

Letter to the Editor

Prime role of IL-17A in neutrophilia and airway smooth muscle contraction in a house dust mite-induced allergic asthma model

To the Editor:

Asthma is a bronchial inflammatory disease leading to airway hyperresponsiveness (AHR) and reversible airflow obstruction. The incidence of asthma has increased considerably over the past 50 years, with approximately 300 million people affected worldwide.¹ Most of the animal studies have demonstrated that CD4 T_H2 cells are central in the orchestration and amplification of allergic asthma. However, negative results obtained from

T_H2-focused human clinical trials have highlighted the importance of considering the large variety of asthma phenotypes.² Indeed, human asthma is highly heterogeneous and is not always only due to T_H2-driven airway inflammation.^{3,4} Recently, many studies have suggested that IL-17A, a cytokine mainly produced by T_H17 cells, is involved in certain severe cases of asthma by regulating neutrophilic inflammation and steroid resistance.⁵ However, support for this theory remains modest because certain reports have demonstrated (1) that IL-17A or T_H17 cells alone are not sufficient to trigger the disease and (2) that, intriguingly, IL-17A can mediate anti-inflammatory mechanisms. Consequently, we aimed to determine the contribution of IL-17A in a mouse model of acute asthma induced by house dust mites (HDMs).

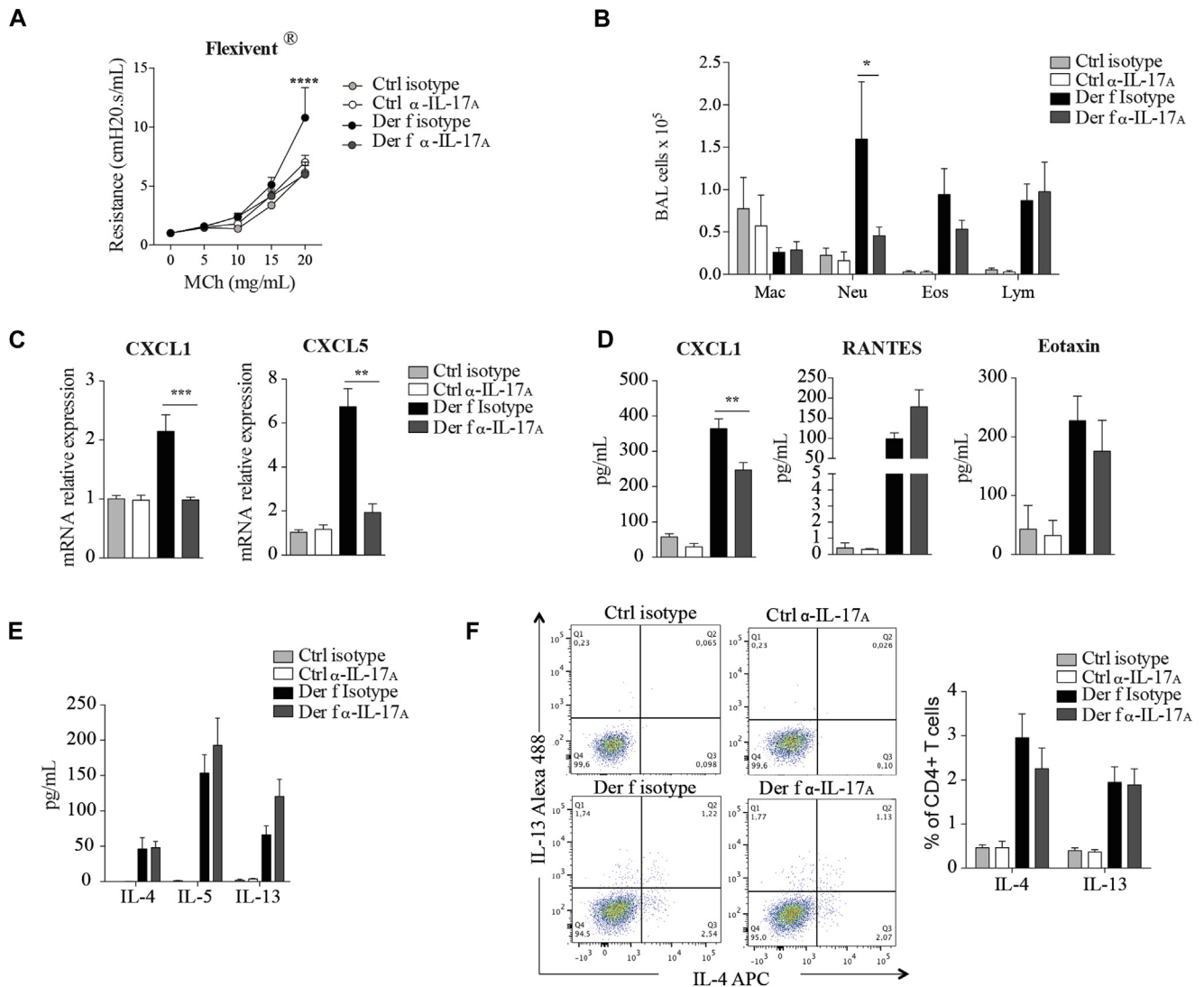
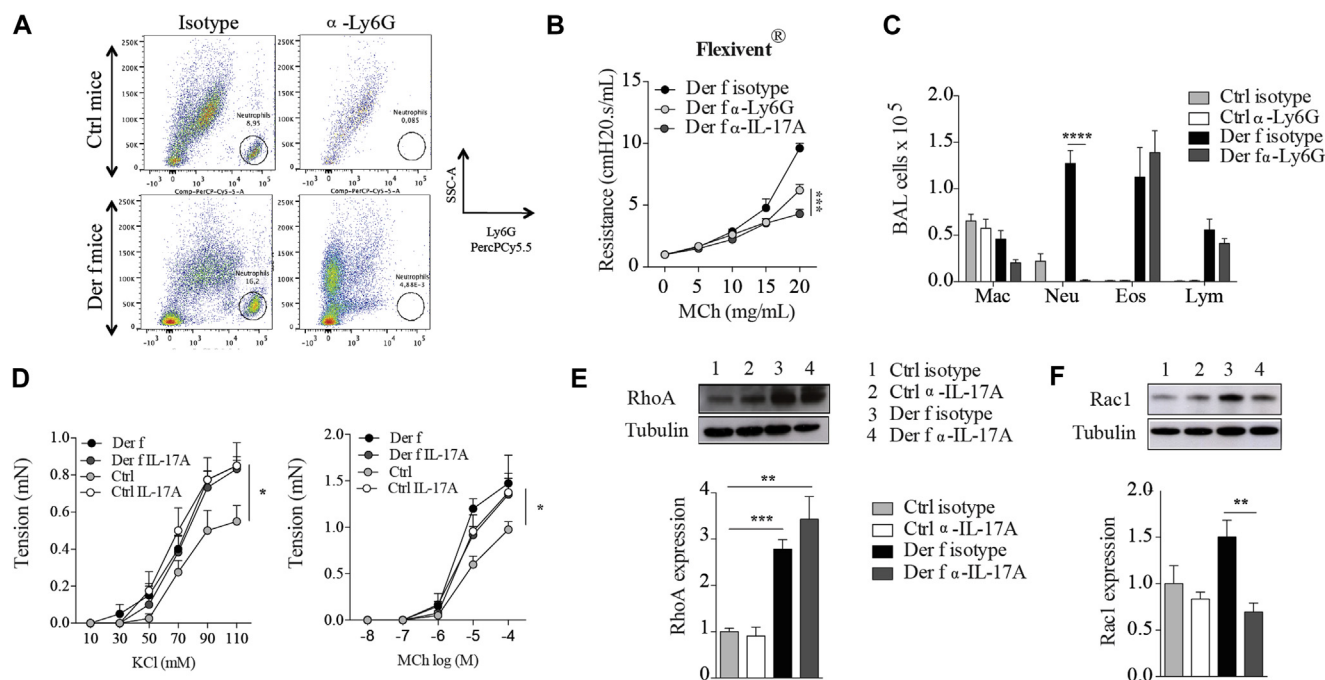


FIG 1. IL-17A neutralization abrogates Der f-induced airway responses. **A**, Measurement of AHR by flexi-vent. **B**, Infiltrated cells in BAL (macrophages [Mac], neutrophils [Neu], eosinophils [Eos], and lymphocytes [Lym]). **C**, CXCL1 and CXCL5 expression in the lung. **D**, CXCL1, RANTES, and eotaxin production in BAL. **E**, Levels of T_H2 cytokines in BAL. **F**, Frequency of IL-4- and IL-13-producing T cells in the lung. Asthmatic mice (Der f) and control mice (Ctrl) were treated with an isotype control or anti-IL-17A (Der f isotype vs Der f α-IL-17A: ****P < .0001, ***P < .001, **P < .01, *P < .05, n = 6-9 mice per group). APC, Allophycocyanin; BAL, bronchoalveolar lavage.



Asthma was induced in mice by percutaneous sensitization and intranasal challenge with an HDM extract (*Dermatophagoides farinae*, or Der f; see Fig E1, A, in this article's [Online Repository at www.jacionline.org](http://www.jacionline.org)). Exposure to Der f resulted in increased airway resistance, notable production of Der f1-specific IgE, and strong cellular infiltration of the airways (Fig E1, B-D). Cell subtype analysis by flow cytometry revealed a mixed influx of eosinophils, neutrophils, and lymphocytes (see Fig E2, A, in this article's [Online Repository at www.jacionline.org](http://www.jacionline.org)). The recruitment of these inflammatory cells was associated with an upregulation of chemokines involved in eosinophil (RANTES and eotaxin) and neutrophil (CXCL1 and CXCL5) attraction (Fig E2, B and C). Having found increased levels of eosinophils and neutrophils, we investigated T_H2 and T_H17 responses. Elevated levels of the cytokines IL-4, IL-5, IL-13, and IL-17 in bronchoalveolar lavage associated with an expansion of T_H2 and T_H17 cells in lung tissues were found in Der f-induced asthmatic mice, confirming mixed T_H2 - T_H17 -driven inflammation in our model (Fig E2, D and E). The levels of IL-6 and IL-1 β , which are known to attract and promote the differentiation of T_H17 cells, were increased in the airways of the asthmatic mice 24 hours after the final challenge (Fig E2, D). These molecules have also been recently described as specific biomarkers for neutrophilic and mixed granulocytic inflammation in severe asthma.⁵

To investigate the implication of T_H17 in the development of AHR and granulocytic infiltration, intranasal injections of a blocking anti-IL-17 antibody were given during the challenges. Neutralization of IL-17 in Der f-treated mice significantly

reduced AHR (Fig 1, A) and dampened neutrophil influx in bronchoalveolar lavage (Fig 1, B). Interestingly, neutralization of IL-17 decreased the expression of the proneutrophil chemokines CXCL1 and CXCL5 in the airways of the anti-IL-17 antibody-treated mice (Fig 1, C and D). In contrast, we found similar levels of eosinophils and T_H2 cytokines in untreated and anti-IL-17 antibody-treated asthmatic mice (Fig 1, D-F), suggesting no synergic effect between T_H2 and T_H17 responses. Because IL-13 has been shown to be crucial in the induction of asthma symptoms,⁶ we evaluated airway inflammation and AHR after IL-13 neutralization. Our results indicated a partial improvement in AHR (see Fig E3, A, in this article's [Online Repository at www.jacionline.org](http://www.jacionline.org)) but no modification of bronchial inflammation (Fig E3, B-D). These results suggest a prime role for the IL-17-neutrophil axis in our model of allergic asthma.

Growing evidence supports an intimate link between neutrophils and severe asthma. However, the contribution of neutrophils to the development of AHR in T_H17 -dependent allergic airway disease remains to be elucidated. Consequently, to discover to what extent the complete disappearance of AHR after IL-17A neutralization was due to the decreased neutrophil influx, neutrophils were depleted by administration of anti-Ly6G (Fig 2, A). Neutrophil depletion partially decreased AHR in Der f-treated mice (Fig 2, B), with no impact on eosinophil levels (Fig 2, C), demonstrating that the effect of IL-17A was definitely not mediated only by neutrophils and suggesting a direct role for IL-17 in bronchial contraction. To confirm this hypothesis, we incubated isolated bronchi from control and asthmatic mice with IL-17A for 12 hours and evaluated the contractile force

generated in response to methacholine (MCh) or potassium chloride (KCl) *ex vivo*. The amplitude of contractile responses to KCl and MCh in bronchi from Der f-treated mice was increased compared with that of responses in bronchi from control mice and was not further enhanced by IL-17A (Fig 2, D). In contrast, IL-17A significantly enhanced both MCh- and KCl-induced contractions in bronchi from control mice, reaching an amplitude similar to that of contractile responses in bronchi from Der f-treated mice (Fig 2, D). Interestingly, this direct role of IL-17 in smooth muscle cell contraction is in accordance with results of a recent clinical trial demonstrating clinically meaningful effects of anti-IL-17A (brodalumab), especially in a group with high reversibility of FEV₁ in response to albuterol, a bronchial smooth muscle relaxant.⁷

The Rho small G-protein family members RhoA and Rac1 are central effectors of smooth muscle contraction signaling pathways.⁸ Interestingly, IL-17 has been described as enhancing smooth muscle contraction by boosting the RhoA pathway in mice.⁹ Our molecular investigations reveal that *in vivo* neutralization of IL-17 does not reduce the overexpression of RhoA in the bronchi of Der f-treated mice (Fig 2, E) but does significantly reverse the upregulation of Rac1 expression (Fig 2, F). Collectively, these data suggest that IL-17 promotes AHR in asthmatic mice by regulating bronchial contraction through a Rac1-dependent signaling pathway.

Overall, our study demonstrates that Der f-induced allergic asthma after percutaneous sensitization and airway challenge induces AHR and mixed T_H2/T_H17-driven inflammation, similar to the inflammatory phenotype observed in certain severe cases of asthma. In this model, IL-17A neutralization is sufficient to prevent Der f-induced exacerbation. Accordingly, our data support the development of therapies targeting IL-17A-mediated mechanisms to dampen airway inflammation and control refractory asthma.

We thank the Cytocell platform of Nantes for assistance with the flow cytometry analysis. Also, we thank the Therassay platform for the Respiratory Equipment (Plethysmography and Flexivent systems). We are grateful to members of the UTE IRT-UN animal facility. We thank Stallergenes for kindly providing Der f extracts.

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The Ministry of Social Development Regional Partner Grants Program supported this study.

Disclosure of potential conflict of interest: The authors declare that they have no relevant conflicts of interest.

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<http://dx.doi.org/10.1016/j.jaci.2014.12.1872>

METHODS

Murine asthma model

Wild-type 6- to 8-week-old BALB/c mice were used (Charles River Laboratories, Saint Germain sur l'Arbresle, France). Allergic asthma was induced in the mice using a total HDM extract (*Dermatophagoides Farinae*, *Der f*) provided by Stallergenes (Antony, France). The total extract was obtained by using a standardized method as previously described.^{E1} The endotoxin level contained in the HDM extract (3714 EU/mL endotoxin) was assessed by using endosafe-PTS (Charles River Laboratories). Anesthetized mice were sensitized on days 0, 7, 14, and 21 by cutaneous application of 500 μ g of HDM extract in 20 μ L of dimethyl sulfoxide (Sigma-Aldrich, St Louis, Mo) on the ears and challenged intranasally with 250 μ g of HDM in 40 μ L of sterile PBS on day 27 and day 34. The mice were given either rat anti-murine IL-17A, anti-IL-13, or anti-Ly6G antibody or an IgG2a isotype control antibody (25 μ g; R&D, Lille, France) administered intranasally on day 27 and day 34 during HDM (or PBS control) challenges in a final volume of 40 μ L. Analyses were performed 24 hours after the last HDM challenge on day 35. The Regional Ethical Committee for Animal Experiments of the Pays de la Loire (CEEA.2012.78) approved all animal protocols.

AHR measurement

Invasive AHR was measured using the forced oscillation technique with flexiVent (SCIREQ). Mice were anaesthetized, paralyzed (rocuronium bromide), tracheotomized, and mechanically ventilated. Increasing doses of MCh were nebulized to the chamber (0, 5, 10, 15, and 20 mg/mL). The flexiWare software was used for data analysis. Parameters collected were normalized to the 0 mg/mL dose.

Bronchoalveolar lavage fluid collection and staining for flow cytometry

Mice received a sublethal dose of pentobarbital (3.3 mg/kg). Bronchoalveolar lavage (BAL) was performed with 1 mL of PBS by means of cannulation of the trachea. The collected fluid was centrifuged, and the total cell numbers were counted with a Kova slide system by optical microscopy. Identification of immune cells was performed in BAL by flow cytometry as previously described.^{E2} Acquisition was made on LSR II (BD Bioscience, Le Pont-de-Claix, France) and analyzed with FlowJo software (Tree Star, Ashland, Ore).

Isolation of lung cells and staining for flow cytometry

Briefly, the lungs were removed, disrupted, and digested at 37°C for 1 hour in 10 mL of PBS containing Dnase I (Roche, Boulogne-Billancourt, France) and collagenase type II (Invitrogen Life Science Technologies, Cergy Pontoise, France). Lung cells were passed through a 40- μ m cell strainer with a syringe plunger, and the strainer was washed with 10 mL of RPMI 1640 media supplemented with 10% FBS, 200 mg/mL penicillin, 200 U/mL streptomycin, 4 mM L-glutamine, and 0.05 mM β -mercaptoethanol. After red blood lysis, 8×10^5 lung cells were transferred to a 96-well round-bottom plate and were stimulated for 5 hours with a mix containing phorbol 12-myristate 13-acetate (50 ng/mL) and ionomycin (500 ng/mL; Sigma-Aldrich) together with either monensin for T_H17 analysis (2 mg/mL; BD Biosciences) or brefeldin A for T_H2 assessment (1 mg/mL; BD Biosciences). For cytokine detection, FcRs were blocked with mouse CD16/CD32 mAbs (ebiosciences, Paris, France). Before surface-specific staining, the cells were stained with Fixable Viability Dye 450 (BD Biosciences) to exclude dead cells. The following antibodies were used for surface staining: CD3 PeCy7 (145-2C11; BD Biosciences) and CD8 APC-H7 (53-6.7; BD Biosciences). The cells were then fixed and permeabilized using a Cytofix/Cytoperm kit (BD Biosciences) and stained intracellularly with anti-IL-4 (11B11; eBiosciences), anti-IL-13 (eBio13A; eBiosciences), and anti-IL-17A (TC11-18H10; BD Biosciences).

ELISA

Blood was removed by cardiac puncture and centrifuged to collect serum. For the measurement of HDM-specific IgE level, the wells were coated with purified natural Der f1 protein (Indoor Biotechnologies, Cardiff, United Kingdom) overnight. After saturation of the wells with 1% BSA for 12 hours, sera were added (1:40) and left overnight. The plates were washed and horseradish peroxidase-conjugated antimouse IgE (MCA 419P; 1:2000; AbD Serotec, Colmar, France) was added for 5 hours followed by incubation with TMB (3,3',5,5'-Tetramethylbenzidine) substrate at room temperature for 20 minutes. Absorbance was measured at 450 nm with VictorTMX3 (PerkinElmer, Courtaboeuf, France). BAL cytokine and chemokine levels were assessed using Luminex technology (Biorad Laboratories, Marnes-la-Coquette, France) with the Pro Mouse Cytokine 23-plex kit. Data analysis was performed using the Bio-Plex Manager Software (version 4.0).

Histology

Paraformaldehyde (4%, 1 mL) was administered intratracheally in the lungs through a flexible catheter. Trachea was then ligatured and lungs excised. Lungs were fixed in paraformaldehyde 4% for at least 48 hours, embedded in paraffin, cut, and stained with periodic acid-Schiff or hematoxylin eosin for morphological studies and inflammation scoring. Inflammation scores were calculated from 0 to 7 by adding bronchial epithelial cells dystrophy grade (0-3) and peribronchial/perivascular inflammatory infiltrate abundance grade (0-4).

Analysis of airway reactivity *ex vivo*

Excised bronchi from control and asthmatic mice were cut into 2-mm sections and placed in physiological saline (130 mM NaCl, 5.6 mM KCl, 1 mM MgCl₂, 2 mM CaCl₂, 11 mM glucose, 8 mM HEPES, pH 7.4, with NaOH). Bronchial segments were incubated with or without recombinant cytokines (100 ng/mL of IL-17A; R&D) for 12 hours with Dulbecco modified Eagle medium at 4°C. The segments were then mounted on a multichannel isometric myograph (Danish Myo Technology, Aahron N, Denmark) containing a Krebs-Henseleit solution maintained at 37°C (118.4 mM NaCl, 4.7 mM KCl, 2 mM CaCl₂, 1.2 mM MgSO₄, 1.2 mM KH₂PO₄, 25 mM NaHCO₃, and 11 mM glucose), and a resting tension of 0.5 mN was applied. We constructed cumulative concentration-response curves in response to KCl and MCh (Sigma Aldrich, Saint-Quentin Fallavier, France). Chambered wire myograph was connected to a digital data recorder (MacLab/4e, ADInstruments, Oxfordshire, United Kingdom), and recordings were analyzed using LabChart (version 7) software.

RNA isolation and real-time quantitative PCR

The lungs were excised from the mice, disrupted, and homogenized in TRIzol reagent (Invitrogen), and RNA was extracted by phenol-chloroform separation. The total quantity of RNA was determined by using a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies Inc, Wilmington, Del). cDNA was synthesized starting from 2 μ g using MMLV Transcriptase according to the manufacturer's instructions. Real-time quantitative PCR was performed on a ViiA7 Fast Real-Time PCR System using commercially available primers (Applied Biosystems, Saint Aubin, France). A pool of cDNA from naive mice and the housekeeping gene *HPRT* were used to normalize RNA expression using the $2^{(-\Delta\Delta Ct)}$ technique.

Western blot

The bronchi were dissected from control and asthmatic mice pretreated with an anti-IL-17A or isotype antibody. Samples were homogenized in lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA, 5 mM MgCl₂, 1% Nonidet P40, 50 mM NaF, 0.5% sodium deoxycholate, 0.2% SDS, and 20 mM L- β -glycerolphosphate) and complete protease inhibitor cocktail

(Sigma). Samples were separated by using 10% SDS-PAGE and transferred to a nitrocellulose membrane (Biorad Laboratories). The membranes were blocked for 1 hour with 5% milk, incubated overnight with primary antibodies, incubated for 1 hour with peroxidase-conjugated secondary antibody, and revealed with Plus-ECL reagent (PerkinElmer).

Statistical analysis

Comparison of experimental values between 2 groups was done by using the Mann-Whitney test. The nonparametric Kruskal-Wallis test with Dunn's posttest was used for comparison of more than 2

groups. Penh and lung resistance results were analyzed using a 2-way ANOVA. All statistical analyses were performed in GraphPad Prism (version 6).

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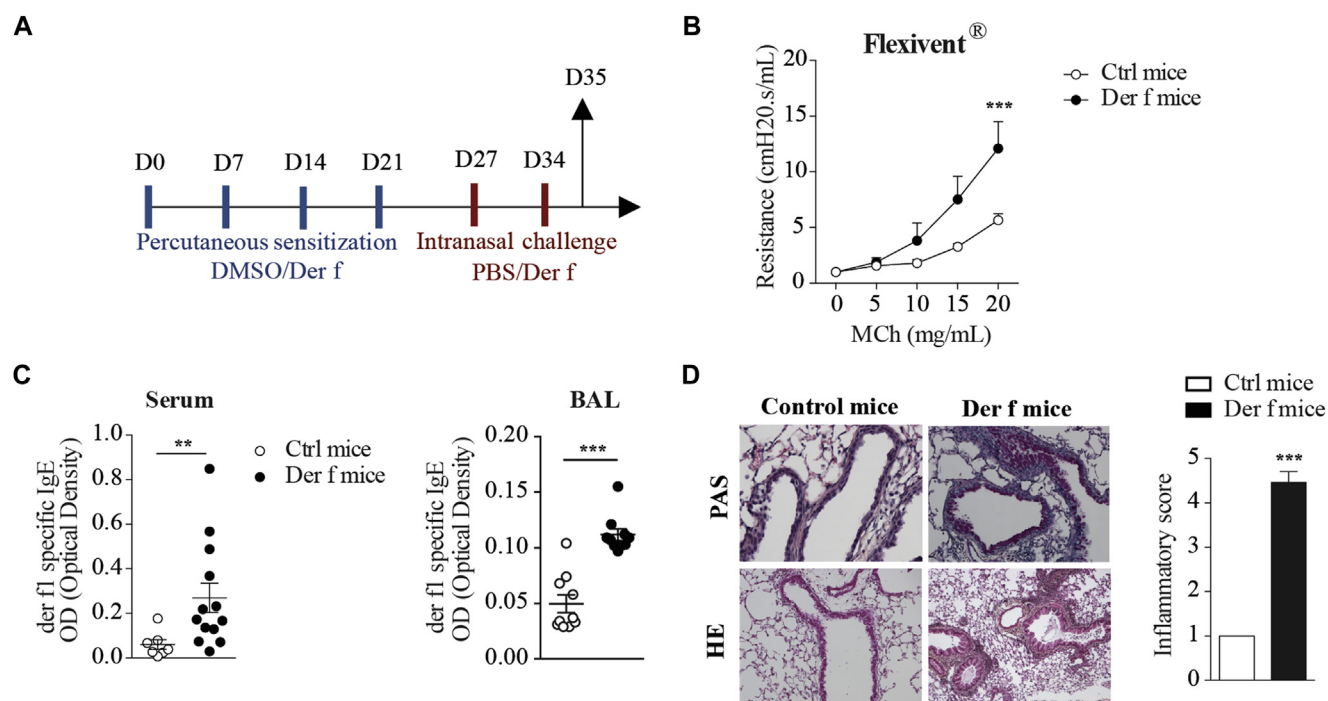


FIG E1. Percutaneous sensitization and intranasal challenge induce AHR and bronchial inflammation. **A**, Experimental model. **B**, Airway resistance measurement by flexiVent ($n = 8$ mice by group). **C**, Derf 1-specific IgE titers in serum and BAL. **D**, Representative PAS and H&E staining in airways (Der f vs Ctrl, $***P < .001$, $**P < .01$). BAL, Bronchoalveolar lavage; DMSO, dimethyl sulfoxide; H&E, hematoxylin eosin; PAS, periodic acid-Schiff.

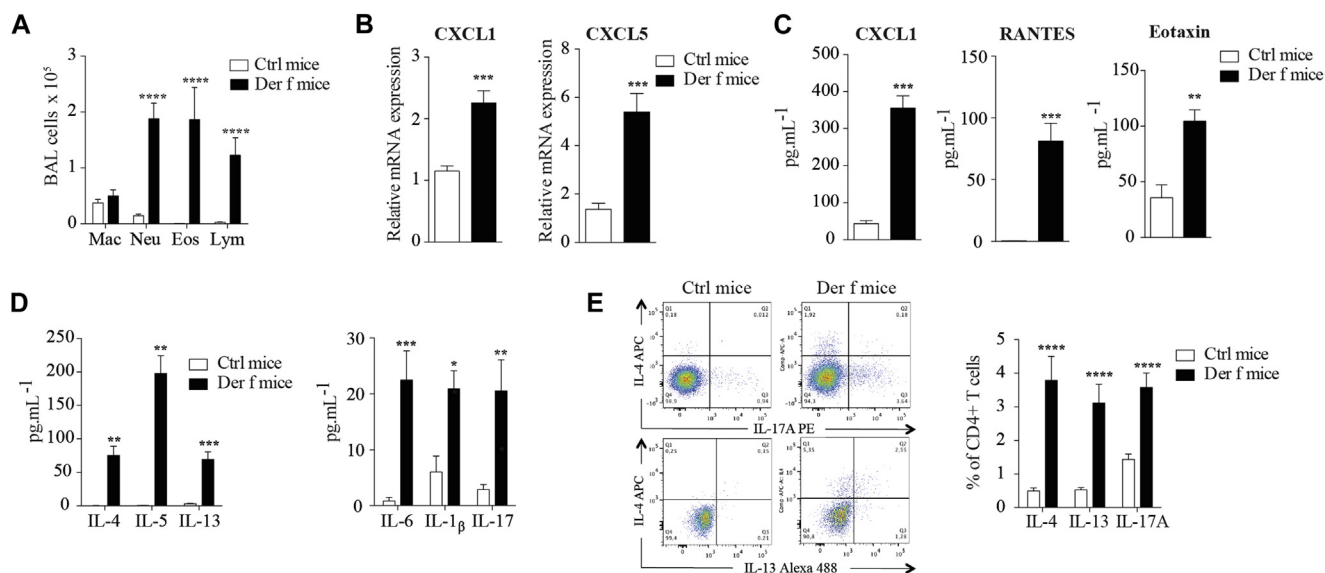


FIG E2. Allergen exposure generates mixed bronchial inflammation. **A**, BAL cellularity (n = 10-15 mice per group; macrophages [Mac], neutrophils [Neu], eosinophils [Eos], and lymphocytes [Lym]). **B**, Lung expression of CXCL1 and CXCL5. **C**, BAL levels of CXCL1, eotaxin, and RANTES. **D**, Secretion of T_H2- and T_H17-related cytokines in BAL (n = 6-8 mice per group). **E**, Intracellular staining for CD4⁺ T cells producing IL-13, IL-4, and IL-17A in the lung (n = 15-20 mice per group). BAL, Bronchoalveolar lavage. ****P < .0001, ***P < .001, **P < .01, and *P < .05.

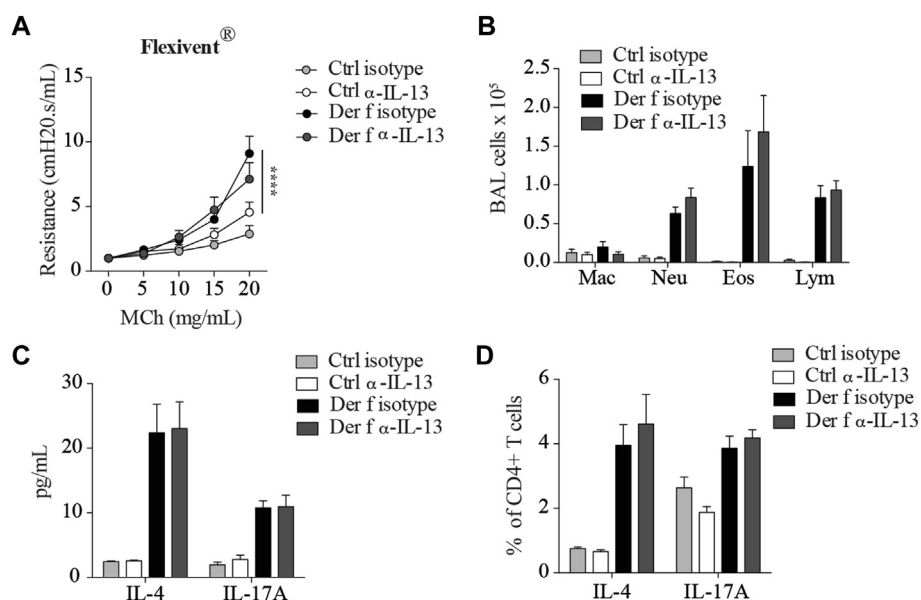


FIG E3. IL-13-blocking has a limited effect on AHR and airway inflammation. **A**, AHR assessment using flexiVent ($n = 6$ mice per group). **B**, Lung and BAL cellularity after antibody treatment ($n = 6-8$ mice per group). **C**, Multiplex cytokine quantification ($n = 5-7$ mice per group). **D**, Detection of intracellular cytokines in CD4⁺ T cells ($n = 6-8$ mice per group). BAL, Bronchoalveolar lavage.