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Article type : Letter to the Editor

Use of spirometry-like measurements to monitor house dust mite-induced experimental asthma in mice

To the editor,

Spirometry is used to diagnose and monitor asthma, a chronic lung disease characterized by respiratory symptoms, airflow limitation, and accumulation of immune system cells, including mast cells in the airways. Airway hyperresponsiveness (AHR) measures an increased airway sensitivity and is related to a decline in lung function assessed by spirometry in population-based studies. Spirometry determines the volume and speed of air that a patient can inhale and exhale, which depends on the size of the lung and is modified by lung diseases. Approaches to acquire accurate measurements of spirometry-like parameters in mice utilize negative pressure-driven forced expiration techniques.^{1,2} These studies successfully recorded changed lung parameters in chronic obstructive lung disease models, but failed to detect basal changes in spirometry-like parameters for house dust mite (HDM)- or ovalbumin-induced allergic airway inflammation models.

In our study, we investigated the effect of body weight and age on lung function in naïve BALB/c mice using an automated system to compute spirometry-like parameters (Buxco Pulmonary Function Test (PFT), DSI). In this system, the mouse is anesthetized, intubated, and connected to a ventilator. The PFT system uses vacuum to exhaust air (negative pressure) as a surrogate for exhaling, and measures the airflow and pressure during a succession of forced pulmonary tests to generate spirometry-like parameters. Weight and age correlated positively with forced expiratory

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flow in 0.1 seconds (FEV 0.1) (Figure 1A, B). Moreover, peak of expiratory flow (PEF), forced vital capacity (FVC), inspiratory capacity (IC), tidal volume (TV), compliance (C_{dyn}), vital capacity (VC), and total lung capacity, but not resistance or functional residual capacity correlated positively with weight (Figure S1). When weight-matched female and male mice were analysed, the females were approximately 20 weeks older than the males and showed higher values for most spirometry-like parameters (Figure S2). Most spirometry-like parameters also increased with age in males (Figure S3). Results showing that lung function relates to differences in mouse strain, sex, and growth were earlier found using whole-body plethysmography.³ We conclude that spirometry-like lung function parameters are highly dependent on age and weight, and that great care needs to be taken when matching groups of mice of the same gender for generating this type of data.

To test whether spirometry-like measurements could distinguish mice with HDM-induced lung inflammation from sex-, weight- and age-matched phosphate-buffered saline (PBS) control mice, an established model was used.⁴ BALB/c mice received seven intranasal administrations of HDM or PBS on days 0, 3, 6, 9, 12, 15, and 18, and lung function was determined on day 19 (Figure 1C). The mice showed reduced FEV 0.1 and PEF after HDM-sensitization (Figure 1E, F). Reductions in FVC, IC, C_{dyn}, and TV were also found in HDM-sensitized mice compared to PBS controls, but no significant reduction in the other parameters was observed (Figure S4). Devos *et al.* recorded spirometry-like parameters in an HDM-model using both forced expiration and forced oscillation technique (FOT) and did not detect basal changes in spirometry-like measurements.¹ However, they used comparably lower HDM-doses over a shorter period of time (13 days) and we speculate that this could be why no basal changes were observed. In our study, HDM induced smooth muscle proliferation, and the number of α -actin⁺ lung cells correlated negatively with FEV 0.1 (Figure S5).

When HDM-sensitized mice were treated with vehicle or dexamethasone before the last four HDM administrations, dexamethasone reduced the number of bronchoalveolar lavage (BAL) eosinophils to a minimum (Figure 1D, Figure S6A), as expected.⁵ Dexamethasone improved FEV 0.1, PEF, FVC, and showed a trend (p=0.06) to improve IC compared to vehicle-treated mice with HDM-induced allergic airway inflammation (Figure 1E-F, Figure S4). Given the tight relationship

between poor lung function and AHR, this finding also supports studies demonstrating that dexamethasone prevents AHR in mouse models of allergic inflammation, e.g.⁵

Our major interest in mast cells and their role in asthma triggered us to test whether dexamethasone affected mast cells in the lung. Three lung mast cell populations, which differ in integrin $\beta 7$ expression, exist in HDM-sensitized mice. The mast cell progenitor (MCp) population was the most expanded, followed by the induced mast cells with intermediate integrin $\beta 7$ expression, which are absent in PBS control mice, and the mature mast cells (Figure 2A). As the anti-Fc ϵ RI antibody MAR-1 was shown to cross-react with Fc γ RI and Fc γ RIV,⁶ the mast cell populations were also quantified using an anti-IgE antibody. However, both strategies resulted in a similar quantification (Figure S6D, E).

Dexamethasone reduced the total number of lung mast cells, the MCp, and mature mast cell populations, and showed a tendency to decrease the induced mast cells ($p = 0.07$) (Figure 2A, B). Indeed, asthma patients on inhaled corticosteroids have lower mast cell numbers within the lung epithelium and smooth muscle than those treated with $\beta 2$ -agonist alone.⁷ Moreover, glucocorticoids increase the apoptosis of skin mast cells *in vivo*, likely due to an inhibitory effect on stem cell factor-production from fibroblast.⁸ Thus, we speculate that the reduction in lung mast cells by dexamethasone is likely due to a combination of reduced influx of MCp and increased apoptosis of lung mast cells. Importantly, not only the mast cell burden, but also mast cell activation was suppressed by dexamethasone. HDM-sensitized mice had two-fold increased levels of mouse mast cell protease-1 (mMCP-1) in the BAL, but dexamethasone treatment normalized the levels (Figure 2D). HDM-sensitization also induced an increase in Fc ϵ RI expression in the lung mast cell populations, which was decreased in mice treated with dexamethasone (Figure 2C). In line with our data, dexamethasone was shown to reduce mast cell-expression of Fc ϵ RI *in vitro*.⁹ Hence, we speculate that the suppressed mMCP-1 levels in dexamethasone-treated HDM-sensitized mice could be explained by reduced lung mast cell numbers and their abolished activation due to decreased Fc ϵ RI.

We previously demonstrated a correlation between a high frequency of human blood MCp and reduced FEV1 and PEF (% of predicted) in patients with allergic asthma.¹⁰ Therefore, we

investigated whether a similar association existed in the mouse model of HDM-induced allergic airway inflammation. A high frequency of lung MCp correlated with reduced FEV 0.1 or PEF (Figure 2E, F), and similar relationships were found between BAL eosinophils and FEV 0.1 or PEF (Figure S6B, C). Altogether, our data suggest that a high number of lung MCp predicts reduced lung function.

To summarize, our study provides insight into the relationship between mouse weight/age and spirometry-like pulmonary function parameters in naïve mice, and demonstrates that such parameters can be reduced by allergic airway inflammation, and improved by dexamethasone. However, other allergic airway inflammation models need to be validated to ensure that the spirometry-like signs of reduced lung function are reproduced. To this end, spirometry-like measurements in mice are valid as a pre-clinical tool when investigating new drug targets or disease mechanisms in asthma.

Acknowledgements

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Author contributions

PAAV and JH conceived the project, designed and analysed experiments. E M-E set-up the asthma model and participated in the establishing the method to perform spirometry-like measurements. PAAV performed the experiments. PAAV and JH wrote the manuscript. All authors critically assessed and approved the manuscript.

Ethical approval

The experiments were approved by Uppsala (5.8.18_05428/2018) and Stockholm (N14/16) animal ethics committees, and conducted according to the approved protocols.

Key words

asthma, house dust mite, lung function, mast cells, mouse model

Conflict of interest statement

The authors declare no conflict of interest.

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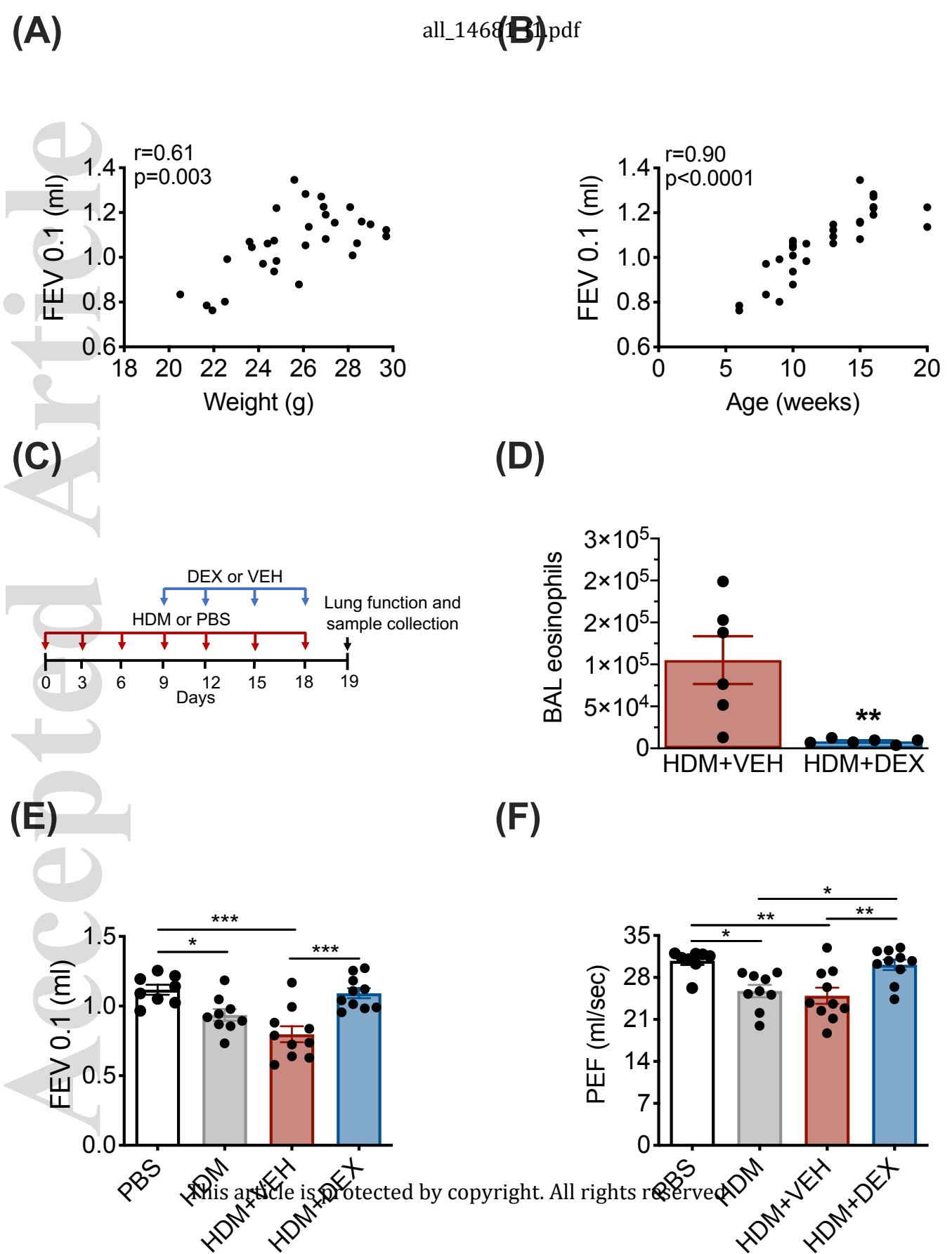
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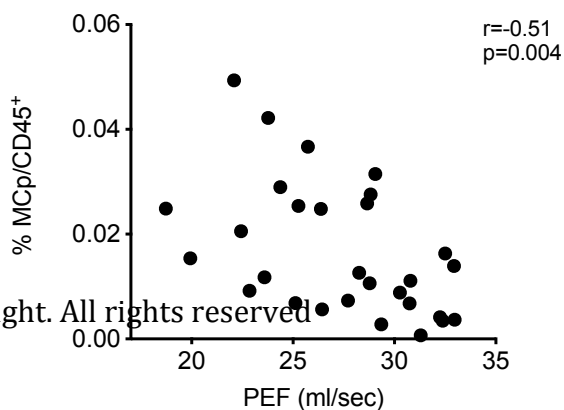
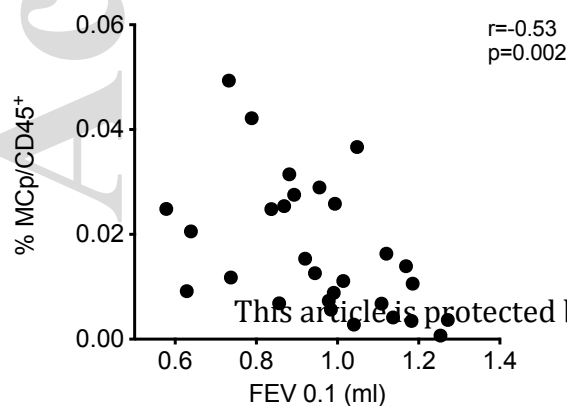
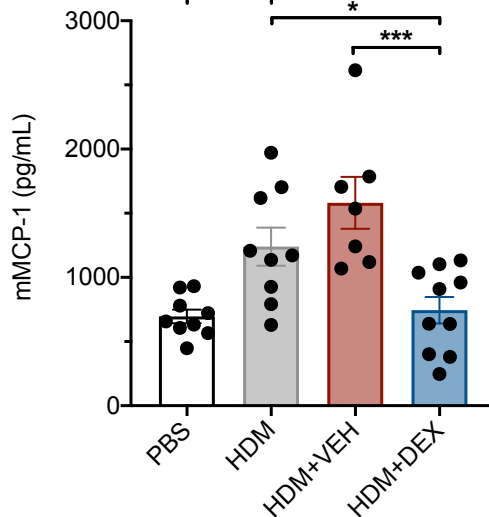
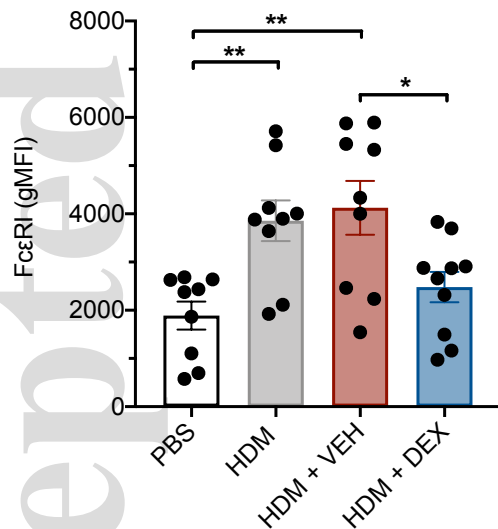
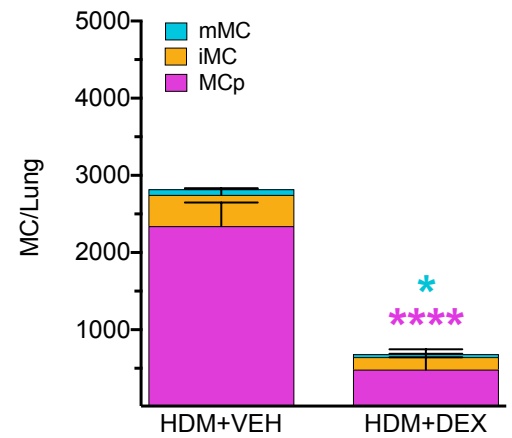
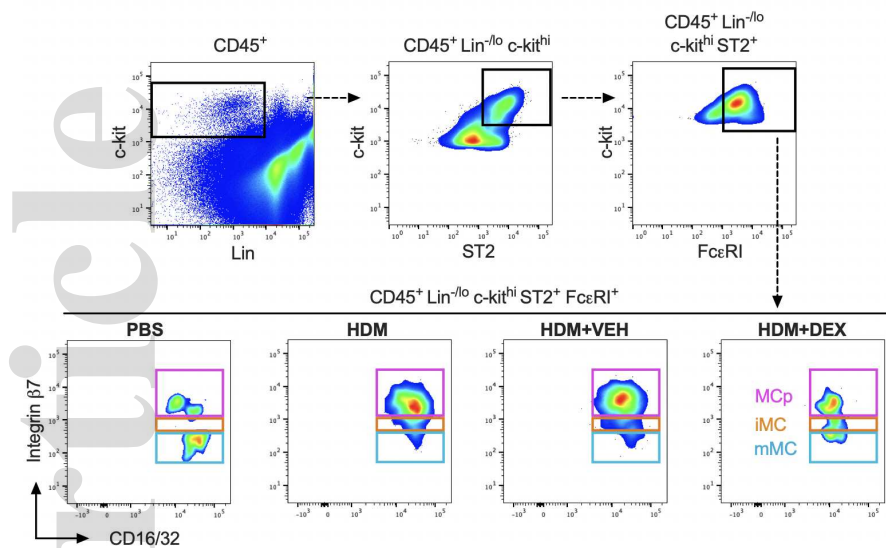
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Figure legends

Figure 1. Spirometry-like parameters are related to mouse weight, reduced by HDM-induced allergic inflammation, and improved by dexamethasone. (A, B) FEV 0.1 was assessed in naïve mice of different weights (A) and age (B). (C-F) Mice were sensitized with HDM or given PBS as controls. HDM mice received dexamethasone (DEX, 3 mg/kg) or vehicle (VEH, 0.9 % NaCl). (D) Eosinophils were quantified in BAL. (E) FEV 0.1 and (F) PEF are shown. Spearman correlation from seven experiments in (A-B). Mean $\pm$ SEM from two (D) or three (E-F) independent experiments. Statistical analyses were performed by an unpaired Student's t test (D) or one-way ANOVA with post-hoc Turkey's test (E-F).

Figure 2. Dexamethasone reduces lung mast cell populations, their expression of Fc ϵ RI, and release of mMCP-1. (A-D) Mice were given PBS or sensitized with HDM alone, or treated with vehicle (HDM+VEH), or dexamethasone (HDM+DEX). (A-C) Mature mast cells (mMC), HDM-induced mast cells (iMC), and MCp were quantified by flow cytometry. (C) Geometric mean fluorescence intensity (gMFI) for Fc ϵ RI in lung mast cells. (D) BAL levels of mMCP-1. (E, F) The frequency of lung MCp in treated and untreated HDM mice correlates with FEV 0.1(E) and PEF (F) using Spearman correlation. (A-F) Data from three independent experiments were pooled. (B-F) The mean $\pm$ SEM is shown, and statistical significance was tested by an unpaired Student's t-test (B) and one-way ANOVA with Turkey's post-hoc test (C, D).





Supplementary methods

Mice

BALB/c mice originally from Bommice, Ry, Denmark and BALB/cJBomTac mice (Taconic) between 6-40 weeks of age were bred and kept at the National Veterinary Institute (Uppsala, Sweden) under a 12 hours dark/light cycle with food and water *ad libitum*. For the weight and age correlation analyses, both strains were used. For HDM-induced allergic airway inflammation experiments the BALB/cJBomTac mice were used. The experiments were approved by Uppsala (5.8.18_05428/2018) and Stockholm (N14/16) animal ethics committees and conducted according to the approved protocols.

HDM-induced allergic airway inflammation and dexamethasone treatment

HDM extract from *Dermatophagoides pteronyssinus* (Greer Laboratories) was reconstituted in 0.1 M PBS. Isoflurane (2 %) anesthetized mice received 50 µg of HDM extract or PBS intranasally (25 µl per nostril) on days 0, 3, 6, 9, 12, 15, and 18. Dexamethasone (3 mg/kg, catalogue # D2915, Sigma-Aldrich) or vehicle (0.9% sterile saline) was administrated intraperitoneally to HDM-sensitized mice on days 9, 12, 15, and 18 (before the HDM administration). The dose of dexamethasone was chosen based on studies reporting an anti-inflammatory effect in mouse models of allergic airway inflammation.^{1,2}

Spirometry-like lung function measurements

Lung function was measured in naïve, HDM-sensitized, or PBS-treated mice one day after the last intranasal administration. Briefly, mice were anesthetized by intraperitoneal injection of ketamine (100 mg/kg, Pfizer; NY, USA), xylazine (20 mg/kg, Bayer), and acepromazine (3 mg/kg, Pharmaxin), intubated by the trachea and placed in Buxco Pulmonary Function Test

(PFT) system chamber (Data Sciences International; DSI). The mice were ventilated at 150 breaths per minute. The PFT system is controlled by the FinePointe software, which utilizes the Boyle's Law, quasistatic pressure-volume curves, and fast-volume manoeuvres to calculate the peak of expiratory flow (PEF), forced expired volume in 100 milliseconds (FEV_{0.1}), forced vital capacity (FVC), inspiratory capacity (IC), resistance (RI), compliance (C_{dyn}), forced residual capacity (FRC), tidal volume (TV), vital capacity (VC), and total lung capacity (TLC). The mice were euthanized after the PFT measurements.

Preparation of BAL and lung single-cell suspension

BAL fluid and lung tissue were collected from mice euthanized by an overdose of isoflurane or neck dislocation after the PFT measurements. BAL was obtained with 1 ml 0.1M PBS via a plastic cannula inserted into the trachea, centrifuged (400 g; 5 min) to obtain cell-free supernatants, which were stored at -20°C. The lungs were perfused through the heart with 10 ml of 0.1 M PBS, dissected and dissociated to single-cell suspensions using the gentle MACS Octo Dissociator and the mouse lung dissociation kit (Miltenyi Biotec). For all experiments except for the quantification of α -actin⁺ cells, debris was removed by Percoll gradient (44%) centrifugation (400 g; 20 min), before the red blood cells were lysed using a mix of 150 mM NH₄Cl, 9.5 mM KHCO₃, and 1.2 mM Na₂EDTA. Finally, the cells were extensively washed with 2 % of heat-inactivated foetal bovine serum in PBS (FACS buffer), and passed through a 70- μ m mesh. The BAL and lung cells were counted using trypan blue staining in a haemocytometer and resuspended in FACS buffer for flow cytometry.

Flow cytometry

The cells were incubated for 30 min on ice with antibodies specified in Table S1 to identify specific cell populations. Isotype controls were used to set the gates for ST2, Fc ϵ RI, and anti-

IgE expression. After staining, the cells were washed twice with FACS buffer before the analysis on the LSR Fortessa cytometer (BD Biosciences). The data were analysed using FlowJo software (TreeStar Inc).

Staining of α -actin cells in lung sections

Mice were euthanized by isoflurane overdose and perfused through the heart with 10 mL of ice cold 0.1M PBS. The lungs were filled with a 1.5 mL of 1:1 OCT compound (VWR International) in PBS, and 10 μ m thickness sections were cut in a cryostat. The slides were rinsed with PBS and incubated with a 5 % normal donkey serum solution containing 0.3 % Triton X-100 in PBS for one hour at room temperature. After that, they were incubated for two hours at room temperature with primary alexa 488-conjugated anti-mouse alpha-actin antibody (1:200, eBioscience), rinsed in PBS, and the nuclei stained with 4',6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich). The slides were mounted with Fluoromount-G (Thermo Fisher Scientific), and images were taken using the 20X objective in an epifluorescence Nikon microscope (Nikon Eclipse 90i Upright).

Quantification of mMCP-1

The levels of mMCP-1 in BAL were quantified using ELISA (88-7503-88; Thermo Fisher Scientific).

Statistical analysis

Statistical analysis was performed using unpaired Student's t-test to compare two groups, or one-way ANOVA with Tukey's multiple comparison test for multiple group comparisons, as described in the figure legends. Correlation analysis was performed by Spearman correlation test. A value of $p < 0.05$ was considered significant and the following designation was used:

* <0.05 , ** <0.01 , *** <0.001 , and **** <0.0001 . The graphs were prepared using GraphPad Prism 8.0.

Method references

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Supplementary table, figures, and legends

Table S1. List of antibodies used for flow cytometry.

Target	Fluorochrome	Clone	Vendor
CD45	Alexa flour 700	30-F11	BD Biosciences
CD3	Pe-Cy5	17A2	BD Biosciences
CD4	Pe-Cy5	GK1.5	eBioscience
CD8b	Pe-Cy5	eBioH35-17.2	eBioscience
CD11b	Pe-Cy5	M1/70	eBioscience
CD19	Pe-Cy5	ebio1D3	eBioscience
B220	Pe-Cy5	RA3-6B2	eBioscience
TER-119	Pe-Cy5	TER-119	eBioscience
Gr1	Pe-Cy5	RB6-8C5	eBioscience
C-kit	Pe-Cy7	2B8	eBioscience
CD16/32	BV605	2.4G2	BD Biosciences
Integrin β 7	FITC	FIB504	eBioscience
Fc ϵ RI	PE	MAR-1	eBioscience
ST2	BV421	DIH9	BioLegend
CD11c	APC	HL3	BD Biosciences
Siglec-F	BV421	E50-2440	BD Biosciences
CD31	Pe-Cy5	MEC 13.3	BD Biosciences
α -Actin	Alexa 488	1A4	eBioscience
anti-IgE	Biotinylated	RME-1	BioLegend
Streptavidin	BV786	-	BD Biosciences

Supplementary figure 1

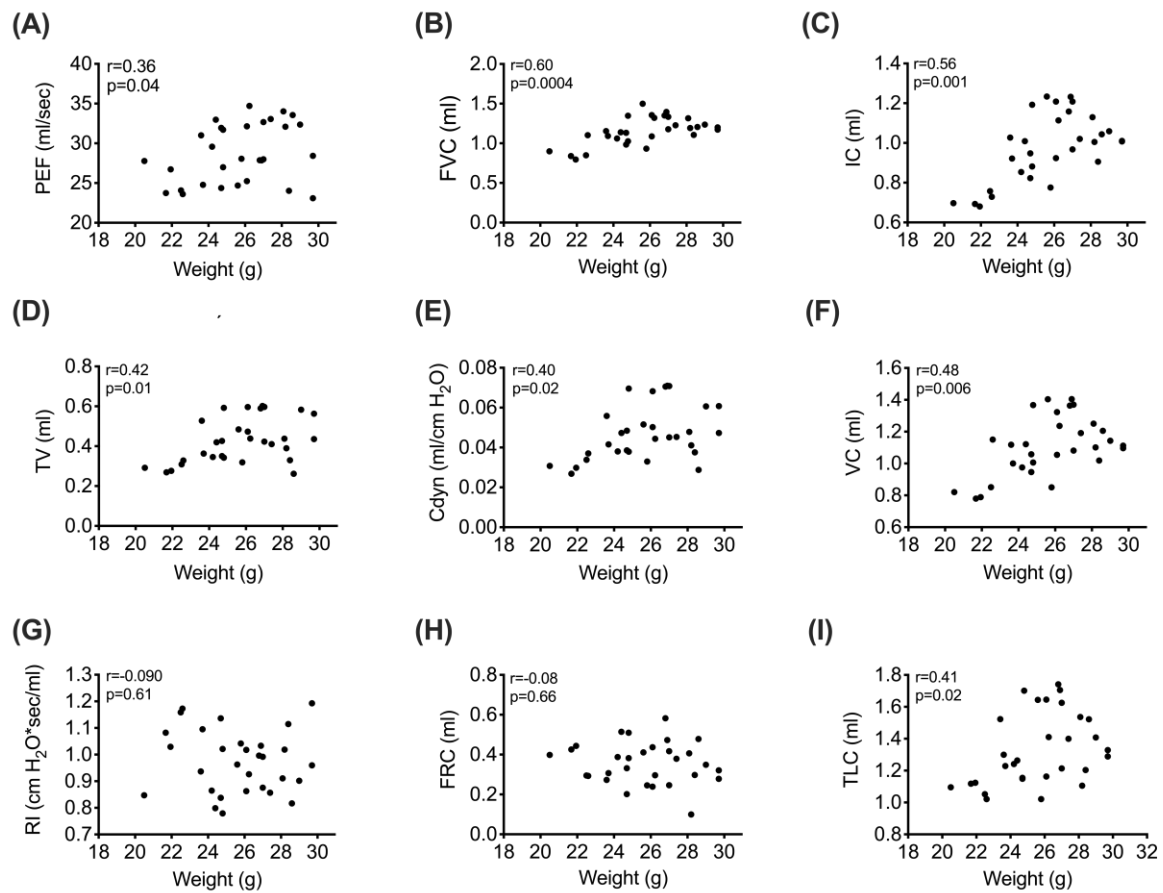


Figure S1. Correlation analysis between different lung function parameters and weight in naïve mice. The lung function parameters (A) PEF, (B) FVC, (C) IC, (D) TV, (E) Cdyn, (F) VC, (G) resistance (RI), (H) forced residual capacity (FRC), and (I) total lung capacity (TLC) were assessed in naïve BALB/c mice of different weights by spirometry-like measurements. Data were analysed using Spearman correlation. Each dot represents one mouse ($n = 30$) from seven independent experiments.

Supplementary figure 2

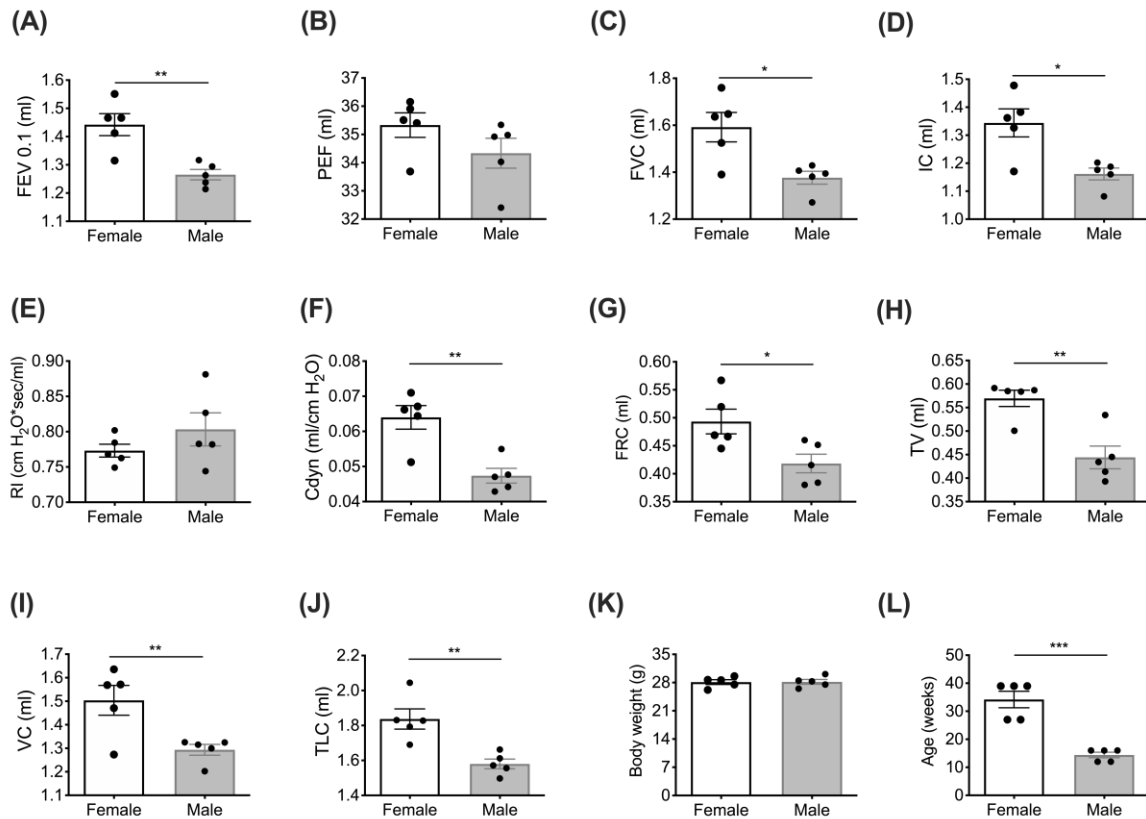


Figure S2. Effect of sex/age on spirometry-like parameters in weight-matched mice. The lung function parameters (A) FEV 0.1, (B) PEF, (C) FVC, (D) IC, (E) RI, (F) Cdyn, (G) FRC, (H) TV, (I) VC, (J) TLC, were assessed in naïve BALB/c mice of similar weight (K) but different ages (L) by spirometry-like measurements. The mean ± SEM is shown, and statistical significance was determined by unpaired Student's t-tests. Each dot represents one mouse (n = 5, per group) from one experiment.

Supplementary figure 3

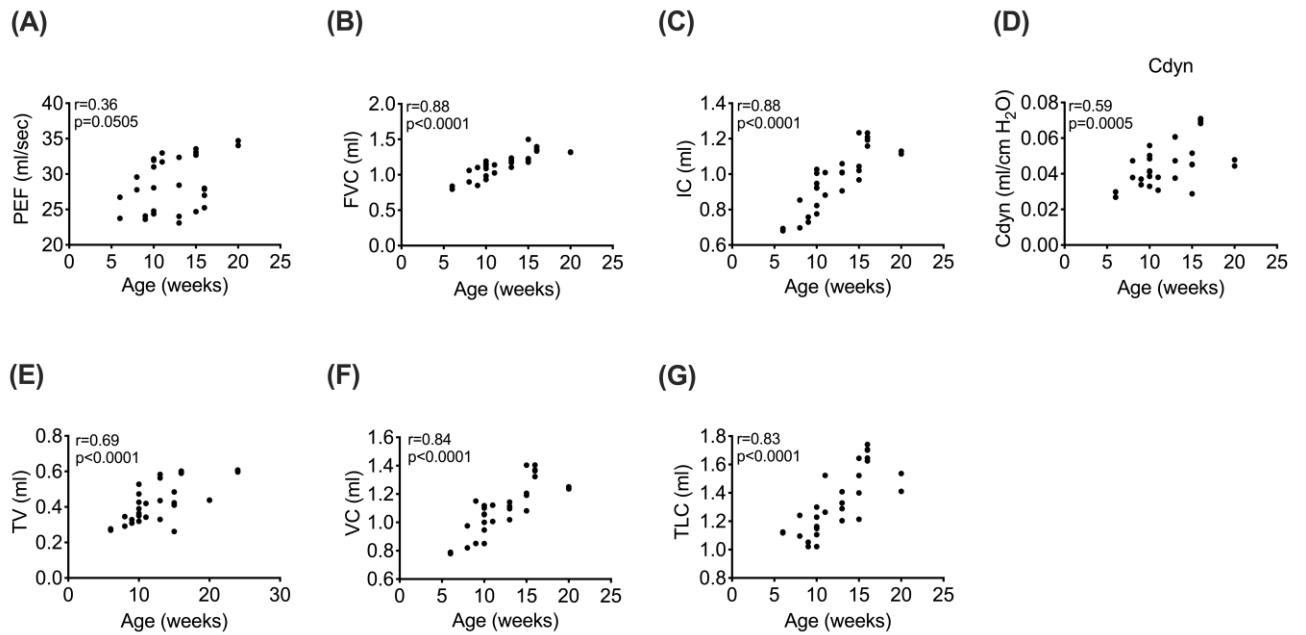


Figure S3. Spirometry-like parameters increase by age in naïve mice. The lung function parameters (A) PEF, (B) FVC, (C) IC, (D) Cdyn, (E) TV, (F) VC, and (G) TLC were assessed in naïve BALB/c mice of different ages by spirometry-like measurements. Data were analysed using Spearman correlation. Each dot represents one mouse ($n = 30$) from seven independent experiments.

Supplementary figure 4

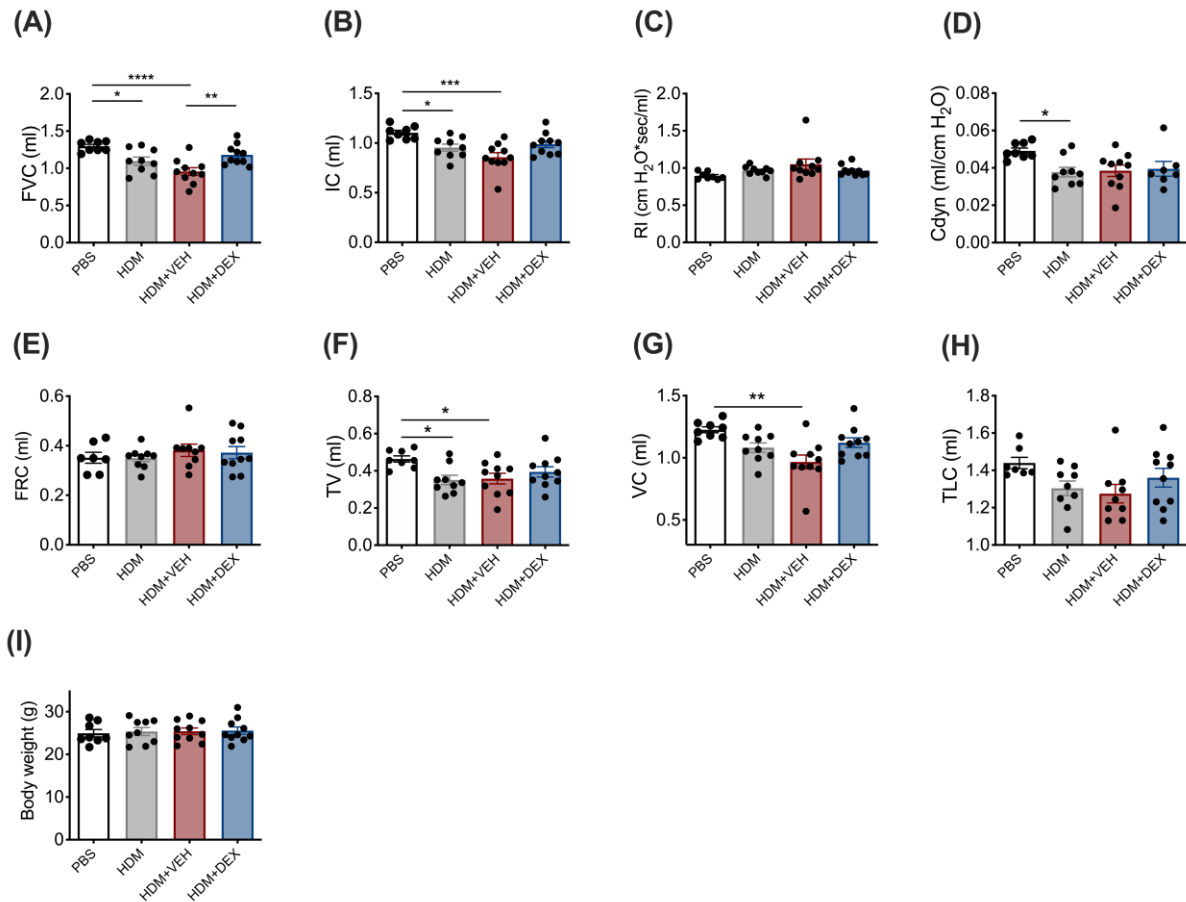


Figure S4. HDM-induced allergic airway inflammation decrease FVC, IC, Cdyn, and TV, and dexamethasone treatment improve FVC. The lung function parameters (A) FVC, (B) IC, (C) RI, (D) Cdyn, (E) FRC, (F) TV, (G) VC, and (H) TLC were assessed in weight-matched (I) PBS-treated mice, mice with HDM-induced allergic airway inflammation (HDM) and vehicle (VEH)- or dexamethasone (DEX)- treated mice with HDM-induced allergic airway inflammation. The data are pooled from three independent experiments, and each dot represents one mouse (n = 8-10/group). The mean ± SEM is shown, and statistical significance was determined by one-way ANOVA followed by Turkey's post-hoc test.

Supplementary figure 5

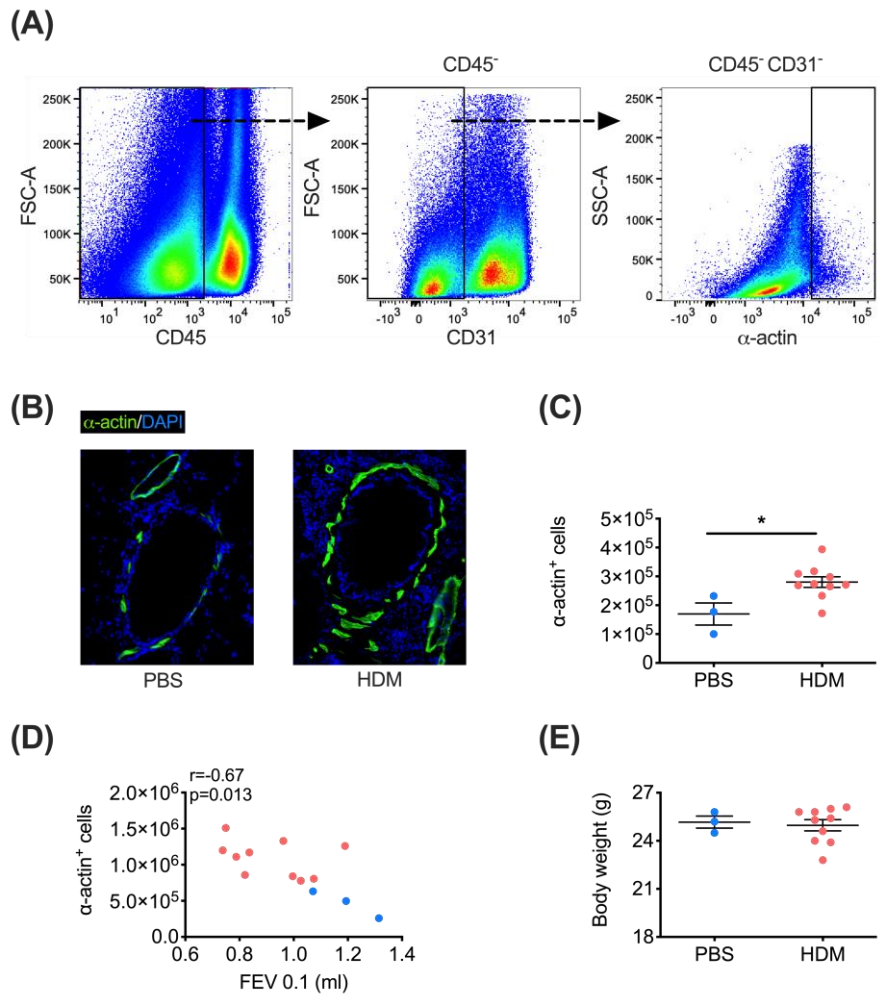


Figure S5. α -Actin⁺ smooth muscle cells are increased in mice with HDM-induced allergic lung inflammation, and α -actin⁺ lung cells correlate negatively with FEV 0.1.

(A) Gating strategy for the quantification CD45⁻ CD31⁻ α -actin⁺ lung cells. (B)

Representative fluorescence microscopy images showing staining of α -actin⁺ smooth muscle cells (green) and DAPI (blue) in the lungs of PBS control and HDM-sensitized mice. (C)

Quantification of total lungs α -actin⁺ smooth muscle cells by flow cytometry. (D, E) The

number of α -actin⁺ smooth muscle cells correlate negatively with FEV 0.1 in mice with HDM-induced allergic airway inflammation, or PBS controls that were weight-matched.

Each dot in (C-E) represents one mouse. The mean $\pm$ SEM is shown in (C) and was analysed by an unpaired Student's t-test, and the data in (D) were analysed by Spearman correlation.

Supplementary figure 6

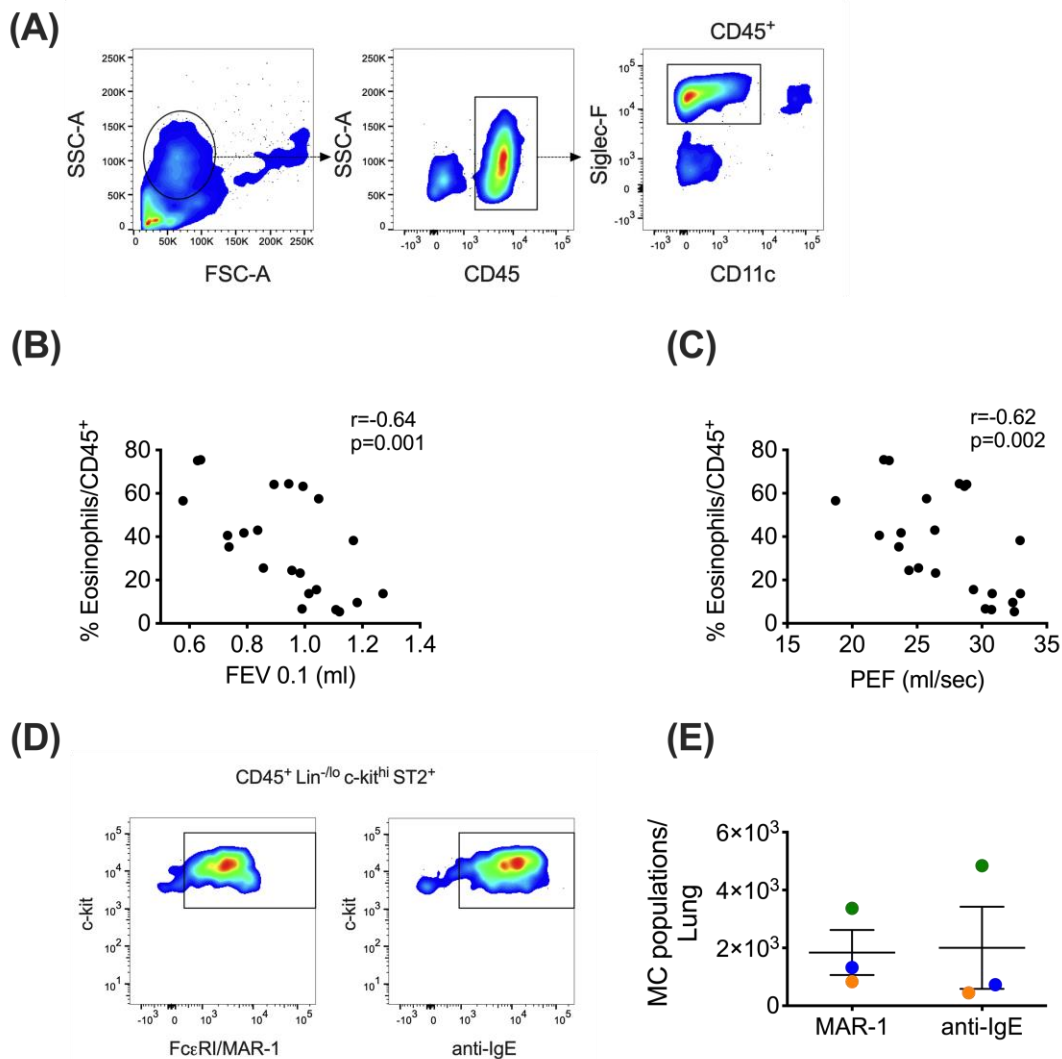


Figure S6. BAL eosinophils correlate negatively with FEV 0.1, and lung mast cells can be correctly identified by the MAR-1 antibody.

(A) Eosinophils were identified as CD45⁺ Siglec-F⁺ CD11c⁻ BAL cells by flow cytometry. (B, C) The frequency of BAL eosinophils correlated negatively with FEV 0.1 (B) and PEF (C) in mice with HDM-induced allergic airway inflammation alone, treated with vehicle or dexamethasone. (D, E) Quantification of lung mast cells in three samples using either an anti-FcεRI antibody (MAR-1) or anti-IgE by flow cytometry (D), which yields similar results (E). Each dot in (B, C) represents one mouse. The mean ± SEM is shown in (E). Data were analysed by Spearman correlation analyses in (B, C) or by paired Student's t-test in (E).