

Combined sensitization of mice to extracts of dust mite, ragweed, and *Aspergillus* species breaks through tolerance and establishes chronic features of asthma

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Background: Existing asthma models develop tolerance when chronically exposed to the same allergen.

Objective: We sought to establish a chronic model that sustains features of asthma long after discontinuation of allergen exposure.

Methods: We immunized and exposed mice to a combination of single, double, or triple allergens (dust mite, ragweed, and *Aspergillus* species) intranasally for 8 weeks. Airway hyperreactivity (AHR) and morphologic features of asthma were studied 3 weeks after allergen exposure. Signaling effects of the allergens were studied on dendritic cells.

Results: Sensitization and repeated exposure to a single allergen induced tolerance. Sensitization to double and especially triple allergens broke through tolerance and established AHR, eosinophilic inflammation, mast cell and smooth muscle hyperplasia, mucus production, and airway remodeling that persisted at least 3 weeks after allergen exposure. Mucosal exposure to triple allergens in the absence of an adjuvant was sufficient to induce chronic airway inflammation. Anti-IL-5 and anti-IL-13 antibodies inhibited inflammation and AHR in the acute asthma model but not in the chronic triple-allergen model. Multiple allergens produce a synergy in p38 mitogen-activated protein kinase signaling and maturation of dendritic cells, which provides heightened T-cell costimulation at a level that cannot be achieved with a single allergen.

Conclusions: Sensitivity to multiple allergens leads to chronic asthma in mice. Multiple allergens synergize in dendritic cell signaling and T-cell stimulation that allows escape from the single allergen-associated tolerance development. (*J Allergy Clin Immunol* 2009;123:925-32.)

Key words: Chronic asthma, mouse, inflammation, airway hyperreactivity, tolerance, dendritic cells

Existing mouse models of asthma have provided a wealth of information.¹ A major drawback of these models has been the transient nature of the airway pathology and hyperreactivity. In these models airway pathologies peak 24 to 72 hours after

Abbreviations used

AEH:	Airway epithelial hypertrophy
AHR:	Airway hyperreactivity
DRA:	Dust mite, ragweed, and <i>Aspergillus</i> species
ERK:	Extracellular signal-regulated kinase
FO:	Forced oscillatory
FoxP3:	Forkhead box P3
Gti:	Tissue resistance
Hti:	Tissue elasticity
MAPK:	Mitogen-activated protein kinase
Mch:	Acetyl- β -methylcholine
NADPH:	Reduced nicotinamide adenine dinucleotide phosphate
OVA:	Ovalbumin
pERK:	Phosphorylated ERK
Raw:	Airway resistance

allergen challenge and resolve in 1 to 2 weeks.^{2,3} Mice chronically exposed to a single allergen frequently have tolerance, thus mimicking the 80% to 85% of the human population that is chronically exposed to allergen yet are asymptomatic.⁴⁻⁶ Eosinophilic inflammation and mucus production are modestly present or significantly reduced in many chronic models. Airway hyperreactivity (AHR) persists in many of these models in substantially attenuated form.⁷ Development of such tolerance is a major impediment to understanding the biology of chronic asthma. This is very important because the brunt of therapeutic intervention is aimed at controlling the chronic pathologic process of asthma. In an attempt to develop a mouse model of chronic asthma, we used natural allergens that cause human asthma because they contain nonimmunogenic components that have adjuvant-like effects.⁸⁻¹⁰ Because most allergic asthmatic patients are sensitized to multiple allergens, we sensitized mice with a combination of 3 allergens: dust mite, ragweed, and *Aspergillus* species. Our results suggest that multiple allergen exposure breaks through tolerance and induces airway pathologies and hyperreactivity that persist long after the allergen exposure.

METHODS

The majority of the methods used in this article can be found in the [Methods](#) section of this article's Online Repository at www.jacionline.org.

Allergens and adjuvant

Allergens used include ovalbumin (OVA; Sigma, St Louis, Mo) and extracts of dust mite (*Dermatophagoides farinae*), ragweed (*Ambrosia artemisiifolia*), and *Aspergillus fumigatus* (Greer Laboratories, Lenoir, NC). Adjuvant was aluminum and magnesium hydroxide (Imject alum; Pierce, Cheshire, United Kingdom; 1:1 vol/vol with allergen). Quantities of allergens for subcutaneous (100 μ L behind the ear) and intranasal (15 μ L in saline)

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allergen challenges are as follows: *D. farinae* (5 µg; LPS content, 3-35 EU by means of LAL assay); ragweed (50 µg; LPS content, 5 EU); *Aspergillus* species (5 µg; LPS content, 0.1 EU); dust mite, ragweed, and *Aspergillus* species (DRA) mix (dust mite, 5 µg; ragweed, 50 µg; and *Aspergillus* species, 5 µg); or OVA (60 µg). The allergen dose was based on the results of a survey of previous publications indicating successful sensitization and elicitation of allergic inflammation in the lungs.⁴⁻¹⁰

Protocol for chronic asthma

Female BALB/c mice were immunized at 12 to 15 weeks of age twice 1 week apart with various combinations of dust mite, ragweed, and/or *Aspergillus* species in alum, as described above. The week after the second immunization, intranasal challenges were performed twice a week for 8 weeks with the immunizing allergens. A control group of mice were immunized with saline in alum and intranasally exposed to saline. Another group of mice were chronically exposed to a single or multiple (DRA) allergens intranasally twice a week for 8 weeks without the preceding subcutaneous immunization in alum. The mice were rested for 3 weeks after the 8-week exposure period before analyses. For anticytokine therapy, the antibody was administered 2 weeks after the last (eighth) week of allergen challenge and assessed on day 21 after allergen challenge. A timeline of manipulations and interventions in the acute and chronic protocol is shown in Fig 1, A.

Protocol for acute asthma

Mice were immunized with OVA or *Aspergillus* species in alum as above twice 1 week apart. The week after the second immunization, animals were challenged intranasally with the immunizing allergens 5 days in a row (OVA model) or twice a week for 2 weeks (*Aspergillus* model). This slight difference in the *Aspergillus* model was included to be consistent with the chronic model, in which allergen exposure is performed twice a week. Airway pathologies and hyperreactivity were assessed at 72 hours after this acute exposure. In another set of experiments in the OVA model, symptoms were left to resolve for 2 weeks before intranasal anticytokine therapy (50 µg of anti-IL-5 or anti-IL-13) was administered, and a secondary (recall) allergen challenge was administered 1 hour later; symptoms were assessed 24 hours after this challenge.

Morphometric measurements

Inflammation was quantified with Metamorph image acquisition and analysis software (Molecular Devices, Eugene, Ore) on hematoxylin and eosin-stained lung sections (5 µm) at ×200 magnification. Airway inflammation and airway epithelial hypertrophy (AEH) were measured as the area of inflammatory infiltrates and area of epithelium per micrometer of basement membrane, respectively, by using a minimum of 7 airways per mouse and 3 to 5 mice per group.

RESULTS

Chronic exposure to a single allergen causes tolerance

We studied the effect of chronic exposure to a single allergen, *A. fumigatus* extract, and compared it with that after acute exposure. We also compared the effect of acute exposure to *Aspergillus* species with that to OVA because the latter is a widely used experimental allergen in mouse studies. We found that *Aspergillus* species immunization followed by a short series of intranasal challenge (acute model) caused significant airway inflammation, epithelial hypertrophy, and AHR to methacholine, and these effects were comparable with those seen after OVA administration (Fig 1, B, upper panel, and Fig 1, C). However, chronic (twice a week for 8 weeks) exposure to *Aspergillus* species followed by a 3-week rest period led to a remarkable reduction in airway

inflammation, AHR (Fig 1, B, middle panel, and Fig 1, C), and epithelial hypertrophy (see Fig E1, A, in this article's Online Repository at www.jacionline.org). Airway inflammation reduced from a 12-fold increase over that seen with the saline control in the acute model to a 3.4-fold increase over that seen with the saline control in the chronic model (Fig 1, C). AHR (Fig 1, D, compare "acute A [+]" with "A [+]") and epithelial hypertrophy (see Fig E1, A) showed a near-complete resolution. The possibility was examined that the mice in the chronic model had a higher level of inflammation immediately after the last allergen exposure and simply did not maintain it over the next 21 days. To this objective, we studied airway inflammation 24 hours after the last allergen exposure in the chronic *Aspergillus* model. The degree of inflammation at 24 hours was no different from that observed on day 21 (see Fig E1, B and E), suggesting that these mice had tolerance in regard to inflammation.

Chronic exposure to multiple allergens with and without immunization with an adjuvant resists tolerance development and induces sustained features of asthma

Because most allergic subjects are sensitized to multiple allergens, we reasoned that sensitization to multiple allergens might alter the outcome of chronic exposure. Therefore we sensitized and exposed mice for 8 weeks to a combination of 2 and 3 common allergens: dust mite, ragweed, and *Aspergillus* species extracts. The combination of allergens, especially the 3-allergen (DRA) combination, induced severe airway inflammation (12.4 µm² area/µm of basement membrane; Fig 1, B, middle panel, and Fig 1, C), epithelial hypertrophy (see Fig E1, A), and AHR (Fig 1, D, compare "DRA [+]" with "A [+]"), which persisted for 3 weeks after the last allergen exposure. Examination of a smaller group of mice 30 days after the last allergen exposure exhibited a level of airway inflammation that was similar to that observed at 3 weeks (see Fig E1, C and E). The combination of 2 allergens (dust mite and ragweed, dust mite and *Aspergillus* species, and ragweed and *Aspergillus* species) produced an intermediate phenotype, with dust mite and *Aspergillus* species and ragweed and *Aspergillus* species (both containing the *Aspergillus* species extract) being more potent than dust mite and ragweed (see Fig E1, D and E). The chronic model showed a higher level of airway resistance (Raw) as opposed to tissue resistance (Gti) and increased hysteresivity when analyzed using forced oscillation method (see Fig E2 in this article's Online Repository at www.jacionline.org).¹¹ If tolerance is defined by the loss of inflammation from the acute to the chronic phase to a level similar to that seen in saline-treated control mice, 93% of mice receiving DRA (n = 14) broke tolerance, whereas no mouse receiving a single allergen and 67% of mice receiving 2 allergens (only mice with *Aspergillus* species extract combinations) broke tolerance. We examined whether mucosal sensitization to DRA without the preceding subcutaneous immunization in alum would induce sustained airway inflammation. Mice chronically exposed to DRA without alum had airway inflammation (Fig 1, B, bottom panel, and Fig 1, C) and AHR (Fig 1, D, compare "DRA [-]" with "R [-]") that persisted for 3 weeks. Mice exposed to ragweed alone did not have inflammation and AHR. To confirm that the mice had systemic sensitization to the allergens after mucosal exposure, we studied splenic T-cell proliferation. Splenic T cells from mice receiving DRA but not mice receiving ragweed

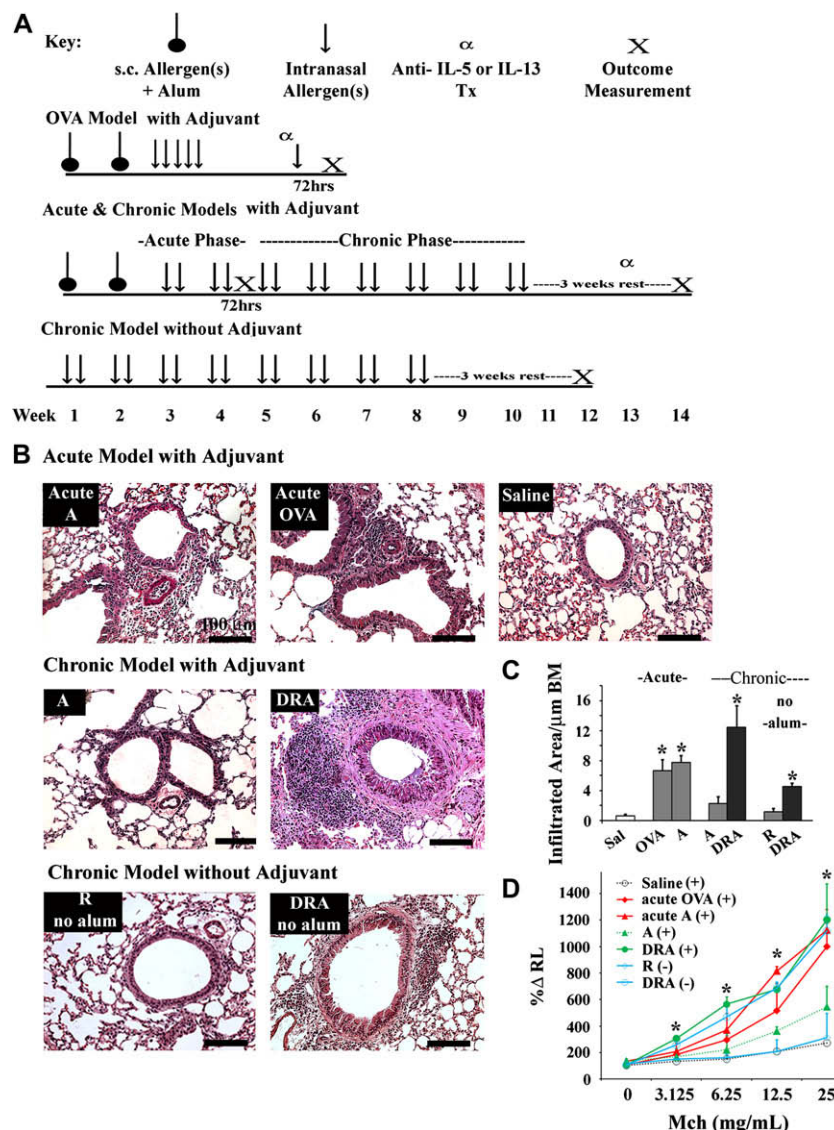


FIG 1. Inflammation and AHR in acute and chronic models. **A**, A timeline of immunization, allergen exposure, rest period, antibody treatment, and time of death in the acute and chronic models (with and without adjuvant) is shown. s.c., Subcutaneous. **B**, Top panel, $\times 200$ hematoxylin and eosin–stained lung sections from the acute *Aspergillus* model (Acute A), the acute OVA model, and saline control. Middle panel, Lung sections from the animals immunized with and exposed to *Aspergillus* species (A) alone or to DRA, as per the chronic protocol with alum. Bottom panel, Lung sections from the animals exposed to ragweed (R) or DRA as per the chronic protocol without alum. Histologic changes were assessed 3 and 21 days after allergen exposure in the acute and chronic models, respectively. Scale bars = 100 μ m. **C**, Quantitative scores from hematoxylin and eosin micrographs (magnification $\times 200$) for area of inflammatory infiltrates per micrometer of basement membrane (BM). N = 3 to 4 per group. * $P < .05$ (unpaired t test) for OVA and *Aspergillus* species (A) versus saline (Sal; acute model) and for DRA versus chronic *Aspergillus* species and chronic ragweed (R) in the chronic models. **D**, Methacholine-induced total lung resistance (RL; percentage change from baseline) was assessed at 72 hours (acute model) or on day 21 (chronic model). +, Immunization with alum; –, no alum. * $P < .05$ (unpaired t test, $n = 4$ –5) for DRA (+) versus chronic A (+) and saline (+); for acute A (+) and acute OVA (+) versus chronic A (+) and saline (+); and for DRA (–) versus R (–).

showed strong antigen-driven proliferation (see Fig E3 in this article's Online Repository at www.jacionline.org).

Characterization of airway inflammation and remodeling

Mice receiving DRA showed a 30-fold increase over the chronic *Aspergillus* species model in toluidine blue–positive intraepithelial

mast cells (Fig 2, A). Airway inflammation was associated with a significant increase in major basic protein–positive eosinophils (Fig 2, B), goblet cell hyperplasia (fluorescent periodic acid–Schiff staining for mucus glycoproteins; Fig 2, C), peribronchial smooth muscle mass (α -smooth muscle actin–positive cells; Fig 2, D), and collagen deposition (Sirius red stain; Fig 2, E) when compared with the single-allergen (*Aspergillus* species) model and saline control values. Peribronchial inflammation in mice

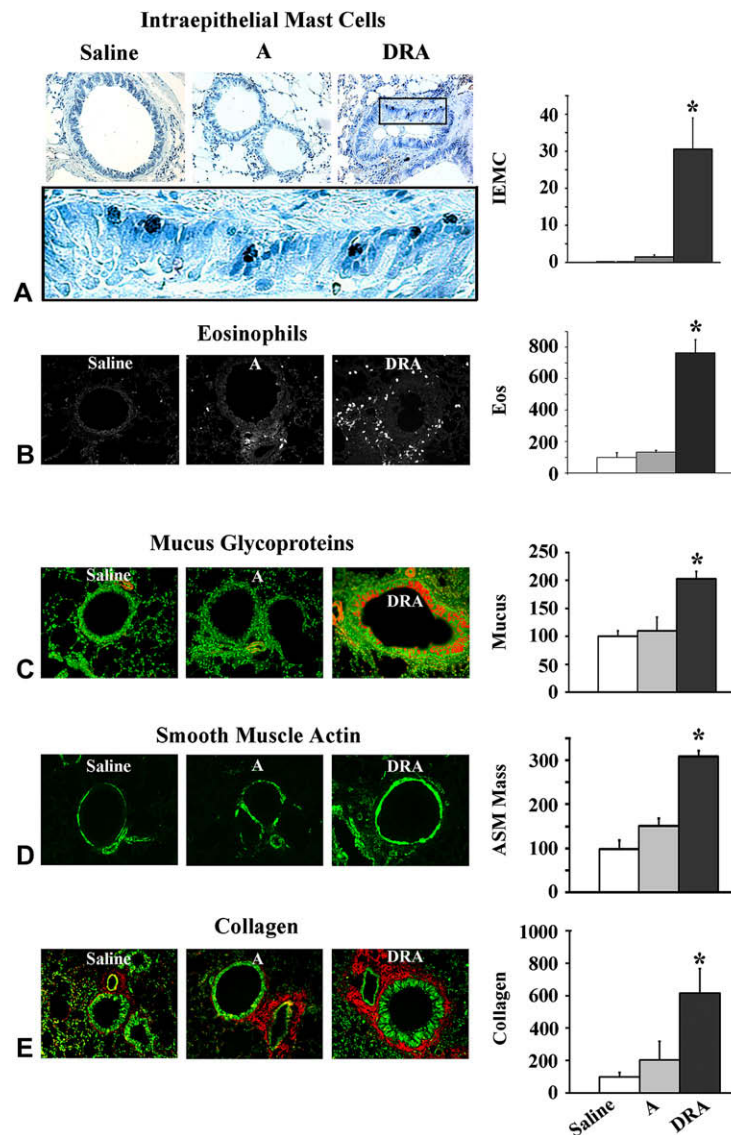


FIG 2. Characterization of airway inflammation and remodeling in the chronic DRA and *Aspergillus* species models. Tissue sections from mice sensitized and exposed to allergens (DRA, *Aspergillus* species [A], or saline) as per the chronic protocol were killed 21 days after the last allergen exposure and stained for the following. **A**, Mast cells (top panel) stained with toluidine blue. The bottom portion of the top panel shows a higher magnification of the boxed DRA airway segment from the upper panel. The bar graph on the right panel reflects quantification of intraepithelial mast cells (IEMC) per 10 airways per mouse. * $P < .002$, ANOVA. **B**, Eosinophil (Eos) immunostaining with a mouse anti-major basic protein antibody. **C**, Fluorescent periodic acid-Schiff staining for mucus glycoproteins (orange stain), which also stains tissue green in the fluorescein isothiocyanate channel and facilitates visualization of the tissue. **D**, Immunofluorescence staining (green) for α -smooth muscle actin (ASM), which stains peribronchial and perivascular smooth muscles and myofibroblasts. **E**, Sirius Red staining for collagenous fibers (red). Bar graphs on the right (Fig 2, B-E) show quantitation of the integrated fluorescent intensity per micrometer of basement membrane. * $P < .01$, ANOVA.

receiving DRA was characterized by the presence of CD45⁺ leukocytes and CD4 and CD8 T cells (see Fig E4 in this article's Online Repository at www.jacionline.org).

Cytokines, chemokines, serum IgE, and vascular cell adhesion molecule

We measured cytokine and chemokine levels in lung homogenates on day 21 after the last allergen exposure in chronic

models. Eotaxin, IL-5, IL-10, IL-13, and IL-17 levels were significantly increased in the lungs of mice receiving DRA compared with levels in those receiving saline and *Aspergillus* species (Fig 3, A). RANTES and TGF- β levels were significantly higher in mice receiving DRA versus those receiving saline but not those receiving *Aspergillus* species. Levels of the other cytokines and chemokines did not show any differences among the study groups. IL-4 was detectable in low quantities and did not show any difference between the study groups (see Fig E5 in

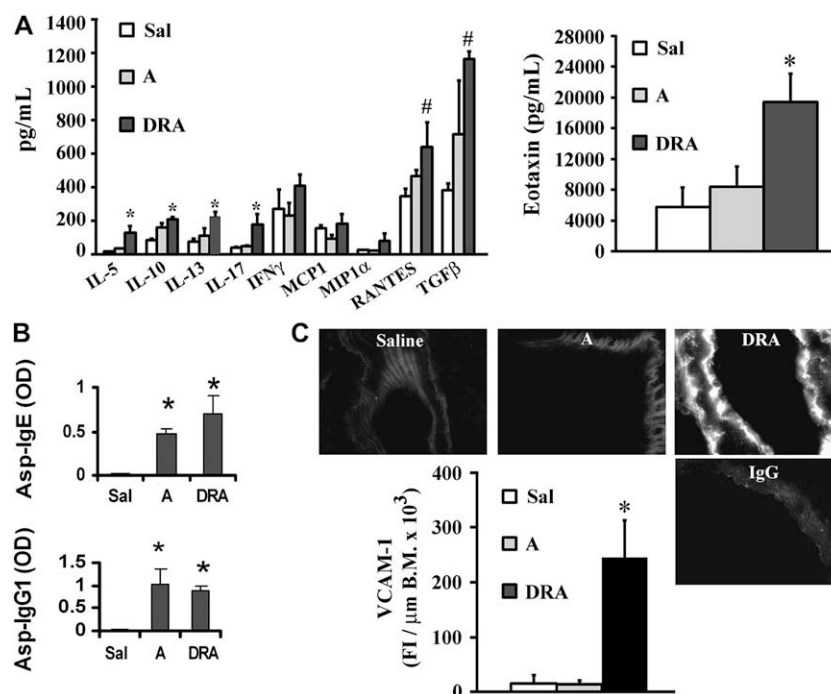


FIG 3. Cytokines, chemokines, serum IgE and IgG1, and adhesion molecules in the chronic asthma model. **A**, Left lung lobes from DRA-, *Aspergillus* species (A)-, or saline (Sal)-treated mice were homogenized, and the cell-free supernatant was assayed by means of multiplex ELISA ("Searchlight" by Endogen) for select cytokines and chemokines. IL-5, IL-10, IL-13, and IL-17 levels were measured separately by using a simple ELISA. N = 3 to 5. * $P < .05$, ANOVA, or # $P < .05$, DRA versus saline unpaired t test. MCP1, Monocyte chemoattractant protein 1; MIP1 α , macrophage inflammatory protein 1 α . **B**, Serum *Aspergillus* species-specific (Asp) IgE and IgG1 levels were measured by using a sandwich ELISA. N = 4 per group. $P < .0001$ versus saline (Sal). **C**, Saline (control)-, single allergen (*Aspergillus* species)-, and DRA-treated mouse lungs obtained 21 days after the last allergen exposure were immunostained for vascular cellular adhesion molecule 1 ($\times 1000$ magnification). The bottom panel shows quantitation of vascular cellular adhesion molecule fluorescence intensity (FI). * $P < 0.01$, ANOVA. N = 3. B.M., Basement membrane.

this article's Online Repository at www.jacionline.org). All sensitized animals had increased levels of *Aspergillus* species-specific IgE and IgG1 antibodies, but differences were not significant (Fig 3, B). T_H2 cells, eosinophils, and basophils selectively use vascular cellular adhesion molecule 1 for adhesion to endothelium and entry into the tissue.^{12,13} Mice receiving DRA had endothelial vascular cellular adhesion molecule 1 expression 25 times higher per micrometer of basement membrane than that expressed in chronically challenged single allergen-immunized and control mice (Fig 3, C).

Lack of efficacy of anticytokine antibodies in the chronic DRA model

Anti-IL-5 and anti-IL-13 antibodies are highly effective in the mouse model of asthma.^{14,15} Human studies with an anti-IL-5 antibody failed to demonstrate clinical usefulness.¹⁶ A recent human trial with a mutant protein that blocks the activation of IL-4 receptor α , a receptor common to both IL-4 and IL-13, showed selective inhibition of the late-phase allergic reaction without affecting AHR, FEV₁, peak expiratory flow, symptom scores, IgE levels, and eosinophil counts.¹⁷ We examined the effect of neutralizing antibodies against IL-5 and IL-13 in the acute (OVA) and chronic asthma models. We chose the acute OVA and not the acute *Aspergillus* model because most previous studies with anti-cytokine antibodies used the former model. Antibody was administered 1 hour before the allergen challenge in the acute

OVA model. In human chronic asthma this pretreatment approach is unrealistic because it is difficult to predict the timing of the next asthma attack. To mimic an intervention in human asthma, we administered antibody 2 weeks after the last allergen challenge (1 week before death) in the chronic asthma model. As reported previously, both anti-IL-5 and anti-IL-13 antibodies prevented airway inflammation and AEH (Fig 4, A and B) and AHR (Fig 5, C) in the acute asthma model.^{14,15} However, both antibodies were totally ineffective in reducing airway inflammation and hyperreactivity in the chronic asthma model (Fig 4, A-C). Thus our chronic model is better suited to predict therapeutic outcome in response to anticytokine therapy.

Mechanism of action of multiple allergens: Synergistic effect on signaling in and maturation of dendritic cells

Tolerance to a single allergen due to chronic exposure has previously been shown to be mediated by natural regulatory T cells and tolerizing dendritic cells.^{5,6,18-21} Interestingly, we observed increased forkhead box P3 (FoxP3)-positive cells in the lungs of mice receiving DRA compared with those of mice receiving *Aspergillus* species (see Fig E6 in this article's Online Repository at www.jacionline.org). This is in agreement with the increased concentrations of IL-10 and TGF- β found in lung tissue from mice receiving DRA. The results suggest that the lack of tolerance in mice receiving DRA is not due to the absence of FoxP3⁺

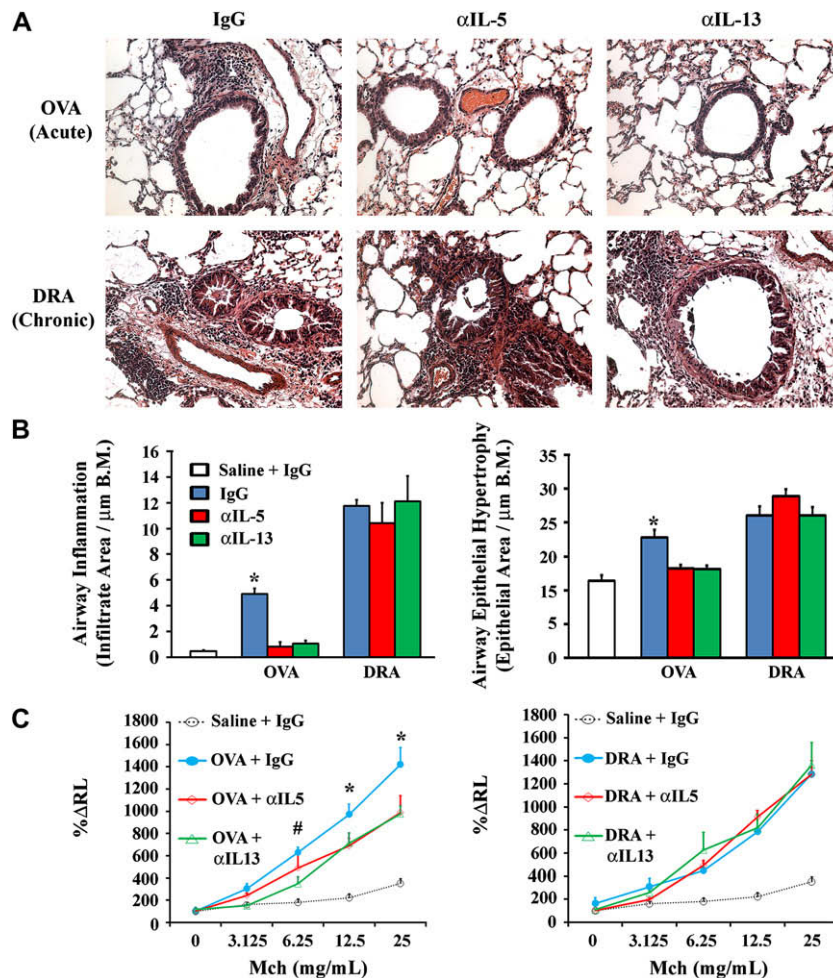


FIG 4. Effect of neutralizing antibodies against IL-5 and IL-13 on airway inflammation and hyperreactivity in the acute OVA and chronic DRA models. OVA-sensitized mice received antibodies 1 hour before a secondary challenge with OVA and were killed 24 hours later. DRA-sensitized mice received antibodies 2 weeks after the last allergen exposure and were killed 1 week later. Each mouse received either 50 μ g of rat IgG2a ($n = 3$) or IgG2b ($n = 3$) as negative control animals or 50 μ g of α IL-5 ($n = 5$) or α IL-13 ($n = 4$) antibodies. **A**, Hematoxylin and eosin micrographs showing airway inflammation. **B**, Quantitation of airway inflammation (left panel) and AEH (right panel). * $P < .008$ for IgG compared with anticytokine cohorts in the OVA group, ANOVA. $N = 4$ to 6. B.M., Basement membrane. **C**, Total lung resistance (RL) in response to nebulized methacholine expressed as a percentage change from baseline. Left panel, OVA-induced acute asthma model; right panel, DRA-induced chronic asthma model. $P < .05$ for *anti-IL-5 and anti-IL-13 or #IL-13 alone, respectively, versus IgG and saline (unpaired t tests).

regulatory T cells. Next we examined the effect of single versus multiple allergens on dendritic cells. We generated CD11c⁺ dendritic cells by culturing bone marrow cells in GM-CSF for 7 days.^{22,23} At the end of the culture period, more than 72% of the cells were CD11c⁺, and a majority of these cells expressed surface MHC class II (see Fig E7, A, left panel, in this article's Online Repository at www.jacionline.org). We observed a dose-dependent increase in extracellular signal-regulated kinase (ERK) 1/2 signaling in these dendritic cells when incubated with a single allergen. Fig E7, B, shows phosphorylated ERK (pERK) 1/2 induction by the *Aspergillus* species extract. Incubation with a single dose (3 μ g/mL) of dust mite, ragweed, and *Aspergillus* species and the equivalent dose of DRA (1 μ g of each allergen; total, 3 μ g/mL) induced a similar level of pERK1/2 in dendritic cells (Fig 5, A, left panel). The effect on p38 signaling was strikingly different. Dust mite, ragweed, and *Aspergillus*

species each at the dose of 3 μ g/mL induced p38 phosphorylation in 22% $\pm$ 3%, 28% $\pm$ 5%, and 25% $\pm$ 3% cells. DRA at the same total dose (ie, one third the dose of each individual allergen) induced p38 phosphorylation in 88% $\pm$ 7% cells, suggesting a synergistic effect (Fig 5, A, right panel, and see Fig E7, A, right panel). We measured the LPS content and protease activity of the allergen extracts. Dust mite and *Aspergillus* species had the highest and lowest levels of LPS, respectively (see the Methods section). Despite the 30- to 50-fold difference in LPS content between *Aspergillus* species and dust mite, the difference in p38 and ERK1/2 phosphorylation among these allergens was negligible. The *Aspergillus* species extract showed the highest level of protease activity among the 3 allergens (see Fig E8 in this article's Online Repository at www.jacionline.org), but this activity also did not make any difference in dendritic cell mitogen-activated protein kinase (MAPK) signaling.

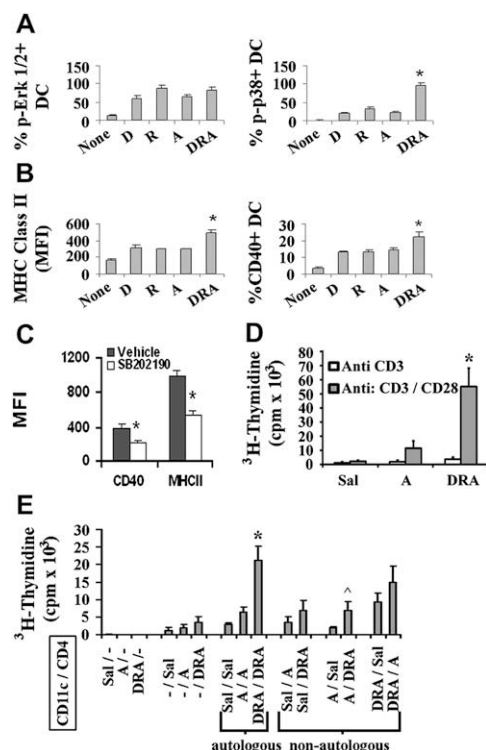


FIG 5. Dendritic cell signaling and T-cell activation. **A**, Effect of single (*D*, dust mite; *R*, ragweed; *A*, *Aspergillus* species) and triple (*DRA*) allergens on ERK1/2 (left) and p38 MAPK (right) phosphorylation in dendritic cells (DC). **P* = .0007, paired *t* test. *N* = 3. **B**, Effect of dust mite, ragweed, *Aspergillus* species, and *DRA* on MHC class II expression (mean fluorescence intensity [MFI]; left panel, **P* = .02 compared with dust mite, ragweed, and *Aspergillus* species [*n* = 3]) and the number of CD40⁺ dendritic cells (right panel, **P* = .03 compared with dust mite, ragweed, and *Aspergillus* species [*n* = 3]). **C**, Effect of pretreatment of dendritic cells with the p38 inhibitor SB202190 or vehicle (dimethyl sulfoxide) on *DRA*-induced CD40 and MHC II expression. *N* = 3. **P* < .04, paired *t* test. **D**, Effect of anti-CD3 and anti-CD3 plus anti-CD28 antibody stimulation on spleen T-cell proliferation (tritiated thymidine incorporation). **P* = .02 for anti-CD3 plus anti-CD28 stimulation, ANOVA. **E**, Effect of dendritic cells on anti-CD3-stimulated T-cell proliferation. Splenic CD4 T cells from saline (Sal)-, *Aspergillus* species-, and *DRA*-treated mice were stimulated with anti-CD3 in the presence or absence of splenic CD11c dendritic cells in autologous and nonautologous manners, and tritiated thymidine incorporation was measured. **P* = .01 and .04 for *DRA*/*DRA* versus autologous and nonautologous cohorts, respectively, ANOVA. ^*P* = .04 for *DRA*/*DRA* versus *Aspergillus* species/*DRA*, unpaired *t* test. *N* = 4.

Next we examined the biologic relevance of this increased p38 signaling caused by *DRA*. The expression level (mean fluorescence intensity) of MHC class II was significantly increased in triple allergen (*DRA*)-treated compared with single allergen-treated cells (Fig 5, *B*, left panel). Unlike MHC II, CD40 was expressed at a low level on dendritic cells, and this level changed very little after allergen stimulation. Individual allergens increased the number of cells expressing CD40 (Fig 5, *B*, right panel), as reported previously.²⁴ However, *DRA* induced a significantly greater number of CD40⁺ cells. The *DRA*-induced increase in MHC class II antigen and CD40 levels was significantly inhibited in cells that were pretreated with the p38 MAPK inhibitor SB202190 (Fig 5, *C*). These results suggest that multiple allergens induce a higher level of dendritic cell signaling, which leads to increased expression of costimulatory molecules.

Next we examined the T-cell receptor (TCR)-mediated proliferative response of CD4 T cells in the presence and absence of

CD11c⁺ dendritic cells from the mice receiving chronic *DRA*, *Aspergillus* species, and saline control. Anti-CD3 stimulation did not induce significant differences in proliferation of T cells from mice receiving *DRA* and *Aspergillus* species, but *DRA* T cells showed increased proliferation compared with *Aspergillus* species and saline T cells when stimulated with anti-CD3 plus anti-CD28 antibodies (Fig 5, *D*). Similarly, when dendritic cells were used in lieu of anti-CD28, DCs from mice receiving *DRA* provided the highest level of costimulation (Fig 5, *E*). In contrast, dendritic cells from mice receiving *Aspergillus* species significantly inhibited *DRA* T-cell proliferation. DCs from mice receiving *DRA* showed a tendency to augment proliferation of T cells from mice receiving saline and those receiving *Aspergillus* species, but the difference did not reach statistical significance.

DISCUSSION

We have developed a mouse model of chronic asthma with the following important new features: (1) resistance to tolerance despite repeated allergen exposure; (2) persistence of eosinophilic inflammation, AHR, and other features of asthma beyond the first 3 weeks after exposure; (3) mucosal sensitization to allergens and induction of chronic inflammation without the need for adjuvants; and (4) resistance to anti-IL-5 therapy, thus mimicking aspects of human chronic asthma. In most previous studies, pathologic features of experimental asthma were investigated within 24 to 72 hours after the allergen exposure. In a few studies in which the pathologic features were observed for longer than 72 hours, near-complete resolution took place within 1 week after the allergen exposure.²⁵ In our model the *Aspergillus* species extract was of pivotal importance. A combination of allergens that contained *Aspergillus* species induced sustained inflammation. Nonetheless, *Aspergillus* species alone was not sufficient to produce the phenotype. Previously, the effect of a combination of 2 allergens (recombinant cockroach and dust mite proteins rBla g 2 and rDer f 1) was studied in the acute asthma model at 72 hours. These mice did not have AHR.²⁶ Dust mite was previously shown to facilitate sensitization to inhaled OVA,²⁷ which is similar to the effect of *Aspergillus* species in our model. It should be noted that there are models of asthma in which chronic exposure to a single allergen leads to persistent, although low-level, inflammation and AHR, however inflammation in these models was studied within 1-3 days following the allergen challenge.^{28,29}

One shortcoming of mouse models is that they do not have sustained AHR. Although methacholine produces meaningful total lung resistance, the response from the parenchyma dominates, whereas Raw is largely absent.³⁰ The *DRA* model distinguishes itself from many other models by the persistence of Raw. One of the remarkable features of our model is the presence of intraepithelial mast cells. The accumulation of intraepithelial mast cells in tracheal sections and peribronchial regions has been reported previously.³¹ Intraepithelial mast cells are ideally situated to encounter inhaled allergens and promote sustained inflammation and AHR.³²

A common practice in preclinical drug development is to pretreat animals with a therapeutic agent before an allergen challenge and then study the outcome. This simple approach has limitations in human asthma. Our model has an advantage in this regard because in its chronic stage it can be used to test therapeutic agents without performing a *de novo* allergen challenge. The result from the anti-IL-5 antibody experiment suggests that our asthma model is a better predictor of outcome in

human asthma. Our results are in agreement with a recent report by Kumar et al,³³ who showed a lack of efficacy of an anti-IL-5 antibody. An anti-IL-13 antibody inhibited airway reactivity only at the highest dose (50 mg/mL) of methacholine but had no effect at lower doses.

The mechanism of persistence of inflammation and AHR in mice receiving DRA is likely related to the effect of multiple allergens on dendritic cells. DRA stimulates p38 MAPK phosphorylation in more dendritic cells than a single allergen at the same protein concentration. This heightened p38 MAPK signaling increases MHC class II and CD40 expression on dendritic cells, which is in agreement with previous reports.^{34,35} We believe that the increased dendritic cell signaling and activation contribute to the heightened costimulatory activity of DRA-stimulated dendritic cells. As a result, T-cell activation remains unabated, and the airway inflammation persists.

The mechanism by which multiple allergens induce a synergy in p38 but not ERK1/2 signaling is unknown. This effect is unlikely to come from the protein load or the protein-derived antigenic epitopes alone because we have used the same amount of protein. It is possible that the synergy results from the adjuvant-like activities of the allergens. Many allergens contain proteases and reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase levels. In one study intranasal OVA failed to induce airway inflammation. However, when administered along with the proteases from *A fumigatus*, it produced significant airway inflammation.¹⁰ Pollen-derived NADPH oxidase has been shown to be essential for induction of airway inflammation by allergens.⁸ NADPH oxidase generates reactive oxygen species, which induce nonspecific inflammation in a p38-dependent manner. These studies suggest additional nonantigenic functions of allergens. We recognize that the allergen extracts we used are mixtures containing multiple proteins and that the effects seen in the model might be due to unknown components of the allergens.

Clinical Implications: We have developed a model of chronic asthma that allows for the study and treatment of long-lasting features of asthma, obviating acute *de novo* allergen challenges.

REFERENCES

1. Taube C, Dakhama A, Gelfand EW. Insights into the pathogenesis of asthma utilizing murine models. *Int Arch Allergy Immunol* 2004;135:173-86.
2. Lommatzsch M, Julius P, Kuepper M, Garn H, Bratke K, Irmscher S, et al. The course of allergen-induced leukocyte infiltration in human and experimental asthma. *J Allergy Clin Immunol* 2006;118:91-7.
3. Whitehead GS, Walker JK, Berman KG, Foster WM, Schwartz DA. Allergen-induced airway disease is mouse strain dependent. *Am J Physiol Lung Cell Mol Physiol* 2003;285:L32-42.
4. Cui ZH, Joetham A, Aydinoglu MK, Hahn YS, Born WK, Gelfand EW. Reversal of allergic airway hyperreactivity after long-term allergen challenge depends on gamma-delta T cells. *Am J Respir Crit Care Med* 2003;168:1324-32.
5. Takabayashi K, Libet L, Chisholm D, Zubeldia J, Horner AA. Intranasal immunotherapy is more effective than intradermal immunotherapy for the induction of airway allergen tolerance in Th2-sensitized mice. *J Immunol* 2003;170:3898-905.
6. Van Hove CL, Maes T, Joos GF, Tournoy KG. Prolonged inhaled allergen exposure can induce persistent tolerance. *Am J Respir Cell Mol Biol* 2007;36:573-84.
7. Siegle JS, Hansbro N, Herbert C, Yang M, Foster PS, Kumar RK. Airway hyperreactivity in exacerbation of chronic asthma is independent of eosinophilic inflammation. *Am J Respir Cell Mol Biol* 2006;35:565-70.
8. Boldogh I, Bacsai A, Choudhury BK, Dharajiya N, Alam R, Hazra TK, et al. ROS generated by pollen NADPH oxidase provide a signal that augments antigen-induced allergic airway inflammation. *J Clin Invest* 2005;115:2169-79.
9. Reed CE, Kita H. The role of protease activation of inflammation in allergic respiratory diseases. *J Allergy Clin Immunol* 2004;114:997-1009.
10. Kheradmand F, Kiss A, Xu J, Lee SH, Kolattukudy PE, Corry DB. A protease-activated pathway underlying Th cell type 2 activation and allergic lung disease. *J Immunol* 2002;169:5904-11.
11. Bates JH, Allen GB. The estimation of lung mechanics parameters in the presence of pathology: a theoretical analysis. *Ann Biomed Eng* 2006;34:384-92.
12. Bochner BS, Luscinskas FW, Gimbrone MA Jr, Newman W, Sterbinsky SA, Derse-Anthony CP, et al. Adhesion of human basophils, eosinophils, and neutrophils to interleukin 1-activated human vascular endothelial cells: contributions of endothelial cell adhesion molecules. *J Exp Med* 1991;173:1553-7.
13. Bereta J, Bereta M, Cohen S, Cohen MC. Regulation of VCAM-1 expression and involvement in cell adhesion to murine microvascular endothelium. *Cell Immunol* 1993;147:313-30.
14. Garlisi CG, Kung TT, Wang P, Minniccozi M, Umland SP, Chapman RW, et al. Effects of chronic anti-interleukin-5 monoclonal antibody treatment in a murine model of pulmonary inflammation. *Am J Respir Cell Mol Biol* 1999;20:248-55.
15. Taube C, Duez C, Cui ZH, Takeda K, Rha YH, Park JW, et al. The role of IL-13 in established allergic airway disease. *J Immunol* 2002;169:6482-9.
16. Flood-Page P, Swenson C, Faerman I, Matthews J, Williams M, Brannick L, et al. A study to evaluate safety and efficacy of mepolizumab in patients with moderate persistent asthma. *Am J Respir Crit Care Med* 2007;176:1062-71.
17. Wenzel S, Wilbraham D, Fuller R, Getz EB, Longphre M. Effect of an interleukin-4 variant on late phase asthmatic response to allergen challenge in asthmatic patients: results of two phase 2a studies. *Lancet* 2007;370:1422-31.
18. Akbari O, DeKruyff RH, Umetsu DT. Pulmonary dendritic cells producing IL-10 mediate tolerance induced by respiratory exposure to antigen. *Nat Immunol* 2001;2:725-31.
19. Koya T, Kodama T, Takeda K, Miyahara N, Yang ES, Taube C, et al. Importance of myeloid dendritic cells in persistent airway disease after repeated allergen exposure. *Am J Respir Crit Care Med* 2006;173:42-55.
20. Kearley J, Barker JE, Robinson DS, Lloyd CM. Resolution of airway inflammation and hyperreactivity after in vivo transfer of CD4+CD25+ regulatory T cells is interleukin 10 dependent. *J Exp Med* 2005;202:1539-47.
21. Hadeiba H, Locksley RM. Lung CD25 CD4 regulatory T cells suppress type 2 immune responses but not bronchial hyperreactivity. *J Immunol* 2003;170:5502-10.
22. Scheicher C, Mehlig M, Zecher R, Reske K. Dendritic cells from mouse bone marrow: in vitro differentiation using low doses of recombinant granulocyte-macrophage colony-stimulating factor. *J Immunol Methods* 1992;154:253-64.
23. Inaba K, Inaba M, Romani N, Aya H, Deguchi M, Ikehara S, et al. Generation of large numbers of dendritic cells from mouse bone marrow cultures supplemented with granulocyte/macrophage colony-stimulating factor. *J Exp Med* 1992;176:1693-702.
24. Lamhamedi-Cherradi SE, Martin RE, Ito T, Kheradmand F, Corry DB, Liu YJ, et al. Fungal proteases induce Th2 polarization through limited dendritic cell maturation and reduced production of IL-12. *J Immunol* 2008;180:6000-9.
25. Kumar RK, Herbert C, Kasper M. Reversibility of airway inflammation and remodeling following cessation of antigenic challenge in a model of chronic asthma. *Clin Exp Allergy* 2004;34:1796-802.
26. Sarpong SB, Zhang LY, Kleeberger SR. A novel mouse model of experimental asthma. *Int Arch Allergy Immunol* 2003;132:346-54.
27. Fattouh R, Pouladi MA, Alvarez D, Johnson JR, Walker TD, Goncharova S, et al. House dust mite facilitates ovalbumin-specific allergic sensitization and airway inflammation. *Am J Respir Crit Care Med* 2005;172:314-21.
28. Johnson JR, Wiley RE, Fattouh R, Swirski FK, Gajewska BU, Coyle AJ, et al. Continuous exposure to house dust mite elicits chronic airway inflammation and structural remodeling. *Am J Respir Crit Care Med* 2004;169:378-85.
29. Wegmann M, Fehrenbach H, Fehrenbach A, Held T, Schramm C, Garn H, et al. Involvement of distal airways in a chronic model of experimental asthma. *Clin Exp Allergy* 2005;35:1263-71.
30. Tomioka S, Bates JH, Irvin CG. Airway and tissue mechanics in a murine model of asthma: alveolar capsule vs. forced oscillations. *J Appl Physiol* 2002;93:263-70.
31. Yu M, Tsai M, Tam SY, Jones C, Zehnder J, Galli SJ. Mast cells can promote the development of multiple features of chronic asthma in mice. *J Clin Invest* 2006;116:1633-41.
32. Masuda T, Tanaka H, Komai M, Nagao K, Ishizaki M, Kajiwara D, et al. Mast cells play a partial role in allergen-induced subepithelial fibrosis in a murine model of allergic asthma. *Clin Exp Allergy* 2003;33:705-13.
33. Kumar RK, Herbert C, Webb DC, Li L, Foster PS. Effects of anticytokine therapy in a mouse model of chronic asthma. *Am J Respir Crit Care Med* 2004;170:1043-8.
34. Escors D, Lopes L, Lin R, Hiscott J, Akira S, Davis RJ, et al. Targeting dendritic cell signaling to regulate the response to immunization. *Blood* 2008;111:3050-61.
35. Luft T, Rodionova E, Maraskovsky E, Kirsch M, Hess M, Buchholtz C, et al. Adaptive functional differentiation of dendritic cells: integrating the network of extra- and intracellular signals. *Blood* 2006;107:4763-9.

METHODS

Definitions

For the purposes of this article, we will arbitrarily define tolerance as a condition in which repeated exposure to an allergen or allergens causes progressively diminished airway response— inflammation, goblet cell hyperplasia, AHR, and T-cell proliferation in an immunized mouse. We refer to periods of 1 week or less as acute. Time periods of 3 weeks or greater will be referred to as chronic. The chronic model will be defined by using 2 criteria: (1) chronic exposure (8 weeks of allergen exposure) and (2) persistence of the features of asthma for at least 3 weeks after the cessation of allergen exposure.

Allergens used

OVA grade V was from Sigma Chemical Co (catalog no. A5503), and defatted extracts of dust mite (catalog no. XBP81D3A25), ragweed (catalog no. XP56D3A25), and *A fumigatus* (catalog no. XPM3C3A25) were from Greer Laboratories (Lenoir, NC).

Protease activity assay

Protease activity was measured in allergen extracts by using the Quanticleave Protease Assay Kit (Pierce). Briefly, allergen extracts (5 μg /mL) were prepared in PBS, and their protease activity was compared with that of a trypsin standard for the ability to cleave fluorescently labeled casein. Results are reported as relative trypsin activity found in 0.5 μg of allergen extract after 1 hour of incubation at room temperature. The fluorescence emission was read at an emission wavelength of 535 nm, with excitation at 485 nm.

Quantitative morphometric airways analysis

A minimum of the 7 most severely visually inflamed airways from each mouse was used to obtain a mouse average provided they fit the following criteria measured with image analysis software (Metamorph). First, airways needed to be entirely contained in a $\times 200$ field of view and completely intact. Second, the airways were considered oblong and excluded from analysis if they deviated from a theoretic circle by more than 20%. Third, exceptions included airways that appeared emphysematic (airway and alveolar contacts severed and airway is collapsed, although these were few in number and are thus averaged out) as appears in Fig 2, A. Airway sizes had a nonnormal distribution. Approximately 80% of the airways could be considered small airways (0–50,000 μm^2 , as arbitrarily defined by Hirota et al^{E1}), with a median size of 24,400 μm^2 .

Two aspects of airway inflammation were assessed. Airway inflammatory infiltrates and AEH were measured as follows. Airway inflammation was measured by color thresholding the 24-bit hematoxylin and eosin picture for the nuclei of inflammatory infiltrates. The area covered by the infiltrate's nuclei was divided by the perimeter of the airway's basement membrane. Thus a quantitative airway inflammation score of 1 is indicative of an airway that has 1 μm^2 of inflammatory infiltrate (nuclear area) per micrometer of basement membrane. AEH was also quantitatively scored and is defined as the area of epithelium (in square micrometers) divided by the basement membrane perimeter (in micrometers). There were no observable differences in inflammatory measures between differently sized airways in this model.

Airway hyperreactivity and lung mechanics

Mice were anesthetized with ketamine (180 mg/kg), xylazine (9 mg/kg), and acepromazine (4 mg/kg). After the animals lost the foot-pad pinch response, a tracheostomy was performed, and the subject was connected to a small-animal ventilator with a computer controlled piston (Flexivent; Scireq, Montreal, Canada) through an 18-gauge cannula and kept on a circulating warm-water pad (34°C). Subjects were ventilated at a frequency of 150 breaths/min with a tidal volume of 0.2 mL while breathing against an artificial positive end-expiratory pressure of 2.5 to 3 cm H₂O. After this, the lungs were inflated 3 times to total lung capacity to standardize volume history between subjects. Before this recruitment of closed airways, there were no differences between groups for any of the parameters measured (ie, no

allergen-induced resistance was detected in any of the groups). Similarly, there were no differences between groups for total lung resistance at baseline after lung inflations ($P = .42$, ANOVA). Resistance measurements were then taken to establish baselines for both the single-compartment model (allows measurement of total lung resistance) and the constant-phase model (compartmentalizes total lung resistance into Raw and tissue resistance/elastance). Resistance measurements were taken with a 2.5-Hz sinusoidal piston volume movement of 0.15 mL, and subsequently, pressure-volume and flow data were fit to the single compartment model (see equation 1) to obtain values for total lung resistance. Lungs were also probed with a forced oscillatory (FO) wave between 1 and 20 Hz over 4 seconds with a peak tidal volume of 0.15 mL and fit to the constant-phase model (see equation 2), which allows distinction between Raw and Gti and also affords the measurement of tissue elasticity (Hti). Freshly prepared methacholine, 0 to 25 mg/mL in 0.9% saline, was delivered through an inline nebulizer. The direct delivery of methacholine to the lung was timed with inspiration. Measurements were taken as described above, alternating between single-frequency sinusoidal measurements and FO waves for each dose of acetyl- β -methylcholine (Mch). After the 25-minute procedure, subjects were opened up and found to have a heart rate of 70 to 80 beats/min.

$$P_{tr}(t) = RV^*(t) + EV(t) + P_{EEP} \quad \text{equation 1}$$

The single-compartment model of the lung (see equation 1) is often used to obtain values for resistance (R) and elastance (E), which can be independently calculated when tracheal pressure (P_{tr}), flow (V^*), and volume (V) perturbations vary with time (t) while a known positive end expiratory-pressure (P_{EEP}) is applied.

$$Z(f) = R_{aw} + i2\pi fI + (G_{ti} - iH_{ti})/(2\pi f)^\alpha \quad \text{equation 2}$$

Raw is the Newtonian or airway resistance, and G and H are frequency-independent measures of tissue (ti) resistance and elastance, respectively. I is airway inertance, and i is the square root of -1 , whereas α is determined by G and H ($\alpha = [2/\pi] \arctan[H_{ti}/G_{ti}]$). FO lung mechanics can be further analyzed to fit a single-compartment lung model with constant-phase tissue impedance at each frequency applied to the lung (see equation 3). When these pulmonary input impedance (Z_{in}) data are solved, we are left with a real part of Z_{in} indicative of the resistance of airways of increasing size across the broadband signal and a summed imaginary part of Z_{in} called the reactance.

$$Z_{in}(f) = R_N(f) + 12\pi fI + (G_{ti} - iH_{ti})/(2\pi f)^\alpha \quad \text{equation 3}$$

Equations 1 and 2 have been thoroughly reviewed by Bates and Lutchen.^{E2} Input impedance has previously been described by Hantos et al.^{E3}

AHR data analysis

Coherence tolerances of 95% and 90% were considered for analysis of the single-compartment and constant-phase models, respectively. The peak 3 resistance measurements for each mouse at each dose were averaged to produce a single value per mouse per dose for each perturbation. Group averages were then expressed as fold increases in baseline resistance or elastance $\pm$ SEM. Statistical significance was established between groups by using both ANOVA and the unpaired Student t tests (JMP and Microsoft Excel) at each Mch dose. For input impedances, averaged raw data were displayed before and after Mch challenge.

Determination of airway cellularity

Mice were immunized and challenged as stated in the Methods section and rested for either 72 hours (acute model) or 21 days (chronic model). Right lung lobes were then fixed in formalin instilled gently through a tracheal cannula, sectioned (5 μm), and stained with toluidine blue (Sigma) to visualize mast cells. Briefly, deparaffinized slides were stained with a 0.1% solution (wt/vol) of acidic toluidine blue (pH 2.3 in 7% ethanol) for 2 minutes, washed 3 times in water, dehydrated, and mounted. Ten random airways per mouse that

satisfied the above criterion were counted for intraepithelial mast cells (dark purple against blue background). As described below for immunofluorescent staining, rabbit anti-mouse major basic protein (from James Lee, Mayo Clinic, Scottsdale, Ariz) was used to visualize eosinophils, and anti-CD45, anti-CD4, or anti-CD8 (fluorescein isothiocyanate conjugated) were used to visualize the presence of the respective leukocytes.

Airway remodeling

Animals were killed 21 days after the last of the chronic allergen challenges, and right lungs were taken as above, fixed in formalin, sectioned (5 μ m), and stained for the following. Goblet cell hyperplasia was determined by staining lung sections with a fluorescent periodic acid–Schiff stain, as reported previously.^{E4} Airway smooth muscle mass was visualized by means of immunofluorescent staining (see below) for α -smooth muscle actin. Collagen deposition in the lamina propria was visualized by staining lung sections with Sirius red (Sigma) and inducing fluorescence in the tetramethyl Rhodamine Iso-Thiocyanate (TRITC) channel (perivascular regions of collagen deposition were excluded from this analysis).^{E5} For analysis of all airway remodeling parameters, the fluorescent intensity of either the epithelium (airway mucus) or the subepithelium (α -smooth muscle actin and collagen) was calculated as integrated fluorescent intensity per micrometer of airway basement membrane and then normalized to saline control values.

Lung cytokine measurements

Lungs were isolated 21 days after the last allergen challenge from mice immunized with alum and challenged with either saline ($n = 3$), *Aspergillus* species ($n = 3$), or DRA ($n = 3$). Whole blood was collected from the inferior vena cava and aorta and used for serum analysis (see below). The lungs were flushed with 3 mL of sterile saline through the right ventricle. The left bronchus was clamped with a hemostat, and the left lung was removed before instilling the right lung with formalin or OCT compound (Ted Pella, Calif) for frozen sections. Left lungs were homogenized in RIPA buffer without any detergent (50 mmol/L Tris-HCl [pH 7.4], 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L phenylmethylsulfonyl fluoride, 1 μ g/mL aprotinin, leupeptin, pepstatin, 1 mmol/L Na_3VO_4 , and 1 mmol/L NaF). Lung lysates were then sent to Endogen for performance of the Searchlight ELISA-spot assay on the following mouse cytokines/chemokines: IL-5, IL-10, IL-13, IFN- γ , JE (MCP1), macrophage inflammatory protein 1 α , RANTES, TGF- β , and eotaxin. IL-5, IL-10, and IL-13 results were confirmed, and IL-4 and IL-17 levels were measured by using a sandwich ELISA assay in our laboratory. Values reported are for lung lysates at 1 μ g/ μ L protein concentration, as measured by the Bradford method (Thermo Scientific, Rockford, Ill).

Measurement of serum *Aspergillus* species-specific IgE and IgG1 antibodies

Serum was collected and diluted to 1 μ g/ μ L (Bradford) in assay diluent. Briefly, ELISA plate wells (Immulon 4 HBX; Fisher Scientific, Pittsburgh, Pa) were coated overnight at 4°C with 10 μ g/mL *Aspergillus* species extract in PBS. Plates were washed 3 times with PBS–Tween 20 (0.05%) and blocked in PBS (10% FBS) for 1 hour at room temperature. The above diluted samples in a 50- μ L volume (50 μ g of protein) were applied in triplicate for 2 hours at room temperature, and plates were washed 5 times. One-step detection was accomplished by means of incubating with biotinylated anti-IgE or IgG1 (BD PharMingen, San Jose, Calif) mixed 1:1000 with streptavidin–horseradish peroxidase (BD PharMingen) for 1 hour at room temperature. Plates were washed 7 times and developed with TMB substrate (Pierce) for 20 minutes. The reaction was stopped by means of the addition of 2N H_2SO_4 and read at 450 nm. Results are reported as raw OD values.

Splenocyte proliferation assay

Splenocytes were isolated from the chronic groups (saline, *Aspergillus* species, and DRA) on day 21 after allergen challenge. Spleens were removed and passed through a 70- μ m steel mesh. Red blood cells were lysed with the hypotonic buffer ammonium chloride (ACK), and leukocytes were

washed several times before positively selecting CD11c⁺ cells (Miltenyi Biotec, Bergisch Gladbach, Germany). The depleted fraction was then used for negative selection of CD4⁺ cells (Miltenyi Biotec). Cells were resuspended in complete RPMI (1 mmol/L sodium pyruvate, nonessential amino acids, 10 mmol/L HEPES, 50 μ mol/L β -mercaptoethanol, and 10% FBS). Two thousand five hundred CD11c⁺ cells and 25,000 CD4⁺ cells were seeded in 200 μ L of cRPMI in a 96-well round-bottom plate (Cellstar, Greiner Bio-One, Monroe, NC) and stimulated with 0.75 μ g/mL anti-CD3 and 2 μ g/mL anti-CD28, where applicable (BD PharMingen). Cells were cocultured for 48 hours before pulsing with 0.5 μ Ci of tritiated thymidine (PerkinElmer, Waltham, Mass) for 16 hours. Cells were harvested and read on a β -counter.

Immunofluorescent staining

Antibodies used for immunofluorescent staining included anti-major basic protein (James Lee), anti-CD4, anti-CD8 (both fluorescein isothiocyanate conjugated and from BD PharMingen), anti-FoxP3 (clone FJK-16s), anti-CD45 (Santa Cruz Biotechnology, Santa Cruz, Calif), anti-vascular cellular adhesion molecule 1 (R&D Systems, Minneapolis, Minn), and anti- α -smooth muscle actin (Dako, Glostrup, Denmark). Briefly, paraffin sections (5 μ m) were deparaffinized in xylene and rehydrated. OCT-treated frozen sections were used for CD4 and CD8 staining. Blocking was performed with 10% goat or donkey serum in PBS (0.05% saponin) at room temperature for 1 hour. The primary antibodies (10 μ g/mL) were then incubated overnight at 4°C with 5% goat/donkey serum in PBS (0.05% saponin). Slides were washed several times in PBS (0.05% saponin) before applying the Alexafluor-488–conjugated secondary antibody (5 μ g/mL) for 1 hour at room temperature in 5% goat/donkey serum. Slides were washed several times as above, mounted, and viewed with an epifluorescence microscope (Nikon Eclipse 2000) equipped with a Cool-snap CCD camera and Metamorph image analysis software (Molecular Devices). Staining for major basic protein required antigen retrieval with porcine trypsin type II (0.2 mg/mL, Sigma), 1 mmol/L CaCl_2 , and 50 mmol/L Tris (pH 7.4) for 30 minutes at 37°C.

Generation of bone-marrow-derived dendritic cells

Bone marrow–derived dendritic cells were derived as previously described.^{E6,E7} Briefly, bone marrow was isolated and erythrocytes were lysed in hypotonic ACK buffer. Cells were cultured at 2×10^5 cells/mL in culture dishes (Fisher Scientific) in cRPMI (see the Methods section for splenocytes) supplemented with 20 ng/mL recombinant mouse GM-CSF. On day 3, immature nonadherent cells were subcultured as on day 0 and used for experiments on day 7.

Flow cytometry

Bone marrow–derived dendritic cells (5×10^5) were incubated at 37°C with increasing concentration of *Aspergillus* species, as indicated in the text, for 10 minutes. Cells were washed 3 times with ice-cold PBS, fixed with 4% paraformaldehyde, and stained for pERK. In another set of experiments, cells were incubated with dust mite, ragweed, and *Aspergillus* species at 3 μ g/mL each or DRA (1 μ g/mL of each allergen; total, 3 μ g/mL) for 10 minutes and then fixed with 4% paraformaldehyde and washed 3 times with ice-cold PBS. Later, cells were stained for phospho-p38 or pERK1/2. Cells were incubated in 24-well plates with dust mite, ragweed, *Aspergillus* species, or DRA at 3 μ g/mL overnight to check for MHC class II and CD40 expression. Cells were washed in PBS containing 5% FCS and 5 mmol/L sodium azide (FACS buffer) and stained for 30 minutes on ice. Cells were incubated with 2.4G2 blocking reagent/Fc blocking antibody for 15 minutes to reduce non-specific binding. Antibodies used were as follows: fluorescein isothiocyanate–labeled anti-MHC II, allophycocyanin-labeled anti-CD11c, and allophycocyanin-labeled anti-CD40 antibody. Cells were analyzed with a FACS Scan (BD Biosciences).

Unique features of airway hyperreactivity in the chronic asthma model

We applied the FO technique and fit to the constant-phase model to separate out the 2 main components of lung resistance, namely Raw and Gti.

Mice receiving DRA had significantly higher increases in baseline Raw at every concentration of Mch tested (Fig 3, A, left panel). Both the chronic DRA and acute *Aspergillus* species models show hyperreactivity in the lung periphery (Fig 3, A, right panel), whereas the acute model showed a much larger Hti (Fig 3, B, left panel) than seen in mice receiving DRA. Consequently, mice receiving DRA have increased hysteresivity (η : ratio of energy resistively dissipated to that elastically stored in the parenchymal tissue [Gti/Hti]) at all concentrations of Mch (Fig 3, B, right panel).^{E8} The FO-derived input impedance data show that there was no significant difference in methacholine-induced hyperreactivity for perturbations up to 2.5 Hz between the chronic DRA and acute *Aspergillus* models (Fig 3, C). However, the DRA chronic model shows a significant increase in resistance at all frequencies greater than 2.5 Hz with respect to the acute model.

Statistical analyses

We used *t* tests (paired and unpaired) and ANOVA to analyze data statistically. A *P* value of less than .05 was considered significant.

REFERENCES

- E1. Hirota JA, Ellis R, Inman MD. Regional differences in the pattern of airway remodeling following chronic allergen exposure in mice. *Respir Res* 2006;7:120-9.
- E2. Bates JH, Lutchen KR. The interface between measurement and modeling of peripheral lung mechanics. *Respir Physiol Neurobiol* 2005;148:153-64.
- E3. Hantos Z, Petak F, Adamicza A, Asztalos T, Tolnai J, Fredberg JJ. Mechanical impedance of the lung periphery. *J Appl Physiol* 1997;83:1595-601.
- E4. Evans CM, Williams OW, Tuvim MJ, Nigam R, Mixides GP, Blackburn MR, et al. Mucin is produced by Clara cells in the proximal airways of antigen-challenged mice. *Am J Respir Cell Mol Biol* 2004;31:382-94.
- E5. Borges LF, Taboga SR, Gutierrez PS. Simultaneous observation of collagen and elastin in normal and pathological tissues: analysis of Sirius-red-stained sections by fluorescence microscopy. *Cell Tissue Res* 2005;320:551-2.
- E6. Inaba K, Inaba M, Romani N, Aya H, Deguchi M, Ikehara S, et al. Generation of large numbers of dendritic cells from mouse bone marrow cultures supplemented with granulocyte/macrophage colony-stimulating factor. *J Exp Med* 1992;176:1693-702.
- E7. Scheicher C, Mehlig M, Zecher R, Reske K. Dendritic cells from mouse bone marrow: in vitro differentiation using low doses of recombinant granulocyte-macrophage colony-stimulating factor. *J Immunol Methods* 1992;154:253-64.
- E8. Bates JH, Allen GB. The estimation of lung mechanics parameters in the presence of pathology: a theoretical analysis. *Ann Biomed Eng* 2006;34:384-92.

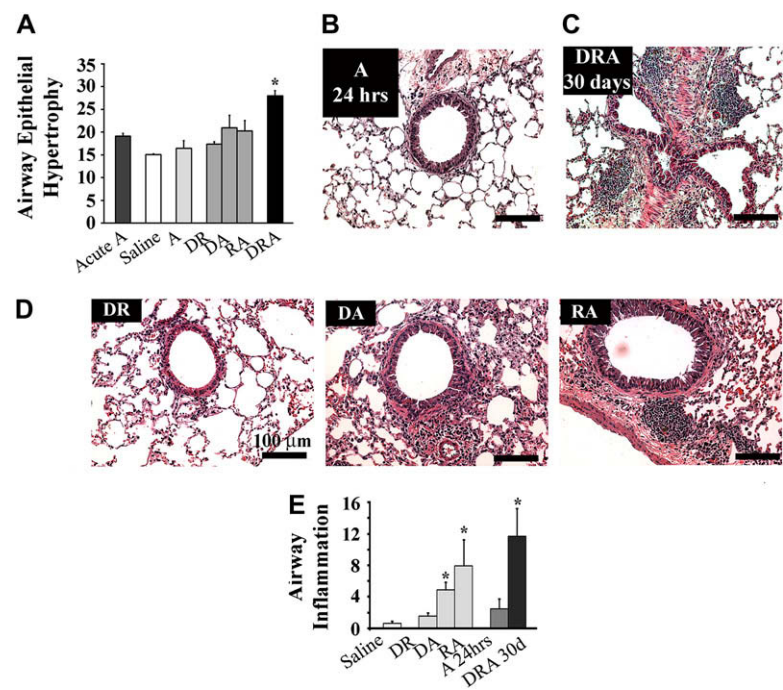


FIG E1. **A**, Morphometric quantitation of AEH. **B**, hematoxylin and eosin stain of a representative lung section from a mouse that was immunized with and exposed to *Aspergillus* species for 8 weeks. The mouse was killed 24 hours after the last allergen exposure (n = 3). **C**, Representative hematoxylin and eosin stain of a lung section from a mouse immunized with and exposed to DRA as per the chronic model with alum and rested for 30 days before death (n = 3). **D**, Hematoxylin and eosin stain of representative lung sections from mice immunized with and exposed to a combination of 2 allergens: dust mite and *Aspergillus* species (DA), dust mite and ragweed (DR), and ragweed and *Aspergillus* species (RA; n = 3). **E**, Morphometric quantitation of airway inflammation from the models presented in Fig E1, A through D (* $P < .05$ compared with saline). A, *Aspergillus* species.

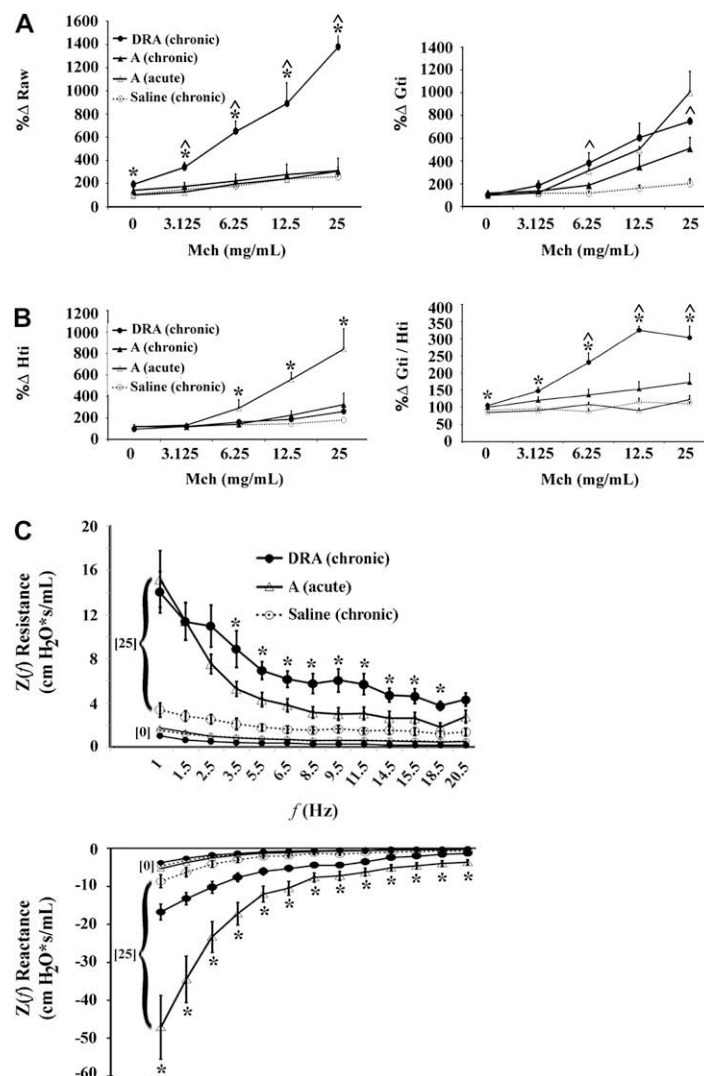


FIG E2. Airway hyperreactivity in acute and chronic asthma models. Raw was measured on anesthetized mice attached to a Flexivent apparatus. Measurements were done 21 days and 3 days after the last allergen exposure in the chronic and acute asthma models, respectively. Results are expressed as percentage change from prechallenge baseline as a function of methacholine concentration for the following. **A**, Left panel, Raw; right panel, Gti; **B**, left panel, Hti; right panel, tissue hysteresivity (Gti/Hti). **C**, Forced oscillation-derived input impedance data for resistance (top panel) and reactance (bottom panel). Raw data from the prechallenge baseline 0 and 25 mg/mL Mch expressed as a function of frequency (f). For Fig E2, A through C, P < .05 for chronic DRA *versus acute *Aspergillus* species or ^versus chronic *Aspergillus* species (unpaired t test). For Fig E2, C, comparisons are at the 25 mg/mL dose only. N = 3 to 5.

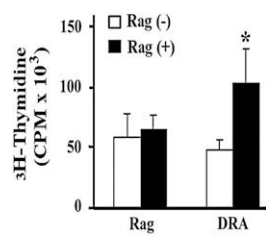


FIG E3. Splenic lymphocyte proliferation in response to allergen stimulation. Splenocytes were stimulated with and without ragweed extract (*Rag*; 50 μ g/mL) and cultured for 72 hours. Tritiated (³H) thymidine incorporation in the last 16 hours of culture was measured (n = 3, **P* < .05).

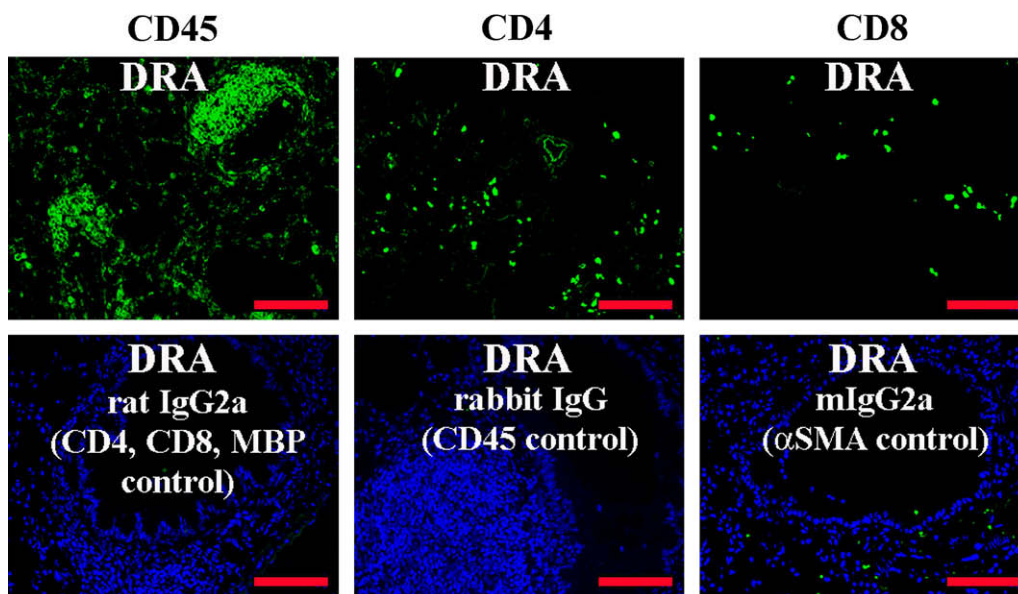


FIG E4. Immunostaining (green) for CD45 (panleukocyte marker)-positive leukocytes and CD4- and CD8-positive T cells in DRA lung sections ($n = 3$). Bottom images show immunostaining (green) with isotype control antibodies and counterstaining with the nuclear stain 4'-6-diamidino-2-phenylindole dihydrochloride (blue). *MBP*, Major basic protein.

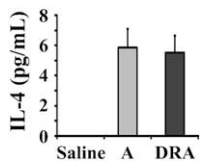


FIG E5. Measurement of IL-4 in lung homogenate from mice immunized with and exposed to saline, *Aspergillus* species (*A*), and DRA.

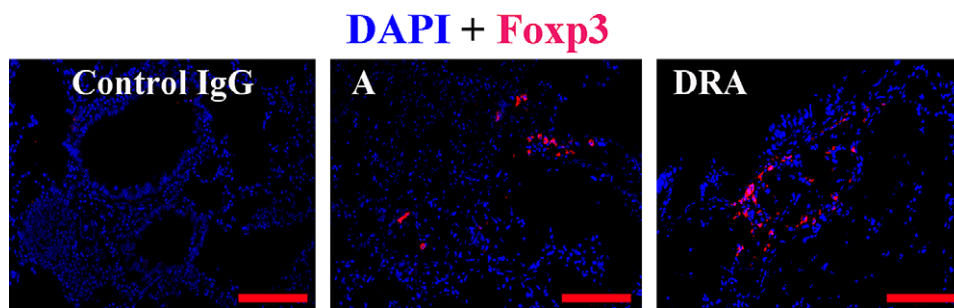


FIG E6. Expression of FoxP3⁺ cells in lung sections from mice receiving *Aspergillus* species (A) and mice receiving DRA. Lung sections from mice that were immunized and exposed to the allergens according to the chronic protocol were immunostained with an Alexa 594-labeled anti-FoxP3 antibody (red) and counterstained with 4'-6-diamidino-2-phenylindole dihydrochloride (DAPI; blue). Representative images from each study group (n = 3) and an IgG control stain are shown.

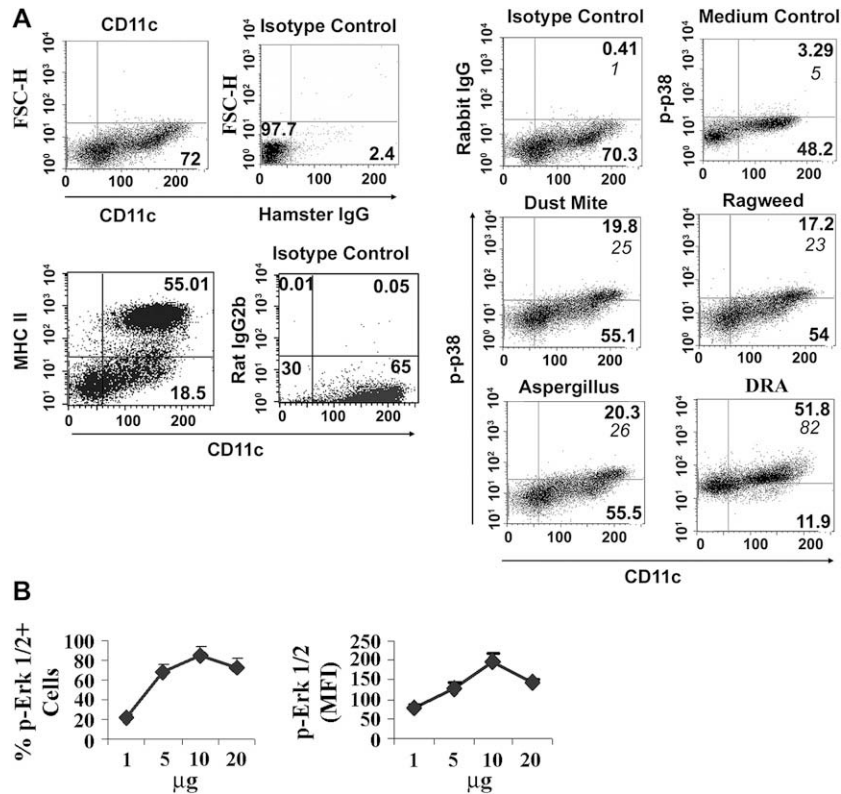


FIG 7. A, Generation of CD11c⁺ dendritic cells. Mouse bone marrow-derived cells were generated by culturing them in GM-CSF for 7 days. Cells were then stained with allophycocyanin-labeled hamster anti-CD11c or control hamster IgG antibody (*upper panel*). *In vitro* grown dendritic cells were double stained with the hamster anti-CD11c antibody and a rat fluorescein isothiocyanate-anti-mouse pan-MHC class II antibody (*lower panel*). A flow cytogram with the rat IgG2b control antibody is shown to the right. *FSC-H*, forward scatter height. *Right panel*, Allergen induction of phosphorylated (*p*) p38 MAPK in dendritic cells. Dendritic cells were treated with medium control or 3 μg/mL dust mite (*D*), ragweed (*R*), or *Aspergillus* species (*A*) or 3 μg/mL DRA (1 μg/mL of each allergen) for 10 minutes and then fixed and double stained with an anti-phospho-p38 MAPK and an anti-CD11c antibodies. The isotype control (rabbit) immunostaining for phospho-p38 antibody is shown in the *top panel*. The *number in each square* represents the percentage of gated cells. The *bottom italicized number in the top right square* represents the percentage of phospho-p38⁺CD11c⁺ cells. **B**, Effect of single allergens (*D*, dust mite; *R*, ragweed; *A*, *Aspergillus* species) and the triple allergen DRA on ERK1/2 (*left*) and p38 MAPK (*right*) phosphorylation in dendritic cells. **P* = .0007 (paired *t* test, *n* = 3). *MFI*, Mean fluorescence intensity.

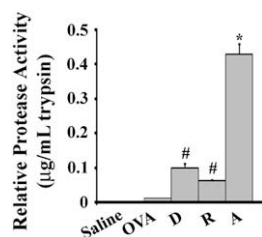


FIG E8. Protease activity of the allergens. Triplicate samples of OVA and the extracts of dust mite (*D*), ragweed (*R*), and *Aspergillus* species (*A*; 5 µg/mL) were used to measure protease activity relative to trypsin. # $P < .05$ and * $P < .01$ versus OVA.