

The extracellular matrix protein mindin regulates trafficking of murine eosinophils into the airspace

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Abstract: Asthma remains a major cause of morbidity and hospitalizations in developed nations. Despite the widespread prevalence of this disease, the genetic and environmental factors that mediate development and progression of allergic airways disease remain poorly understood. Pulmonary recruitment of eosinophils is believed to contribute to many cardinal features of allergic airways disease. Therefore, it is paramount to understand host factors that contribute to pulmonary eosinophil recruitment into the lungs. Mindin is a component of pulmonary extracellular matrix, which can regulate inflammatory cell recruitment. We characterized the role of mindin in the severity of allergic airways disease using established murine models. There were no baseline differences in wild-type and mindin-deficient animals in cell counts or airway physiology. Using the OVA murine model of allergic airways disease, we observed that mindin-deficient animals have less-severe allergic airways disease with fewer airspace eosinophils and lower lung-lavage levels of inflammatory Th₂ cytokines such as IL-13 and IL-4. Furthermore, mindin-deficient animals have reduced airway hyper-responsiveness after methacholine challenge. To determine the role of mindin in eosinophil trafficking, independent of antigen immunization or T lymphocyte activation, we instilled IL-13 directly into the lungs of mice. In this model, mindin regulates eosinophil recruitment into the airspace. In vitro experiments demonstrate that mindin can enhance eotaxin-mediated eosinophil adhesion and migration, which are dependent on the expression of integrins $\alpha_M\beta_2$ and $\alpha_4\beta_1$. In conclusion, these data suggest that mindin participates in integrin-dependent trafficking of eosinophils and can contribute to the severity of allergic airways disease. *J. Leukoc. Biol.* 85: 000–000; 2009.

Key Words: integrin · environmental airways disease · asthma · IL-13 · spondin2

INTRODUCTION

The prevalence of asthma has doubled over the past 20 years, and this disease now affects 7–11% of the population of the United States [1] and Europe [2, 3]. Allergic asthma is characterized by pulmonary eosinophilic inflammation, proinflammatory cytokines, and airway hyper-responsiveness (AHR). Recent evidence supports improved clinical outcomes when asthma treatment decisions are based on monitoring sputum eosinophil counts rather than conventional guidelines [4]. Studies in humans and murine models support a role of eosinophils in exacerbation and progression of allergic airways disease. Absence of eosinophils, through administration of antibodies [5] or genetic engineering of eosinophil-deficient mice [6, 7], protects against the development of AHR. Trafficking of eosinophils into the bronchoalveolar (BAL) compartment involves proinflammatory cytokines (IL-4, IL-5, and IL-13) and chemokines (RANTES and eotaxin). Adhesion of eosinophils is dependent on surface expression of at least seven integrin heterodimers [8] and binding to diverse ligands, including VCAM-1, ICAM-1, fibrinogen, and vitronectin [9]. Identifying novel proteins, which contribute to eosinophil trafficking, will improve our understanding of host factors regulating the pathogenesis of allergic airways disease.

Mindin (also known as spondin2) is an extracellular matrix (ECM) protein and a member of the mindin-F-spondin family [10]. Mindin is a highly evolutionarily conserved ECM protein with divergent functions in vertebrates. There are three fundamental reasons why we considered mindin as a candidate modifier gene in allergic airways disease. First, mindin is highly expressed in tissues relevant to allergic asthma including spleen and lung [11]. Second, mindin-deficient mice demonstrate impaired biological function of macrophages and mast cells [11], and each of these cell types is known to regulate the severity of allergic inflammation. Third, mindin mediates neutrophil recruitment into the lung, dependent on integrin binding [12]. Based on these observations, we hypothesized that

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mindin would modulate the severity of allergic airways disease through the regulation of eosinophil trafficking into the lung.

To test this hypothesis, we used two different *in vivo* murine models of allergic airways inflammation, as well as *in vitro* studies of eosinophil migration. In the OVA model, we demonstrated that mindin-deficient animals have attenuated an antigen-specific response in the lung, including eosinophilic inflammation, inflammatory cytokines, and AHR. To determine whether these defects were independent of immunization and T lymphocyte activation, we challenged mice directly with intratracheal IL-13. We demonstrated that mindin-deficient animals had reduced recruitment of eosinophils into the airways after challenge with IL-13. *In vitro* studies using mindin-coated Transwell plates demonstrated the role of mindin as a matrix in eosinophil chemotaxis. Furthermore, eosinophil adhesion and migration on mindin-coated plates are, in part, dependent on integrin binding. These findings suggest that mindin can function as an ECM for eosinophil trafficking and may contribute to the severity of allergic airways disease.

MATERIALS AND METHODS

Mice

Dr. Y-W. He generated and backcrossed mindin-deficient mice for seven generations onto a C57BL/6J background [11]. Dr. James Lee (Mayo Clinic, Scottsdale, AZ, USA) generated IL-5 transgenic mice [13]. Age- and gender-matched C57BL/6J mice were used as control animals. Experimental protocols were reviewed and approved by the Institutional Animal Care and Use Committee at Duke University Medical Center (Durham, NC, USA) and were carried out in accordance with the standards established by the U.S. Animal Welfare Acts.

OVA sensitization and challenge

Mice were sensitized on Days 0 and 7 by *i.p.* injections of 10 μ g OVA (Sigma Chemical Co., St. Louis, MO, USA), complexed with aluminum hydroxide (Pierce, Rockford, IL, USA), as described previously [14]. Beginning on Day 14, mice were exposed daily for 20 min to an aerosol of 1% OVA (Sigma Chemical Co.), generated using an Ultra-Neb99 (DeVilbiss Healthcare, Somerset, PA, USA). Mice received a daily antigen exposure for 2 or 7 days. Animals were phenotyped 24 h after the last aerosol challenge.

AHR

Direct measurements of respiratory mechanics in response to methacholine were made using the flexivent system (Scireq, Montreal, Canada) and reported as R_T cmH₂O/ml/s. Anesthesia was achieved with 60 mg/kg pentobarbital sodium injected *i.p.* and then given a neuromuscular blockade (0.8 mg/kg pancuronium bromide). Mice were ventilated with a computer-controlled ventilator (flexiVent, Scireq) with a tidal volume of 7.5 ml/kg and a positive end-expiratory pressure of 3 cmH₂O. Measurements of respiratory mechanics were made by the forced oscillation technique. Response to aerosolized methacholine (0 mg/ml, 10 mg/ml, 25 mg/ml, and 50 mg/ml) was determined by resistance measurements every 30 s for 5 min, ensuring the parameters calculated had peaked. Total lung capacity breaths were administered after each dose of methacholine to maintain patent airways and return the measurements back to baseline. The resistance measurements were then averaged at each dose and graphed (R_T cmH₂O/ml/s) along with the initial baseline measurement.

Analysis of airway inflammation, whole blood, and serum total IgE

Whole lung lavage cell differentials were determined as described previously [14]. Whole lung was fixed in formaldehyde at 25 cmH₂O pressure and

paraffin-embedded. Tissue was sectioned and stained with H&E. Interstitial inflammation was quantified in five to eight images of small airways from each fixed lung. A luminex instrument (BioRad, Hercules, CA, USA) was used to evaluate protein concentrations of IL-4, IL-5, RANTES, eotaxin, and keratinocyte-derived chemokine (KC; BioRad). An ELISA kit was used to evaluate IL-13 (R&D Systems, Minneapolis, MN, USA). The lower limit of detection for this assay by cytokine is IL-4 (3 pg/ml), IL-5 (2 pg/ml), RANTES (5 pg/ml), eotaxin (148 pg/ml), KC (3 pg/ml), and IL-13 (9 pg/ml). Serum total IgE levels were detected by ELISA (PharMingen, San Diego, CA, USA). Complete blood counts were performed with an Abbott Cell-Dyn 3700 hematology analyzer, previously optimized for the C57BL/6 strain of mice by the Duke Veterinary Diagnostic Laboratory. Automated differentials were confirmed by manual differential.

Semiquantitative real-time PCR

Total RNA was extracted from whole lung homogenates using the RNeasy mini kit (Qiagen Inc., Valencia, CA, USA), according to the manufacturer's protocol. RNA samples were then reverse-transcribed into cDNA using SuperScript II RT (Invitrogen Corp., Carlsbad, CA, USA), following the manufacturer's instruction. All real-time quantitative PCRs were performed using ABI SDS 7500 and SYBR Green reagent (Applied Biosystems, Foster City, CA, USA). Primers were produced by IDT, Inc. (Coralville, IA, USA), and sequences include eotaxin-2 forward, 5'-GGTTCAGAGGCACATACAAAAC-3', eotaxin-2 reverse, 5'-AAACCTCGGTGCTATTGCCAC-3'; GAPDH forward, 5'-ATCATCTCCGCCCTTCTG-3', GAPDH reverse, 5'-GGTCATGAGCCCTTCCACAAT-3' [15]. cDNA sample was amplified under the following conditions: 2 min, 50°C; 10 min, 95°C; 15 s, 95°C; and 1 min, 60°C, for 40 cycles. Gene expression values were normalized by the housekeeping gene GAPDH.

IL-13 challenge

Mice were given mouse recombinant IL-13 (Invitrogen-Biosource International, Carlsbad, CA, USA) in PBS intratracheal (3.9 μ g) on Days 1, 3, and 6. On Day 7, all animals were phenotyped for cellular inflammation in whole lung lavage. Cytokine levels in lavage were determined by luminex (BioRad).

In vitro eosinophil migration assay

Eosinophils were isolated by using the MACS cell separation system (Miltenyi Biotec, Auburn, CA, USA) from blood of IL-5 transgenic mice, generated by Dr. James Lee. Briefly, 500 μ l blood in 2% heparin was pre-separated by 60% Percoll (Sigma Chemical Co.) gradient in HBSS, 15 mM HEPES (pH 7.4). Isolated cells were mixed with anti-Ter-119-PE antibody, anti-CD90-PE antibody, and anti-CD45R-PE antibody and then labeled with anti-PE MicroBeads. Cells were applied to a LD column, and negative cells were collected. Purified cells were 95% eosinophils by cyto-spin and H&E. Trypan blue staining showed 98% cell viability.

The eosinophil migration assay was performed in a 24-well Transwell plate with a pore size of 5.0 μ m. Uncoated plates were used to evaluate eosinophil migration in response to soluble mindin or lavage supernatant (200 μ l/well). In other experiments, each well was precoated with 5 μ g mindin protein (recombinant protein generated as described previously [11]), fibronectin (Sigma Chemical Co.), or BSA (Sigma Chemical Co.) overnight at 4°C. Wells without coating were used as negative control. Eosinophils (1×10^6) in 100 μ l RPMI 1640 were loaded to the upper well. RPMI 1640 (600 μ l), with or without 30 nM mouse recombinant eotaxin (R&D Systems), was added into the lower chambers. Plates were incubated at 37°C, 5% CO₂, for 90 min and 4 h. Migrated cells in the lower chamber were collected and counted. For integrin-blocking assay, eosinophils were incubated with blocking antibodies (20 μ g/ml; Biolegend, San Diego, CA, USA) for 20 min at 37°C, 5% CO₂, prior to being added to the upper well. Similarly, after 90 min and 4 h, cells that migrated toward eotaxin in the lower chamber were collected and counted.

Eosinophil adhesion assay

The eosinophil adhesion assay was performed using a precoated, 96-well, noncell culture plate as described previously [9, 12] with slight modifications. Briefly, the plate was precoated with 20 μ g/ml mindin or fibronectin in 0.1 M sodium carbonate buffer (pH 9.6) overnight. After washing with PBS three times, the plate was blocked with 200 μ l heat-inactivated FBS for 30 min at 37°C. Purified normal eosinophils (100 μ l) from IL-5 mice (2×10^5 /well) in

RPMI 1640/10% FBS were preincubated with 30 nM eotaxin and 10 μ g/ml integrin block antibodies for 30 min at 37°C before being added to each well. The plate with cells was incubated for 1 h at 37°C and then washed. To quantify adherent eosinophils, the plate was washed, and the supernatant was removed. The plate was centrifuged for 10 min, and then, 100 μ l peroxidase substrate 3,3',5,5'-tetramethylbenzidine reagents (BD Biosciences, San Jose, CA, USA) with 0.2% Triton X-100 were added to each well, and the color was allowed to develop for 30 min at room temperature. H₂SO₄ (50 μ l 4 M) was added to stop the reaction. The plate was read at 450 nm in a microplate reader (EL 808 Ultra microplate reader, Bio-Tek Instruments, Winooski, VT, USA). The number of adherent cells was calculated according to a standard curve made by measuring serial-diluted eosinophils in noncoated wells.

Statistics

Data are expressed as mean $\pm$ SEM. Significant differences between groups were identified by two-tailed ANOVA for multiple comparisons. Primary comparisons were made between mindin^{+/+} and mindin^{-/-} animals for each experimental condition using the two-tailed Student's *t*-test. A *P* value of less than 0.05 was considered statistically significant. Software used was SPSS (Chicago, IL, USA) and GraphPad (San Diego, CA, USA).

RESULTS

Mindin-dependent, antigen-specific allergic inflammation

Composition of the ECM can impact lung structure, which can alter physiologic function in the lung. For this reason, it was important to determine if baseline physiologic function was maintained in mindin-deficient animals. We demonstrate that mindin-deficient animals have preserved populations of air-space cells when compared with wild-type (Fig. 1A). Furthermore, we demonstrate that airway response to methacholine (Fig. 1B) and the compliance of the lung (Fig. 1C) are unaltered in naïve, mindin-deficient mice.

To determine the role of mindin in an antigen-specific response, we immunized and challenged mice to OVA. We demonstrate that after immunization with alum and aerosol antigen challenge for 2 or 7 days, mindin-deficient animals were protected from the development of allergic airways disease. Mindin-dependent differences were robust after 7 days of exposure to OVA. Mindin-deficient animals had reduced eosinophil recruitment into the airspace when compared with wild-type (Fig. 1A). No significant differences were observed in macrophages or lymphocytes in the lavage fluid. Mindin-deficient animals were also protected from AHR to methacholine (Fig. 1B) and from the reduction in lung compliance after antigen challenge (Fig. 1D). In addition to cellular inflammation and AHR, B lymphocyte class-switching is a classic manifestation of atopy and allergic inflammation. Mindin-deficient animals had a trend toward lower levels of serum IgE (C57BL/6, 4556 $\pm$ 664 pg/ml vs. mindin^{-/-}, 2857 $\pm$ 593 pg/ml; *n*=10; *P*=0.057). Furthermore, reduced cellular airway inflammation was associated with a significant reduction in Th₂ proinflammatory cytokines (Fig. 2, A–C). The level of RANTES and eotaxin in the lavage was below the sensitivity of the protein assay. No mindin-dependent differences in whole lung mRNA of eotaxin2 or protein level of leukotriene B₄ (LTB₄) in the lavage was observed (data not shown). However, we additionally observed reduced levels of the chemokine KC in the lavage fluid from OVA-exposed, mindin-deficient mice (Fig. 2D). We did not observe significant mindin-dependent differences in the number of

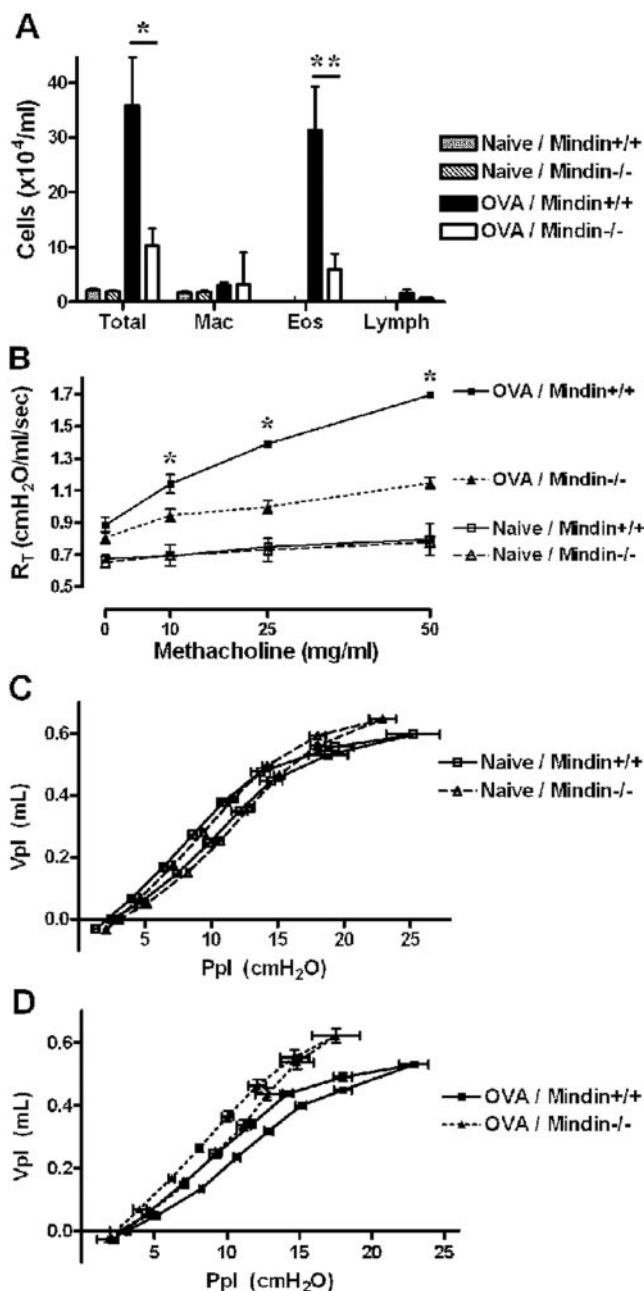


Fig. 1. Mindin-deficient mice are protected against allergic airways disease after OVA immunization and 7-day OVA challenge. (A) Total cells and absolute cell differentials in BAL fluids (BALF) of nonexposed and OVA-challenged, mindin-deficient (open bars) and wild-type mice (closed bars; *n*=9–10/group; mindin^{+/+} vs. mindin^{-/-}; *, *P*<0.05). Mac, Macrophage; Eos, eosinophil; Lymph, lymphocyte. (B) Forced oscillometry was used to determine baseline AHR to aerosolized methacholine (*n*=4–6/group; mindin^{+/+} vs. mindin^{-/-}; *, *P*<0.05). Lung compliance was measured in mice by direct measurements in unexposed (C) and OVA-exposed (D) animals (*n*=4–6/group). Vpl, ; Ppl, .

circulating eosinophils in the blood (Fig. 3, A and B) or infiltrating inflammatory cells around the airways by histology (Fig. 3C). To determine whether this phenotype was specific to prolonged exposure to antigen, animals were exposed to OVA for only 2 days. Similarly, exposure to OVA for only 2 days resulted in mindin-dependent alterations in the severity of allergic airways disease. Mindin-deficient animals demonstrate a trend toward

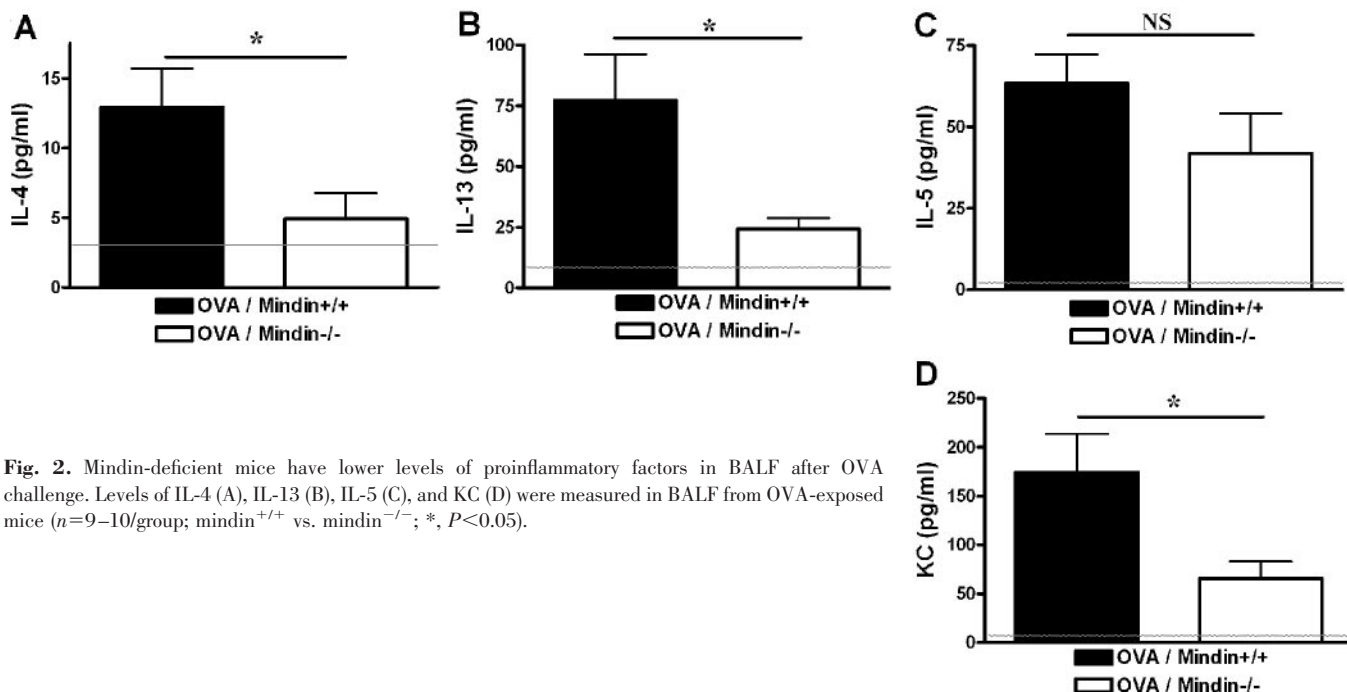


Fig. 2. Mindin-deficient mice have lower levels of proinflammatory factors in BALF after OVA challenge. Levels of IL-4 (A), IL-13 (B), IL-5 (C), and KC (D) were measured in BALF from OVA-exposed mice ($n=9-10$ /group; $\text{mindin}^{+/+}$ vs. $\text{mindin}^{-/-}$; *, $P<0.05$).

reduced absolute number of eosinophils (Fig. 4A), reduced percentage of eosinophils (Fig. 4B), and less-robust AHR after exposure to 50 mg/ml methacholine (Fig. 4C). The level of IL-4 [C57BL/6, $9.6 (\pm 4.49)$, vs. $\text{mindin}^{-/-}$, $3.0 (\pm 0.98)$; $P=0.15$] and IL-13 [C57BL/6, $148.89 (\pm 15.87)$, vs. $\text{mindin}^{-/-}$, $118.10 (\pm 14.80)$; $P=0.17$] in the lavage was independent of mindin. However, we observed lower levels of IL-5 lavage fluid [C57BL/6, $100.0 (\pm 21)$, vs. $\text{mindin}^{-/-}$, $47.24 (\pm 6.34)$; $n=7$; $P=0.02$] after 2 days of OVA challenge. These data support that mindin contributes to an antigen-specific allergic response including eosinophil airspace recruitment, AHR, and secretion of some proinflammatory cytokines.

Mindin-dependent, antigen-independent airway inflammation

Administration of IL-13 to airways of mice generates many of the cardinal features of allergic asthma including eosinophil

infiltration and Th_2 cytokine production [16, 17]. Although IL-13 is released primarily by T lymphocytes, the biological response to direct IL-13 challenge is largely independent of B lymphocytes or T lymphocytes [18]. Additionally, this model does not require sensitization to antigen, which minimizes the influence of dendritic cell (DC) and T lymphocyte interactions on the phenotype. Therefore, to specifically determine the role of mindin in eosinophil recruitment into the airways, independent of lymphocytes or sensitization, animals were challenged directly with recombinant murine IL-13. Similar to the OVA model, mindin-deficient animals demonstrate significantly impaired recruitment of eosinophils into the airspace after IL-13 challenge (Fig. 5A). No significant differences were observed in macrophages or lymphocytes in the lavage fluid. This finding supports a primary, mindin-dependent defect in eosinophil recruitment into the airspace. Interestingly, IL-13-treated, mindin-deficient animals also demonstrated a significant re-

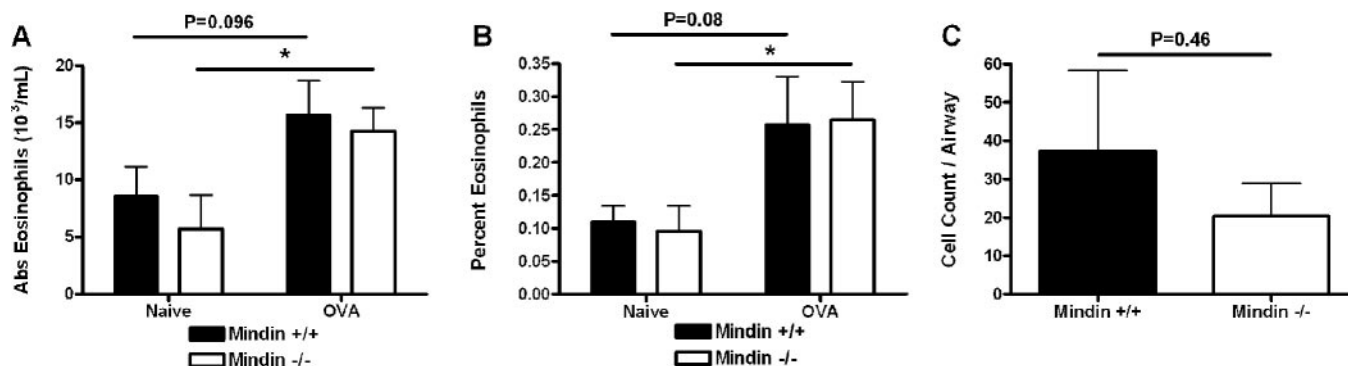


Fig. 3. Level of circulating eosinophils and interstitial eosinophils appear independent of mindin. Animals immunized and challenged to OVA for 7 days were evaluated for blood eosinophils and airway interstitial inflammation. Levels of circulating eosinophils were quantified in the whole blood and presented as absolute number (Abs) of eosinophils (A) or percent eosinophils (B; $n=7$ /group; $\text{mindin}^{+/+}$ vs. $\text{mindin}^{-/-}$; *, $P<0.05$). Histology samples were evaluated semiquantitatively for interstitial inflammation around small airways (C; $n=3$ /group).

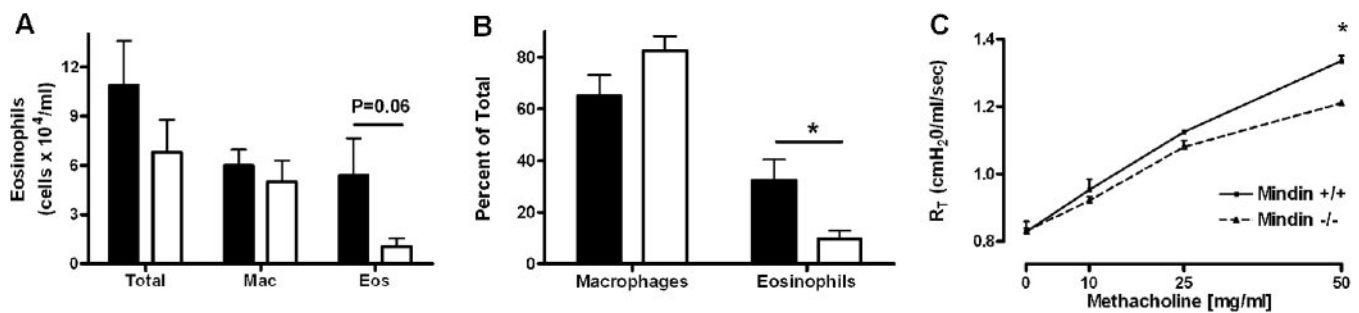


Fig. 4. Mindin-deficient mice are protected against allergic airways disease after OVA immunization and 2-day OVA challenge. (A) Total cells and absolute cell differentials in BALF of OVA-challenged, mindin-deficient (open bars) and wild-type mice (closed bars; $n=7$ /group; mindin^{+/+} vs. mindin^{-/-}; *, $P<0.05$). (B) Forced oscillometry was used to determine baseline AHR to aerosolized methacholine ($n=4$ /group; mindin^{+/+} vs. mindin^{-/-}; *, $P=0.0002$).

duction in the level of proinflammatory Th₂ cytokines (Fig. 5, B and C). We additionally observed reduced levels of other proinflammatory factors in the lavage fluid obtained from mindin-deficient mice including IL-12p70 [C57BL/6, 11.6 (± 1.8), vs. mindin^{-/-}, 5.1 (± 1.2); $n=7$; $P<0.05$] and KC (Fig. 5D). These results support the role of mindin in eosinophil activation and trafficking into the airspace in a manner independent of antigen-specific immunization.

In vitro eosinophil migration and adhesion are enhanced by mindin-integrin interactions

To specifically determine the role of mindin in eosinophil migration, we performed ex vivo eosinophil migration assays. Initially, we tested whether soluble mindin would act as a chemoattractant for eosinophil Transwell migration. Unlike eotaxin, addition of

soluble mindin to the lower well did not significantly impact eosinophil migration on uncoated plates (data not shown). This finding suggests that mindin does not function directly as a chemoattractant for eosinophils. To address specifically whether soluble factors contribute to altered eosinophil migration, eosinophil chemotaxis to lavage supernatant obtained from saline or OVA-challenged mice (wild-type or mindin-deficient) on uncoated plates (**Fig. 6A**) was measured. Although eosinophils did migrate toward supernatant obtained from OVA-challenged mice (when compared with saline), there were no differences in observed migration dependent on mindin. These observations suggest that alterations in soluble factors within the alveolar compartment do not contribute significantly to mindin-dependent alterations in cellular recruitment and suggest a direct effect of mindin on eosinophil trafficking.

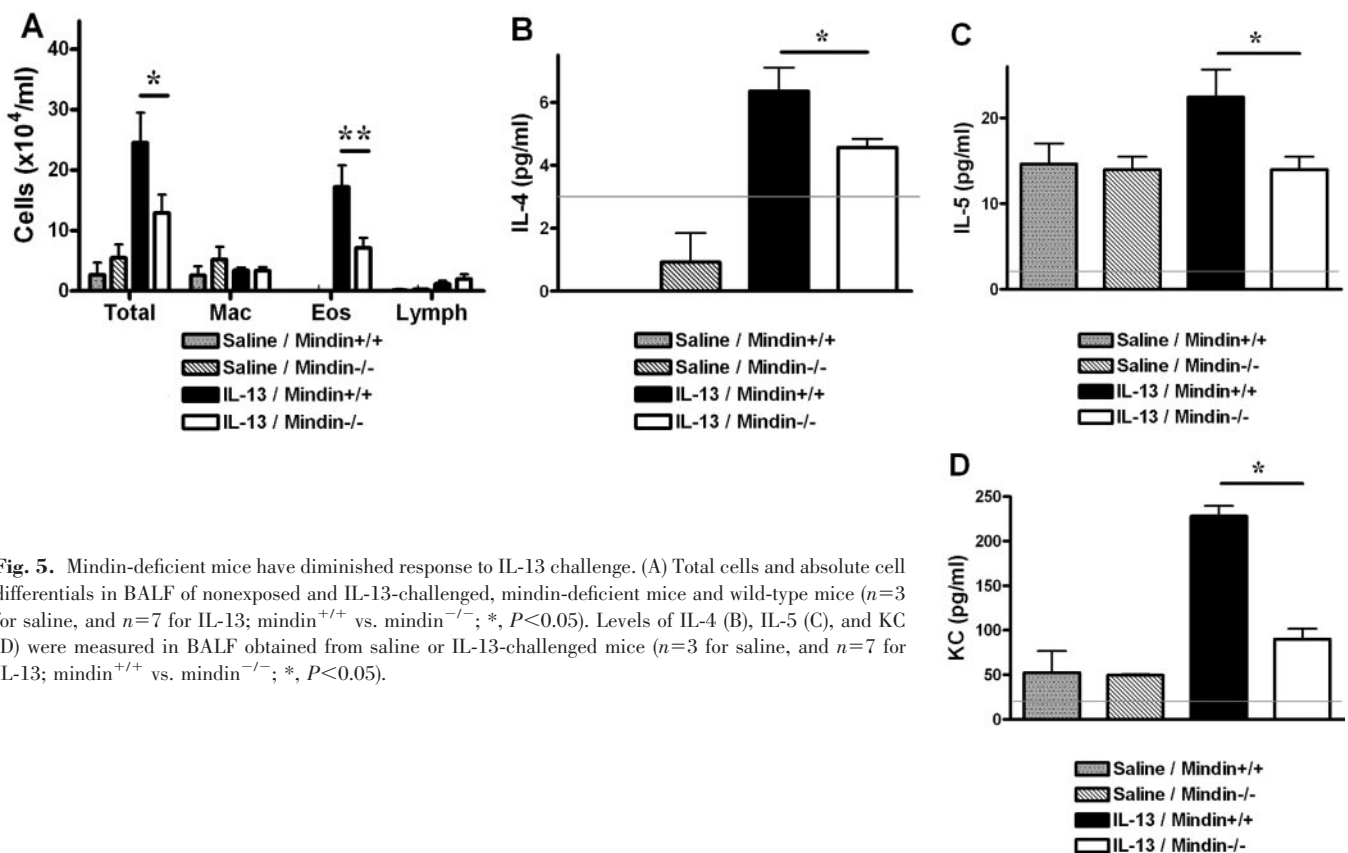


Fig. 5. Mindin-deficient mice have diminished response to IL-13 challenge. (A) Total cells and absolute cell differentials in BALF of nonexposed and IL-13-challenged, mindin-deficient mice and wild-type mice ($n=3$ for saline, and $n=7$ for IL-13; mindin^{+/+} vs. mindin^{-/-}; *, $P<0.05$). Levels of IL-4 (B), IL-5 (C), and KC (D) were measured in BALF obtained from saline or IL-13-challenged mice ($n=3$ for saline, and $n=7$ for IL-13; mindin^{+/+} vs. mindin^{-/-}; *, $P<0.05$).

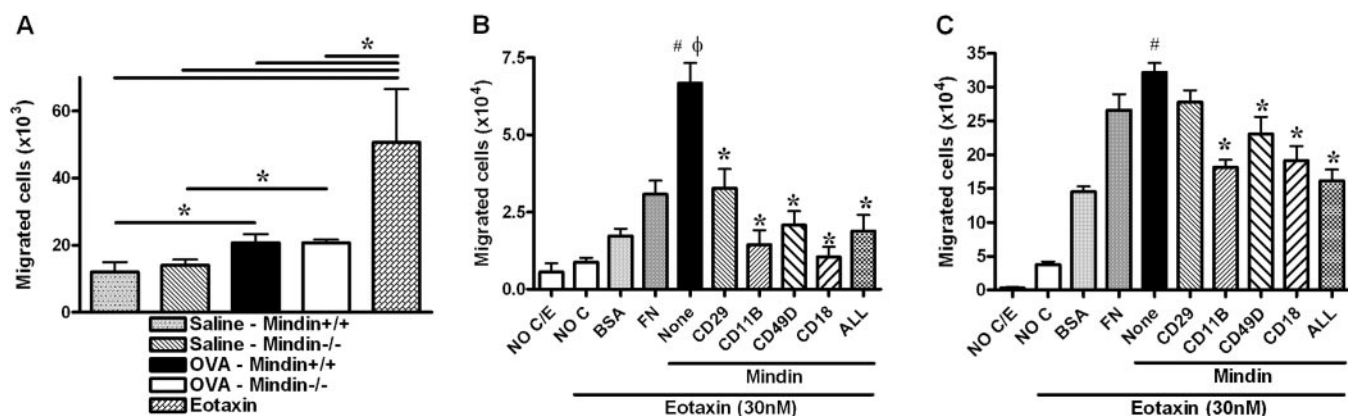


Fig. 6. Mindin-integrin interaction facilitates normal eosinophil migration. (A) Uncoated Transwell plates were used to evaluate migration of murine eosinophils after challenge with cell-free lavage supernatant obtained from saline or OVA-challenged mindin^{+/+} or mindin^{-/-} animals ($n=3/\text{group}$; *, $P<0.05$). (B) Next, Transwell plates were precoated with or without mindin protein, BSA, or fibronectin (FN), and chemotaxis of murine eosinophils was measured toward eotaxin gradient. Anti-integrin CD29 (B_1), CD11b (α_M), CD49d (α_4), and CD18 (B_2) mAb were used to block the binding between mindin and eosinophils; the groups include no coating and no eotaxin control (NO C/E), no coating (NO C), and a mixture of 20 $\mu\text{g}/\text{ml}$ of all four antibodies (ALL). Migrated cells in the lower chamber at 90 min incubation ($n=5/\text{group}$; *, $P<0.05$, vs. mindin-coated wells; #, $P<0.01$, vs. BSA; ϕ , $P<0.01$, vs. fibronectin). (C) Migrated cells in the lower chamber at 4 h incubation ($n=5/\text{group}$; *, $P<0.05$, vs. mindin-coated wells; #, $P<0.01$, vs. BSA).

Recent observations support that mindin can serve as an integrin ligand, facilitating neutrophil and macrophage migration from the bloodstream to lung tissue [12]. Therefore, we considered whether mindin would also interact with integrins expressed on eosinophils and contribute to eosinophil trafficking. To determine specifically whether reduced eosinophil recruitment in the animal models was a result of impaired cell migration dependent on mindin, Transwell plates were precoated with fibronectin (as a positive control) or mindin. Eosinophils were then exposed to an eotaxin chemokine gradient. We observed that mindin and fibronectin enhance eosinophil chemotaxis to eotaxin at 90 min (Fig. 6B) and 4 h (Fig. 6C). Mindin-coated wells demonstrated higher numbers of eosinophils than fibronectin-coated wells at 90 min. However, there were no mindin-dependent differences observed at 4 h when compared with fibronectin. To determine whether integrin interaction with mindin mediates eosinophil migration, integrin-blocking mAb were applied to the chemotaxis assay. Mindin-dependent eosinophil migration was blocked by anti-integrin α_4 (CD49D), α_M (CD11b), β_1 (CD29), and β_2 (CD18) mAb at 90 min (Fig. 6B). Anti-integrin β_1 mAb did not block further cell migration after 4 h incubation (Fig. 6C). We observed different blocking efficiency among these antibodies. At 90 min incubation, anti-integrin β_1 only blocked mindin-mediated eosinophil migration to the level mediated by fibronectin, and other blocking antibodies reduced mindin-promoted eosinophil migration to the baseline level associated with negative control (BSA) precoating. At 4 h incubation, anti-integrin α_M , β_2 mAb, and a four-antibody mixture reduced mindin-mediated eosinophil migration to the level similar to BSA precoating, and anti-integrin α_4 only inhibited 30% of eosinophils migrating through mindin matrix. These studies suggest that mindin enhances eosinophil migration through a mechanism dependent on integrins $\alpha_M\beta_2$ and $\alpha_4\beta_1$.

To determine whether defects in migration were related to eosinophil-mindin adhesion, we performed a specific adhesion

assay (Fig. 7). In the absence of eotaxin, minimal numbers of eosinophils were adherent; however, there were significantly more adherent eosinophils in the mindin-coated wells compared with all other substrates. In the presence of eotaxin, there was slightly enhanced eosinophil adhesion on wells coated with BSA or fibronectin, but mindin-coated wells demonstrated significantly more adherent eosinophils compared with all other substrates. Enhanced mindin-dependent adhesion was blocked by anti- β_1 and anti- α_M to the levels observed with BSA or fibronectin. Together, our *in vitro* studies support that mindin can enhance eosinophil migration and adhesion in a manner dependent on integrins.

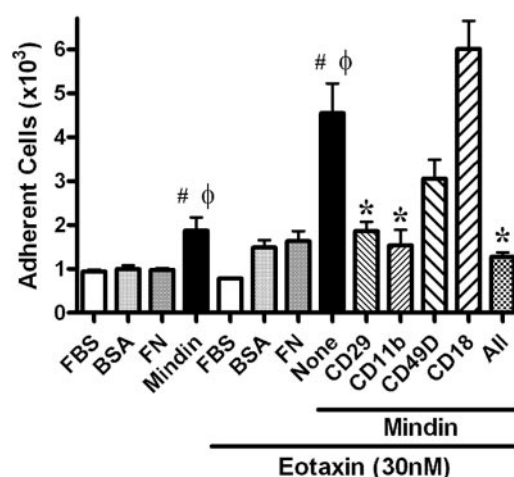


Fig. 7. Mindin-integrin interaction facilitates eosinophil adhesion. Plates were precoated with or without mindin protein, FBS, BSA, or fibronectin, and murine eosinophils were challenged to eotaxin. Anti-integrin CD29 (B_1), CD11b (α_M), CD49d (α_4), and CD18 (B_2) mAb were used to block the binding between mindin and eotaxin-challenged eosinophils; the groups include no coating and no eotaxin control, no coating, and a mixture of 20 $\mu\text{g}/\text{ml}$ of all four antibodies ($n=3/\text{group}$; *, $P<0.05$, vs. mindin-coated wells; #, $P<0.01$, vs. BSA; ϕ , $P<0.01$, vs. fibronectin).

DISCUSSION

In this report, we demonstrate that mindin is an important component of the ECM that contributes to eosinophil trafficking into the airspace. We also demonstrate that the severity of antigen-dependent and antigen-independent allergic airways disease is regulated by mindin, which contributes significantly to many cardinal features of allergic airways disease including eosinophil recruitment, cytokine production, and AHR. In vitro studies support the critical role of mindin in eosinophil migration and adhesion, which, in part, is integrin-dependent. Our data in aggregate suggest that mindin is an ECM protein that plays a central role in eosinophil trafficking into the airspace of mice.

Eosinophils are implicated in the pathogenesis of parasitic helminthes infections and allergic diseases [19]. Previous work demonstrates that tissue recruitment and activation of eosinophils contribute directly to the secretion of many proinflammatory cytokines (IL-2, IL-4, IL-5, IL-10, IL-12, IL-13, IL-16, IL-18, TGF- α/β), chemokines (RANTES, eotaxin), and lipid mediators (platelet-activating factor and LTC₄) [20]. Furthermore, eosinophils can contribute to innate and adaptive immune responses. Specifically, eosinophils contribute to T lymphocyte proliferation and function, thus facilitating response to bacterial pathogens [21] or the development of allergic inflammation [22]. Our data, using two separate murine models of allergic inflammation, support that mindin contributes to the severity of airways disease through the combination of impaired recruitment of eosinophils and defects in the level of soluble, proinflammatory cytokines.

Mindin was found recently to be necessary for efficient DC priming of T cells [23]. Therefore, some of the observed differences in cytokine and eosinophil levels in our OVA model could be explained by mindin-dependent defects in DC-T cell priming. However, we also observed inhibition of cytokine production in the IL-13 model, which is believed to be independent of DC-T cell interaction. We believe that mindin influences the response to allergic airways disease on many levels, dependent and independent of T lymphocyte activation. In vivo studies have demonstrated mindin-dependent differences in proinflammatory cytokine production by different cell types. For example, mindin can amplify macrophage and mast cell production of the proinflammatory cytokines IL-6 and TNF- α and can promote tissue recruitment of neutrophils and macrophages [11]. Indeed, we demonstrated decreased expression of cytokines such as KC and IL-12p70 in mindin-deficient mice in the antigen-dependent OVA model and in the antigen-independent IL-13 model. Epithelial cells are a known source of KC. This suggests that mindin interaction with cell types other than eosinophils or lymphocytes could influence the severity of allergic airways disease. Furthermore, previous work demonstrates that the response to direct IL-13 airway challenge is independent of lymphocytes [18]. IL-13 challenge not only facilitates eosinophil recruitment but can also directly stimulate cytokine production by airway epithelial cells, macrophages, and airway smooth muscle cells [24–27]. The response of cells to IL-13 can be regulated by the extracellular microenvironment. For example, isolated airway smooth muscle cells from asthmatic subjects exhibit amplified secretory

responses to IL-13 as a result of an interaction between integrins and ECM proteins surrounding those cells [28]. Therefore, we speculate that mindin, as an ECM protein, could participate in the activation of IL-13-induced intracellular signaling. The cell type and mechanism of mindin-mediated cytokine secretion remain unknown and will be the topic of future investigations. Although we cannot exclude the possibility that reduced proinflammatory cytokines/chemokines contribute to fewer recruited eosinophils in the airspace, we also show that mindin has a direct effect on eosinophil migration and adhesion in vitro. Therefore, it is plausible that a decrease in recruited eosinophils contributes to lower levels of cytokines in the lavage fluid, observed in mindin-deficient mice.

Our results demonstrate that mindin enhances eosinophil adhesion and migration in response to eotaxin. Mindin-dependent eosinophil migration can be blocked effectively with anti-integrin α_4 , α_M , and β_2 antibodies, suggesting that mindin could function as a ligand to specific integrins during eosinophil binding. Murine eosinophils primarily express integrins $\alpha_4\beta_1$, $\alpha_M\beta_2$, and $\alpha_4\beta_7$. Integrin $\alpha_4\beta_7$ is thought to be important in eosinophil migration in the intestine but not in lung tissue [29]. Eosinophil recruitment from the bloodstream to the lung depends primarily on the expression and activation of integrin $\alpha_4\beta_1$ and $\alpha_M\beta_2$ [30–32]. Each of these integrins has differential functions in the regulation of eosinophil chemotaxis. Integrin $\alpha_4\beta_1$ mediates rolling and adhesion of eosinophils to VCAM-1, which is expressed on the endothelia in response to Th₂ cytokines [33, 34]. Integrin $\alpha_M\beta_2$ primarily facilitates transendothelial and interstitial migration [35, 36], promotes eosinophil migration [37], and is up-regulated in asthmatic human lung eosinophils [9, 38]. We demonstrate that mindin facilitates eotaxin-induced eosinophil adhesion and migration in a manner partially dependent on $\alpha_4\beta_1$ and $\alpha_M\beta_2$. The primary defect in the mice is airspace recruitment of eosinophils. There were not significant differences between the level of interstitial or blood eosinophils. Therefore, these observations suggest that mindin could contribute to eosinophil migration across the airway epithelium. Although the specific site of action is not yet realized, our findings suggest that impaired eosinophil trafficking in mindin-deficient mice is the result of defective interaction between mindin and eosinophil-expressed integrins.

Taken together, this study demonstrates that the ECM protein, mindin, contributes to many cardinal features of allergic asthma, including eosinophil recruitment, cytokine secretion, and AHR. Our study provides evidence supporting a novel role of mindin to normal eosinophil trafficking into the airspace. Our observations suggest that mindin can contribute to the pathogenesis of allergic airways disease, in part, through regulation of eosinophil trafficking into the airspace.

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REFERENCES

- (2002) Asthma prevalence and control characteristics by race/ethnicity—United States, 2002. In *Morbidity and Mortality Weekly Report from the CDC*, vol. 53, 145–148.
- Chinn, S., Jarvis, D., Burney, P., Luczynska, C., Ackermann-Liebrich, U., Anto, J. M., Cerveri, I., De Marco, R., Gislason, T., Heinrich, J., Janson, C., Künzli, N., Leynaert, B., Neukirch, F., Schouten, J., Sunyer, J., Svanes, C., Vermeire, P., Wjst, M. (2004) Increase in diagnosed asthma but not in symptoms in the European Community Respiratory Health Survey. *Thorax* **59**, 646–651.
- Janson, C., Chinn, S., Jarvis, D., Burney, P. (1997) Physician-diagnosed asthma and drug utilization in the European Community Respiratory Health Survey. *Eur. Respir. J.* **10**, 1795–1802.
- Green, R. H., Brightling, C. E., McKenna, S., Hargadon, B., Parker, D., Bradding, P., Wardlaw, A. J., Pavord, I. D. (2002) Asthma exacerbations and sputum eosinophil counts: a randomized controlled trial. *Lancet* **360**, 1715–1721.
- Justice, J. P., Borchers, M. T., Crosby, J. R., Hines, E. M., Shen, H. H., Ochkur, S. I., McGarry, M. P., Lee, N. A., Lee, J. J. (2003) Ablation of eosinophils leads to a reduction of allergen-induced pulmonary pathology. *Am. J. Physiol. Lung Cell. Mol. Physiol.* **284**, L169–L178.
- Lee, J. J., Dimina, D., Macias, M. P., Ochkur, S. I., McGarry, M. P., O'Neill, K. R., Protheroe, C., Pero, R., Nguyen, T., Cormier, S. A., Lenkiewicz, E., Colbert, D., Rinaldi, L., Ackerman, S. J., Irvin, C. G., Lee, N. A. (2004) Defining a link with asthma in mice congenitally deficient in eosinophils. *Science* **305**, 1773–1776.
- Humbles, A. A., Lloyd, C. M., McMillan, S. J., Friend, D. S., Xanthou, G., McKenna, E. E., Ghiran, S., Gerard, N. P., Yu, C., Orkin, S. H., Gerard, C. (2004) A critical role for eosinophils in allergic airways remodeling. *Science* **305**, 1776–1779.
- Barthel, S. R., Johansson, M. W., McNamee, D. M., Mosher, D. F. (2008) Roles of integrin activation in eosinophil function and the eosinophilic inflammation of asthma. *J. Leukoc. Biol.* **83**, 1–12.
- Barthel, S. R., Jarjour, N. N., Mosher, D. F., Johansson, M. W. (2006) Dissection of the hyperadhesive phenotype of airway eosinophils in asthma. *Am. J. Respir. Cell Mol. Biol.* **35**, 378–386.
- Higashijima, S., Nose, A., Eguchi, G., Hotta, Y., Okamoto, H. (1997) Mindin/F-spondin family: novel ECM proteins expressed in the zebrafish embryonic axis. *Dev. Biol.* **192**, 211–227.
- He, Y. W., Li, H., Zhang, J., Hsu, C. L., Lin, E., Zhang, N., Guo, J., Forbush, K. A., Bevan, M. J. (2004) The extracellular matrix protein mindin is a pattern-recognition molecule for microbial pathogens. *Nat. Immunol.* **5**, 88–97.
- Jia, W., Li, H., He, Y. W. (2005) The extracellular matrix protein mindin serves as an integrin ligand and is critical for inflammatory cell recruitment. *Blood* **106**, 3854–3859.
- Borchers, M. T., Ansay, T., DeSalle, R., Daugherty, B. L., Shen, H., Metzger, M., Lee, N. A., Lee, J. J. (2002) In vitro assessment of chemokine receptor-ligand interactions mediating mouse eosinophil migration. *J. Leukoc. Biol.* **71**, 1033–1041.
- Hollingsworth, J. W., Whitehead, G., Lin, K., Nakano, H., Gunn, M., Schwartz, D., Cook, D. (2006) TLR4 signaling attenuates ongoing allergic inflammation. *J. Immunol.* **176**, 5856–5862.
- Wang, X., Seed, B. (2003) A PCR primer bank for quantitative gene expression analysis. *Nucleic Acids Res.* **31**, e154.
- Wills-Karp, M., Luyimbazi, J., Xu, X., Schofield, B., Neben, T. Y., Karp, C. L., Donaldson, D. D. (1998) Interleukin-13: central mediator of allergic asthma. *Science* **282**, 2258–2261.
- Wills-Karp, M. (2004) Interleukin-13 in asthma pathogenesis. *Immunol. Rev.* **202**, 175–190.
- Grunig, G., Warnock, M., Wakil, A. E., Venkayya, R., Brombacher, F., Rennick, D. M., Sheppard, D., Mohrs, M., Donaldson, D. D., Locksley, R. M., Corry, D. B. (1998) Requirement for IL-13 independently of IL-4 in experimental asthma. *Science* **282**, 2261–2263.
- Rothenberg, M. E., Hogan, S. P. (2006) The eosinophil. *Annu. Rev. Immunol.* **24**, 147–174.
- Kita, H. (1996) The eosinophil: a cytokine-producing cell? *J. Allergy Clin. Immunol.* **97**, 889–892.
- Mawhorter, S. D., Kazura, J. W., Boom, W. H. (1994) Human eosinophils as antigen-presenting cells: relative efficiency for superantigen- and antigen-induced CD4+ T-cell proliferation. *Immunology* **81**, 584–591.
- MacKenzie, J. R., Mattes, J., Dent, L. A., Foster, P. S. (2001) Eosinophils promote allergic disease of the lung by regulating CD4(+) Th2 lymphocyte function. *J. Immunol.* **167**, 3146–3155.
- Li, H., Oliver, T., Jia, W., He, Y. W. (2006) Efficient dendritic cell priming of T lymphocytes depends on the extracellular matrix protein mindin. *EMBO J.* **25**, 4097–4107.
- Faffe, D. S., Whitehead, T., Moore, P. E., Baraldo, S., Flynt, L., Bourgeois, K., Panettieri, R. A., Shore, S. A. (2003) IL-13 and IL-4 promote TARC release in human airway smooth muscle cells: role of IL-4 receptor genotype. *Am. J. Physiol. Lung Cell. Mol. Physiol.* **285**, L907–L914.
- Lukacs, N. W. (2001) Role of chemokines in the pathogenesis of asthma. *Nat. Rev. Immunol.* **1**, 108–116.
- Zhu, Z., Ma, B., Zheng, T., Homer, R. J., Lee, C. G., Charo, I. F., Noble, P., Elias, J. A. (2002) IL-13-induced chemokine responses in the lung: role of CCR2 in the pathogenesis of IL-13-induced inflammation and remodeling. *J. Immunol.* **168**, 2953–2962.
- Zimmermann, N., Hershey, G. K., Foster, P. S., Rothenberg, M. E. (2003) Chemokines in asthma: cooperative interaction between chemokines and IL-13. *J. Allergy Clin. Immunol.* **111**, 227–242.
- Chan, V., Burgess, J. K., Ratoff, J. C., O'Connor, B. J., Greenough, A., Lee, T. H., Hirst, S. J. (2006) Extracellular matrix regulates enhanced eotaxin expression in asthmatic airway smooth muscle cells. *Am. J. Respir. Crit. Care Med.* **174**, 379–385.
- Brandt, E. B., Zimmermann, N., Muntel, E. E., Yamada, Y., Pope, S. M., Mishra, A., Hogan, S. P., Rothenberg, M. E. (2006) The $\alpha 4\beta 7$ -integrin is dynamically expressed on murine eosinophils and involved in eosinophil trafficking to the intestine. *Clin. Exp. Allergy* **36**, 543–553.
- Sedgwick, J. B., Jansen, K. J., Kennedy, J. D., Kita, H., Busse, W. W. (2005) Effects of the very late adhesion molecule 4 antagonist WAY103 on human peripheral blood eosinophil vascular cell adhesion molecule 1-dependent functions. *J. Allergy Clin. Immunol.* **116**, 812–819.
- Popper, H. H., Pailer, S., Wurzing, G., Feldner, H., Hesse, C., Eber, E. (2002) Expression of adhesion molecules in allergic lung diseases. *Virchows Arch.* **440**, 172–180.
- Ohkawara, Y., Yamauchi, K., Maruyama, N., Hoshi, H., Ohno, I., Honma, M., Tanno, Y., Tamura, G., Shirato, K., Ohtani, H. (1995) In situ expression of the cell adhesion molecules in bronchial tissues from asthmatics with air flow limitation: in vivo evidence of VCAM-1/VLA-4 interaction in selective eosinophil infiltration. *Am. J. Respir. Cell Mol. Biol.* **12**, 4–12.
- Yamamoto, H., Sedgwick, J. B., Busse, W. W. (1998) Differential regulation of eosinophil adhesion and transmigration by pulmonary microvascular endothelial cells. *J. Immunol.* **161**, 971–977.
- Bochner, B. S., Klunk, D. A., Sterbinsky, S. A., Coffman, R. L., Schleimer, R. P. (1995) IL-13 selectively induces vascular cell adhesion molecule-1 expression in human endothelial cells. *J. Immunol.* **154**, 799–803.
- Jia, G. Q., Gonzalo, J. A., Hidalgo, A., Wagner, D., Cybulsky, M., Gutierrez-Ramos, J. C. (1999) Selective eosinophil transendothelial migration triggered by eotaxin via modulation of Mac-1/ICAM-1 and VLA-4/VCAM-1 interactions. *Int. Immunol.* **11**, 1–10.
- Ebisawa, M., Liu, M. C., Yamada, T., Kato, M., Lichtenstein, L. M., Bochner, B. S., Schleimer, R. P. (1994) Eosinophil transendothelial migration induced by cytokines. II. Potentiation of eosinophil transendothelial migration by eosinophil-active cytokines. *J. Immunol.* **152**, 4590–4596.
- Matsumoto, K., Sterbinsky, S. A., Bickel, C. A., Zhou, D. F., Kovach, N. L., Bochner, B. S. (1997) Regulation of $\alpha 4$ integrin-mediated adhesion of human eosinophils to fibronectin and vascular cell adhesion molecule-1. *J. Allergy Clin. Immunol.* **99**, 648–656.
- Georas, S. N., Liu, M. C., Newman, W., Beall, L. D., Stealey, B. A., Bochner, B. S. (1992) Altered adhesion molecule expression and endothelial cell activation accompany the recruitment of human granulocytes to the lung after segmental antigen challenge. *Am. J. Respir. Cell Mol. Biol.* **7**, 261–269.