression of Smad2. Mediator levels were determined by ELISA in lung homogenate. Data shown represent means ± SEM (N = 6). * = $p < 0.05$ compared with PBS controls.

Figure E3. Neutralising activin A did not impact upon inflammatory cell recruitment to the lung. (A) Total cell counts in the lung and BALF. (B) Differential counts in the lung digest, M macrophage, E eosinophil, N neutrophil, L/M lymphocyte monocyte Data shown represent means ± SEM (N = 6). * = $p < 0.05$ compared with PBS controls.