

Neutrophils Regulate Airway Responses in a Model of Fungal Allergic Airways Disease¹

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Neutrophils infiltrate airway walls in patients with allergic airway diseases and in animal models of these illnesses, but their contribution to the pathogenesis of airway allergy is not established. We hypothesized that, in a mouse model of airway allergy to the ubiquitous environmental mold, *Aspergillus fumigatus*, airway neutrophils contribute to disease severity. Ab-mediated neutrophil depletion resulted in reduced airway hyperresponsiveness and remodeling, whereas conditional transgenic overexpression of the neutrophil chemotactic molecule, CXCL1, in airway walls resulted in worsened allergic responses. This worsened phenotype was associated with a marked increase in the number of airway neutrophils but not other lung leukocytes, including eosinophils and lymphocyte subsets, and depletion of neutrophils in sensitized mice with transgenic overexpression of CXCL1 resulted in attenuated airway responses. The number of lung neutrophils correlated with lung matrix metalloproteinase 9 (MMP-9) activity both in the context of neutrophil depletion and with augmented neutrophil recruitment to the airways. Although wild-type and MMP-9-deficient neutrophils homed to the inflamed airways to a similar extent, transfer of wild-type, but not MMP-9-deficient, neutrophils to MMP-9-deficient animals resulted in augmented allergic airway responses. Taken together, these data implicate neutrophils in the pathogenesis of fungal allergic airway disease. *The Journal of Immunology*, 2006, 176: 2538–2545.

Allergic airway diseases are among the most common ailments in industrialized societies, affecting some 10% of the population (1) and occur in response to a myriad of inhaled Ags. The pathogenesis of these illnesses centers on protracted airway inflammation characterized by accumulation of multiple leukocyte subsets and remodeling of the airway walls, and manifests as airflow limitation and hyperresponsiveness to non-specific stimuli (2). There has been substantial progress in understanding of the aberrations in acquired immunity that underlie the immunopathology of allergic airway diseases, but there is a paucity of information regarding the contribution of ongoing innate immune mechanisms that may be contributing to airway inflammation (3).

Neutrophils are critical components of the effector arm of innate immunity that accumulate prominently in the context of acute inflammation, but also infiltrate tissues in the context of chronic inflammatory conditions, including allergic airway diseases: accumulation of neutrophils in airway walls has been noted in mouse models of allergic airway disease (4, 5) and in patients with allergic airways disease (6, 7), where their presence correlates with severity of airway obstruction (8–11). In addition, mediators of neutrophil chemotaxis, such as ELR⁺ CXC chemokines and leukotriene-B₄, are expressed in both human allergic airway disease

and animal models (12, 13). The precise contribution of neutrophils to the pathogenesis of allergic airway disease is not clearly defined: given the correlation of airway neutrophilia with severity of airway obstruction in humans, neutrophils have been postulated to promote more severe airway inflammation by mediating direct tissue injury or by elaborating proinflammatory mediators (14, 15). Conversely, it is possible that the accumulation of neutrophils is an epiphenomenon that does not directly contribute to pathogenesis. Lastly, neutrophils could conceivably serve to attenuate the inflammatory response in this context: for instance, neutrophils have been noted to skew acquired immune responses to a Th-1 phenotype in some inflammation models (16, 17). Neutrophils may also serve to clear some antigenic stimuli from the lungs by virtue of their antimicrobial properties; this may be particularly relevant in allergic airway responses to fungal Ags, because neutrophils are recognized as important in innate immunity to fungi (18, 19).

We sought to differentiate among these possibilities using a well-characterized model of airway allergy to a ubiquitous environmental mold, *Aspergillus fumigatus*. *Aspergillus* species are unique in causing several distinct and disparate human diseases, the pathogenesis of which is linked to aberrations in the host's immune responses to the microorganism (20). In immunocompromised hosts, neutrophils have long been recognized as critical to defenses against invasive tissue infections caused by *Aspergillus* species (21). This organism is also an important cause of allergic airways disease, including in a subset of cases of human asthma (22) and a distinct clinical entity, allergic bronchopulmonary aspergillosis (23, 24). Tissue neutrophil accumulation correlates with disease severity in patients with allergic airways disease to *Aspergillus* (7, 8, 25), but its role in disease pathogenesis is not known.

We hypothesized that airway neutrophils contribute to disease severity in a mouse model of allergic airways disease to *A. fumigatus*. We tested this hypothesis by examining the consequences

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of neutrophil depletion and augmented neutrophil chemotaxis to the airway in this model.

Materials and Methods

Animals

C57BL/6, FVB, and matrix metalloproteinase 9 (MMP-9)³-deficient mice (FVB background) were purchased from Charles River Laboratories or The Jackson Laboratory. Generation of transgenic mice with tetracycline-inducible airway-specific overexpression of CXCL1 has been described previously (26). Briefly, each animal was heterozygous for two transgenes: an activator construct encoding the tet-activator protein under control of the airway-specific promoter, CC10; and a reporter construct, consisting of the tetracycline-responsive DNA element driving the transcription of CXCL1. Six to 12-wk-old age- and gender-matched animals were used in experiments. All animals were maintained under specific pathogen-free conditions and in compliance with institutional animal care regulations.

In vivo procedures

We used a previously described model of *Aspergillus*-induced allergic airways disease (27–29). Briefly, animals were given 10 μ g of a commercial preparation of *A. fumigatus* cellular Ags (from *A. fumigatus* mycelia grown statically on enriched trypticase media, extracted in 0.01 M ammonium bicarbonate and dialyzed against distilled water; Greer Laboratories) in IFA by i.p. and s.c. routes on day –35, then 10 μ g of Ag in PBS intranasally on days –21, –14, and –7, followed by intratracheal inoculation of 5×10^6 *A. fumigatus* conidia on day 0. Conidia were prepared by culturing *Aspergillus fumigatus* strain 13073 (American Type Culture Collection) on Sabouraud's dextrose agar plates for 7–14 days at 37°C, washing the plate surface with sterile 0.1% Tween 80 in PBS, and filtering and counting the spores under a hemacytometer.

Selective neutropenia was induced using a modification of a previous protocol (30) by i.p. administration of 80 μ g of purified RB6-8C5 mAb or isotype control mAb (clone A95-1; BD Biosciences) on day –1 and every 48 h thereafter. In preliminary studies, this protocol resulted in sustained peripheral blood neutropenia (circulating absolute neutrophil count <50 cells/ μ l) for 4 wk with no visible signs of ill health and no effect on other leukocyte subsets in the blood, spleen, or lungs; similar administration of isotype mAb had no detectable effect on lung function or histology in the *Aspergillus* airway allergy model (data not shown).

In experiments with CXCL1-transgenic animals, sustained transgene activation was achieved by adding 2 mg/ml doxycycline (dox; Sigma-Aldrich) to the drinking water, starting immediately after intratracheal administration of conidia and continued for up to 30 days; gender-matched littermate CXCL1-transgenic mice given ordinary drinking water were used as controls. Similar administration of dox to wild-type animals with airway allergy to *Aspergillus* did not affect airway hyperreactivity nor number of bronchoalveolar leukocytes (data not shown).

Airway hyperresponsiveness was measured at various time points, as described (31, 32). Briefly, the breathing patterns of conscious and unrestrained animals were noninvasively monitored in a whole body plethysmograph (Buxco Electronics). The enhanced pause (P_{enh}), the indicator of airflow obstruction calculated from the plethysmograph pressure-time waveform (33), was monitored continuously and values for each mouse averaged for a 3-min period immediately following exposure to aerosolized methacholine (Sigma-Aldrich). The correlation of P_{enh} with airway resistance, as measured invasively in mechanically ventilated animals, has been validated (33, 34).

In neutrophil transfer studies, mature bone marrow neutrophils were prepared as described (35, 36). Obtained cells were ~90% band forms or segmented polymorphonuclear cells by light microscopy and were >99% viable by trypan blue exclusion. Mice were injected with 10^7 cells in 100 μ l of PBS via a lateral tail vein. In preliminary studies, isolated neutrophils were bactericidal in vitro and homed to the lungs in the context of inflammation but not in normal animals when delivered i.v. (data not shown). In some studies, transferred neutrophils were first labeled with the vital cytoplasmic fluorochrome, CFSE (Invitrogen Life Technologies), by incubating the cells in 5 μ M CFSE in PBS with 5% FCS for 10 min, and washing the cells three times. Uniform staining of cells was routinely achieved by this protocol, as assessed by epifluorescent microscopy.

Harvest of samples

Blood was obtained from the right ventricle into heparinized syringes and plasma was separated. Bronchoalveolar lavage (BAL) fluid and whole lungs were obtained and prepared for histology and various assays, as described previously (26). Photomicrographs were obtained on a Nikon Eclipse E600 microscope with an in-line digital camera (Nikon Coolpix 990) and assembled into multipanel figures using Photoshop software (version 8.0; Adobe Systems).

ELISA and hydroxyproline assays

ELISA was performed using complementary Ab pairs against murine IL-4, IL-5, IL-13, IgE (BD Biosciences), and IFN- γ and CXCL1 (R&D Systems) according to manufacturers' instructions. Lung collagen content was measured using a previously described assay for hydroxyproline (27, 37).

Zymography

Gelatin zymography was used to quantify MMP activity, as described (38). Gels were scanned on a Fluorchem Imaging System (Alpha Innotech) and analyzed using Molecular Analyst software (version 1.4; Bio-Rad). MMP-9 gelatinolytic activity was measured in lung homogenates as density of a lysis band at 92-kDa, using recombinant mouse MMP-9 (R&D Systems) as positive control.

Quantification of lung leukocyte subsets

Lung single-cell suspensions were prepared as previously described (39, 40). For each sample, total number of cells was determined under a hemacytometer, and differentials cell counts performed on cells were cyto-centrifuged onto glass slides and stained by a modified Wright's stain (Diff-Quik; Dade Behring). The following Abs were used for flow cytometry (all from BD Pharmingen): anti-CD3-allophycocyanin (clone 17A2), anti-CD4-biotin (GK1.5), and anti-CD8-PE (53–5.8), anti-CD11b-allophycocyanin and -FITC (M1/70), anti-CD11c-biotin (HL3), anti-CD45-PerCP (clone 30-F11), anti-CD45R-PE (RA3-6B2), Gr1-FITC and -biotin (RB6-8C5), anti-Ly6G-PE (1A8). Samples were analyzed on a FACSCalibur cytometer using CellQuest software (BD Biosciences). Neutrophils were identified by microscopy, as CD11b⁺Gr1^{high} cells, or as CD11b⁺Ly6G⁺ cells (41, 42); eosinophils were identified microscopically; plasmacytoid DC as CD11c^{int}Gr1^{int} (43), T cell subsets as CD3⁺CD4⁺ or CD3⁺CD8⁺ cells, B cells as CD45R⁺ cells with lymphocyte light-scatter characteristics, monocyte/macrophages as CD11b⁺Gr1^{neg-low} cells with monocyte/macrophage light-scatter characteristics (42). Absolute numbers of various leukocytes in each sample were determined from the percentage of each cell subset and the total cell count for the sample.

Statistical analysis

Data were analyzed on a Macintosh Powerbook G4 computer with appropriate statistics software (Prism, version 4.0a; GraphPad Software). All data were expressed as mean $\pm$ SEM. Two-way ANOVA was used to compare values between two groups over multiple time points. Other data were compared using the unpaired two-tail Mann-Whitney (nonparametric) test. Probability values <0.05 were considered statistically significant.

Results

Neutrophil depletion attenuates airway allergy to *Aspergillus*

To determine that selective neutrophil depletion is achievable in this model, we began by comparing lung leukocyte populations at various time points after administration of conidia to sensitized animals, with administration of neutrophil-depleting mAb (RB6-8C5) or the equivalent amount of an isotype mAb against an irrelevant Ag. Lungs of animals that received neutrophil-depleting mAb contained fewer neutrophils as identified by microscopy and flow cytometry (Fig. 1). Repeated administration of the Ab every 2 days resulted in sustained depletion of neutrophils, but did not affect the number of lung eosinophils or monocyte/macrophages (Fig. 2). Because administration of large quantities of this mAb has previously been reported to deplete plasmacytoid dendritic cells (43), we also examined this cell population and found them not to be affected. Administration of neutrophil-depleting Ab did not influence numbers of lung lymphocyte subsets on day 7, but resulted in fewer lung CD8 T cells and B cells on day 14.

³ Abbreviations used in this paper: MMP-9, matrix metalloproteinase 9; P_{enh} , enhanced pause; BAL, bronchoalveolar lavage; dox, doxycycline.

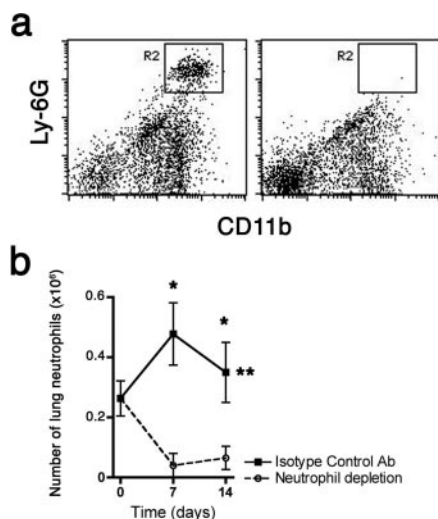


FIGURE 1. Neutrophil depletion in a model of allergic airway disease. *a*, Representative flow cytometry data of lung single-cell suspensions of sensitized mice, 7 days after intratracheal challenge with *A. fumigatus* conidia. Panels are gated on CD45⁺ cells, and 50% of collected events are shown. Gate R2 represents neutrophils. *b*, Day 0 represents mice that were sensitized for 5 wk but not challenged with *A. fumigatus* conidia. Data shown represent mean $\pm$ SEM; $n = 4$ for each group at each time point; *, $p < 0.05$ compared with sensitized mice treated with isotype control mAb at the same time point; **, $p < 0.05$ comparing trend between the two groups over time.

We next examined the effect of neutrophil depletion on the phenotype of murine *Aspergillus*-induced allergic airways disease. We have previously shown that naive mice with neutrophil depletion develop a severe invasive pneumonia after inoculation with *Aspergillus* conidia (30). In distinct contrast to this, sensitized animals challenged with conidia remained healthy and had no deaths over 28 days, both in the context of neutrophil depletion and treatment with isotype Ab. In addition, on day 7 after inoculation with conidia, no viable fungi were recovered from the lungs of either group ($n = 4$ mice per group). However, neutrophil depletion resulted in a sustained reduction in airway hypersensitivity as compared with animals treated with isotype control mAb (Fig. 3*a*). Neutrophil depletion also resulted in reduced peribronchial fibrosis on histology and 65% reduction in lung hydroxyproline, a surrogate for collagen content (Fig. 3, *b* and *c*).

FIGURE 2. Effect of sustained neutrophil depletion on other lung leukocyte subsets in mice with allergic airway disease. Cells were enumerated in whole lung digests. Day 0 represents mice that were sensitized for 5 wk but not challenged with *A. fumigatus* conidia. Data shown represent mean $\pm$ SEM of whole lung leukocytes; $n = 4$ for each group at each time point; *, $p < 0.05$ compared with sensitized mice treated with isotype control mAb at the same time point.

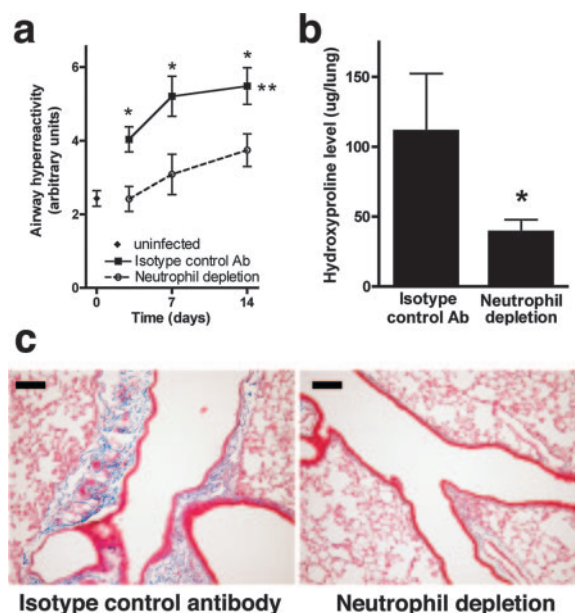
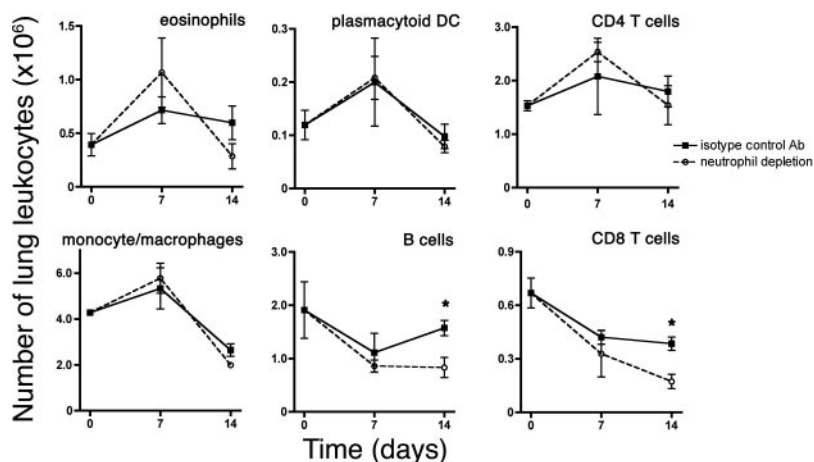


FIGURE 3. Effect of neutrophil depletion on allergic airway responses to *Aspergillus*. *a*, Changes in P_{enh} as measured in awake, unrestrained mice by whole body plethysmography following exposure to aerosolized methacholine. “Uninfected” indicates sensitized mice before intratracheal administration of *A. fumigatus* conidia on day 0. Data represent mean $\pm$ SEM; $n = 4$ mice per group at each time point; *, $p < 0.05$ compared with mice treated with neutrophil-depleting mAb; **, $p < 0.05$ comparing trend between two groups over time. *b*, Whole lung hydroxyproline content on day 14 after conidia challenge. Data represent mean $\pm$ SEM; $n = 6$ mice per group; *, $p < 0.05$ compared with mice treated with the isotype control mAb. *c*, Representative lung Masson trichrome stain on day 28 after conidia challenge. Mice treated with the isotype control mAb showed increased peribronchial fibrosis (light blue staining). Scale bars are 100-μm long (original magnification $\times 100$).

Enhanced recruitment of neutrophils to the airways augments the severity of airway allergy

To confirm the relevance of neutrophils to disease severity in *Aspergillus*-induced allergic airways disease by another method, we next examined the effect of augmented neutrophil deployment to the airways in the context of airway allergy. This was achieved using transgenic animals with tetracycline-inducible airway-specific overexpression of the neutrophil chemoattractant, CXCL1. The effect of acute administration of tetracycline analogues in this

mouse has previously been reported to result in overexpression of CXCL1 in the airway-lining Clara cells, but not in other organs, resulting in a selective recruitment of neutrophils (26). Prolonged administration of dox via drinking water to naive CXCL1-transgenic animals resulted in a ~100-fold increase in bronchoalveolar concentration of CXCL1 and number of BAL neutrophils (Fig. 4), but no alterations in other lung leukocyte populations and without visible signs of ill health. In addition, prolonged dox treatment by this method did not affect lung function nor bronchoalveolar leukocyte populations in wild-type animals with *Aspergillus*-induced airway allergy (data not shown), but resulted in sustained overexpression of CXCL1 in transgenic mice with *Aspergillus*-induced airway allergy (Fig. 4).

Transgenic airway-specific overexpression of CXCL1 in mice with *Aspergillus*-induced allergic airways disease resulted in marked and sustained worsening of airway hyperreactivity as compared with transgenic animals without transgene activation (Fig. 5a). Furthermore, transgene activation resulted in 47% greater lung hydroxyproline content and increased peribronchial fibrosis on histology (Fig. 5, b and c). Examination of lung leukocyte subsets at various time points showed that, as compared with littermate animals not treated with dox, activation of the transgene resulted in sustained increase in the number of lung neutrophils without affecting lung eosinophils, monocyte/macrophages, or lymphocyte subsets (Fig. 6). Interestingly, the sustained increase in

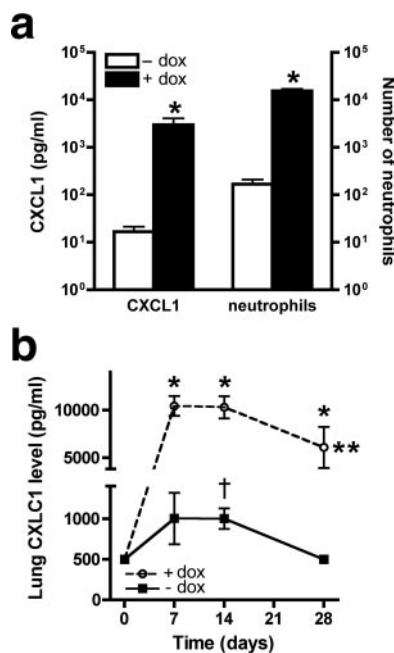


FIGURE 4. Effect of transgene activation in mice with inducible airway-specific transgenic expression of CXCL1. *a*, BAL lavage fluid neutrophil count and CXCL1 concentration in nonsensitized transgenic mice with or without transgene activation. Data represent mean $\pm$ SEM; $n = 4$ –11 mice/group; “+dox”, sustained transgene activation by adding 2 mg/ml dox to drinking water for 28 days; “-dox” transgenic mice without transgene activation, given ordinary drinking water. *, $p < 0.05$ compared with mice without dox treatment. *b*, CXCL1 concentrations at various time points in sensitized transgenic mice either with or without transgene activation. CXCL1 levels were measured in whole lung homogenates. Day 0 represents mice that were sensitized for 5 wk but not challenged with *A. fumigatus* conidia. Data represent mean $\pm$ SEM; $n = 5$ mice per group per time point; “dox”, sustained transgene activation as described above; *, $p < 0.05$ compared with mice without transgene activation at the same time point; **, $p < 0.05$ comparing trend between two groups over time; †, $p < 0.05$ compared with time point 0.

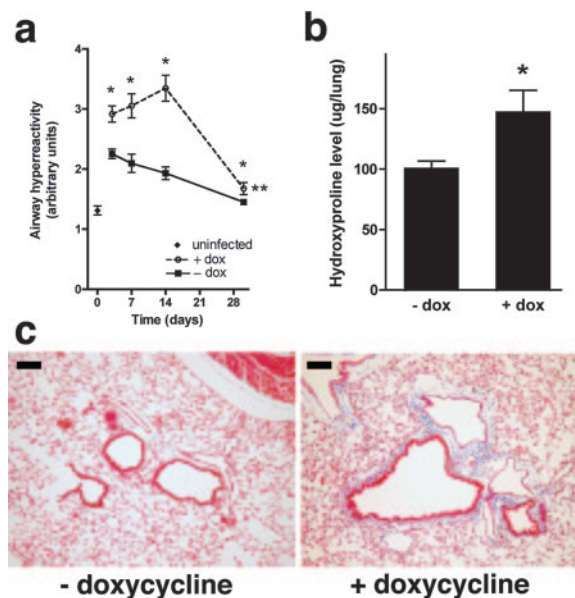


FIGURE 5. Effect of sustained transgenic overexpression of CXCL1 on allergic airway responses to *Aspergillus*. *a*, P_{enh} as measured in unstrained, awake mice by whole body plethysmography following exposure to aerosolized methacholine. “Uninfected,” sensitized mice before intratracheal administration of *A. fumigatus* conidia on day 0; “+dox”, sustained transgene activation by administration of dox via drinking water for 28 days; “-dox” transgenic mice without transgene activation, given ordinary drinking water. Data represent mean $\pm$ SEM; $n = 4$ mice per group at each time point. *, $p < 0.05$ compared with transgenic mice without transgene activation; **, $p < 0.05$ comparing trend between two groups over time. *b*, Whole lung hydroxyproline content on day 14 after conidia challenge. Data represent mean $\pm$ SEM; $n = 6$ mice per group; *, $p < 0.05$ compared with transgenic mice without transgene activation. *c*, Representative lung Masson trichrome stain on day 28 after conidia challenge. Transgenic mice with transgene activation (+dox) show increased peribronchial fibrosis (light blue staining). Scale bars are 100- μ m long (original magnification $\times 100$).

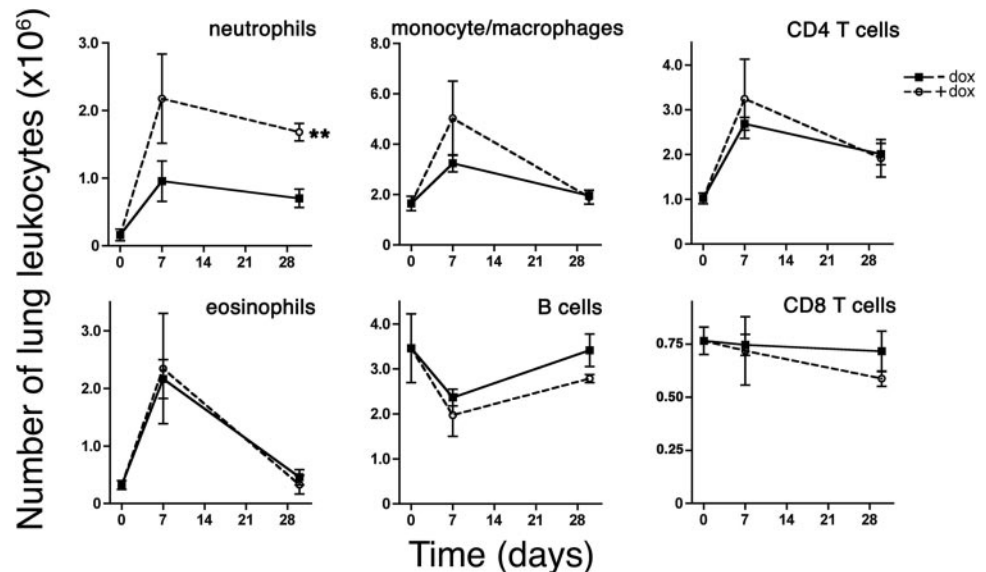
the number of neutrophils was associated with marked augmentation of airway hyperreactivity at earlier time points, and more modest increase in airway hyperreactivity and increased airway remodeling at later time points.

We then sought to ascertain that the augmented disease phenotype associated with CXCL1 overexpression is attributable to the increase in lung neutrophils, rather than some other effect of CXCL1. We thus compared airway hyperreactivity in transgenic animals without transgene activation, with transgene activation, and with both transgene activation and neutrophil depletion on day 7 after administration of conidia (Fig. 7). We found that the enhanced airway hyperresponsiveness in the setting of CXCL1 overexpression was abrogated by depletion of neutrophils, indicating that neutrophils are required for CXCL1-mediated enhancement of airway hyperreactivity.

Lung neutrophils do not influence Th cytokine balance in airway allergy

To assess the mechanism underlying neutrophil-mediated worsening of *Aspergillus*-induced allergic airways disease, we examined the lung levels of Th-1 and -2 cytokines in the context of neutrophil depletion and transgenic CXCL1 overexpression. Surprisingly, lung levels of IL-4, IL-5, IL-13, and IFN- γ were remarkably similar and were unaffected by lung neutrophil content (Fig. 8, left panels). Similarly, serum levels of total IgE were high but were not affected by the presence of neutrophils (Fig. 8, right panels). This

FIGURE 6. Effect of sustained CXCL1-transgene activation on lung leukocyte subsets in mice with allergic airway disease. Cells were enumerated in whole lung digests. Day 0 represents mice that were sensitized for 5 wk but not challenged with *A. fumigatus* conidia. “+dox”, sustained transgene activation by administration of dox via drinking water for 28 days; “-dox” transgenic mice without transgene activation, given ordinary drinking water. Data shown represent mean $\pm$ SEM of whole lung leukocytes; $n = 4$ for each group at each time point; **, $p < 0.05$ compared with sensitized mice without transgene activation over time.



suggests that the detrimental effect of neutrophils in *Aspergillus*-induced airway allergy was independent of Th cytokine balance.

The detrimental effect of neutrophils in Aspergillus-induced airway allergy is dependent on MMP-9

Several groups have shown that mice deficient in MMP-9 have reduced disease severity in different models of airway allergy (44–47). Because neutrophils are a major source of MMP-9 in inflamed tissues (48), we examined the contribution of neutrophil-derived MMP-9 in this system. We began by comparing lung MMP-9 activity in animals with airway allergy to *Aspergillus* in the context of neutrophil depletion and in the setting of augmented neutrophil recruitment to the airways. On day 14 after challenge with conidia, sensitized mice with neutrophil depletion had 76% lower lung MMP-9 activity as compared with isotype mAb-treated controls, whereas transgenic airway overexpression of CXCL1 resulted in a ~5-fold increase in lung MMP-9 activity in sensitized animals (Fig. 9).

We then examined whether the correlation between severity of airway allergy and airway neutrophils is attributable to neutrophil-

derived MMP-9. To achieve this, we transferred mature neutrophils from wild-type or MMP-9-deficient donors to mice with *Aspergillus*-induced airway allergy. Consistent with prior in vitro observations (49, 50), neutrophils from wild-type donors were found to home to the lungs in mice with *Aspergillus*-induced airway allergy but not in normal mice (Fig. 10a). In addition, wild-type and MMP-9-deficient neutrophils were recruited to the lungs of allergic recipients to a similar extent, suggesting that MMP-9 is not essential for neutrophil migration into airway walls in this model. Finally, we assessed the effect of neutrophil transfer on the severity of airway allergic responses (Fig. 10b). In the context of airway allergy to *Aspergillus*, transfer of wild-type neutrophils to MMP-9-deficient hosts resulted in enhanced airway hyperresponsiveness as compared with the transfer of MMP-9-deficient neutrophils to MMP-9-deficient hosts. Moreover, airway responses in MMP-9-deficient recipients of wild-type neutrophils was similar to that of sensitized wild-type animals. These results indicate that in this model, neutrophil MMP-9 is sufficient to render MMP-9-deficient mice susceptible to more severe airway allergy. More broadly, this provides evidence that neutrophils contribute to the pathogenesis of airway allergy via an MMP-9-dependent mechanism.

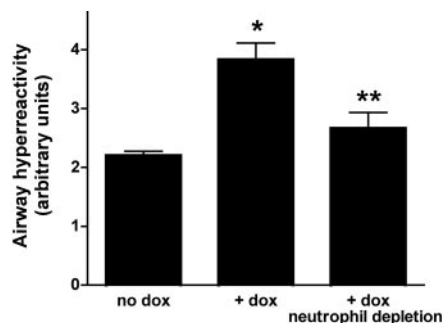
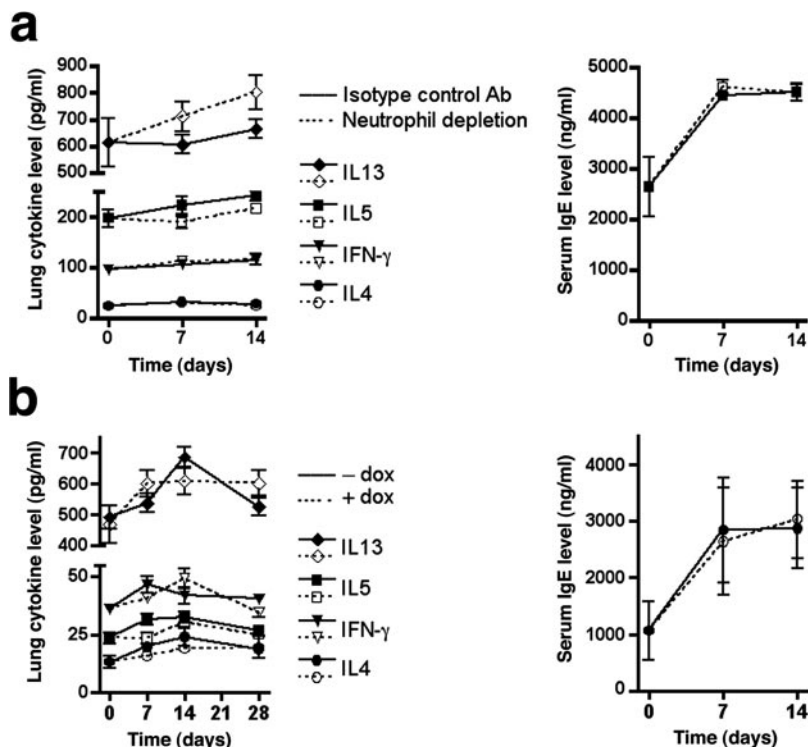


FIGURE 7. Effect of neutrophil depletion in the context of transgenic overexpression of CXCL1 on allergic airway responses to *Aspergillus*. Data represent mean $\pm$ SEM as measured by whole body plethysmography following exposure to aerosolized methacholine on day 7 after administration of conidia; $n = 4$ mice per group; “No dox” transgenic mice without transgene activation, given ordinary drinking water; “With dox”, sustained transgene activation by administration of dox via drinking water; “neutrophil depletion,” animals given neutrophil depleting mAb; *, $p < 0.05$ compared with transgenic mice without transgene activation; **, $p < 0.05$ compared with transgenic mice with transgene activation.

Discussion

The infiltration of eosinophils into the airway walls and lumen has long been recognized as a defining feature of asthma, and absence of eosinophils has been shown to modulate key features of airway allergy in experimental models (51, 52). In contrast, the role of neutrophils in airway allergy has received increasing attention relatively recently. There is a robust correlation between airway neutrophils and human asthma (recently reviewed in Ref. 53): the severity of airway disease appears to correlate with number of neutrophils in the airway in both asthma (54) and allergic bronchopulmonary aspergillosis (8, 25), and neutrophils are overrepresented in patients with asthma exacerbations, severe or poorly controlled asthma, and in those who die of asthma (9, 11, 55–60). In the context of this strong association noted in human disease, our study found long-term elevation in lung neutrophils in a mouse model of chronic airway allergy to clinically relevant Ags of a ubiquitous environmental mold. Moreover, we found that selective depletion of neutrophils ameliorated, and enhanced neutrophil influx augmented, the severity of airway responses independent of

FIGURE 8. Effect of neutrophils on lung cytokines and serum IgE levels in *Aspergillus*-induced airway allergy. *a* and *b*, Mice with mAb-mediated neutrophil depletion and sustained airway-specific transgenic expression of CXCL1, respectively. Cytokines were measured in whole lung homogenates. Data represent mean $\pm$ SEM; $n = 6$ mice in each group at each time point. Day 0 represents mice that were sensitized for 5 wk but not challenged with *A. fumigatus* conidia. “+dox”, sustained transgene activation by administration of dox via drinking water; “-dox” transgenic mice without transgene activation, given ordinary drinking water.



the accumulation of other leukocytes, thus providing evidence for a causal role for neutrophils in the pathogenesis of this illness.

To selectively augment neutrophil recruitment to the airways in the context of airway allergy, we used a conditional airway-specific transgenic system. Similar transgenic approaches have proven to be powerful tools in investigating the role of specific mediators by airway-targeted expression in mouse models of asthma (32, 47). Transgenic overexpression of the ELR⁺ CXC chemokine, CXCL1, allowed for selective recruitment of neutrophils, because this chemokine has been shown to be a potent and selective neutrophil chemoattractant both in vitro and in other transgenic systems (61–63). In addition, this family of ligands is relevant to airway allergy: ELR⁺ CXC chemokines are prominently expressed in both human and mouse allergic airways diseases (9, 64,

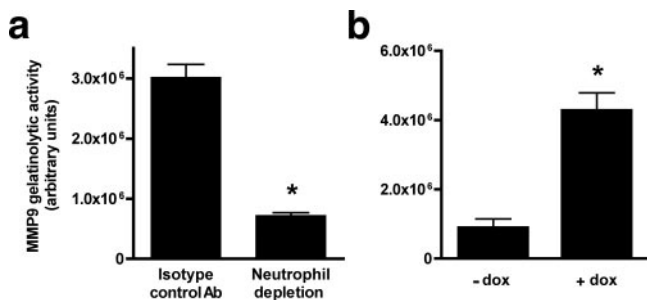


FIGURE 9. Effect of neutrophils on lung MMP-9 activity in airway allergy to *Aspergillus*. Gelatinolytic activity of the 92 kDa (active form) of MMP-9 was measured in whole lung homogenates on day 14 after conidia challenge in (*a*) sensitized mice treated with neutrophil-depleting and isotype control mAb and (*b*) sensitized CXCL1 transgenic mice with or without transgene activation. “+dox”, sustained transgene activation by administration of dox via drinking water; “-dox” transgenic mice without transgene activation, given ordinary drinking water. Data represent mean $\pm$ SEM; $n = 5$ –6 mice in each group; *, $p < 0.05$ for each comparison.

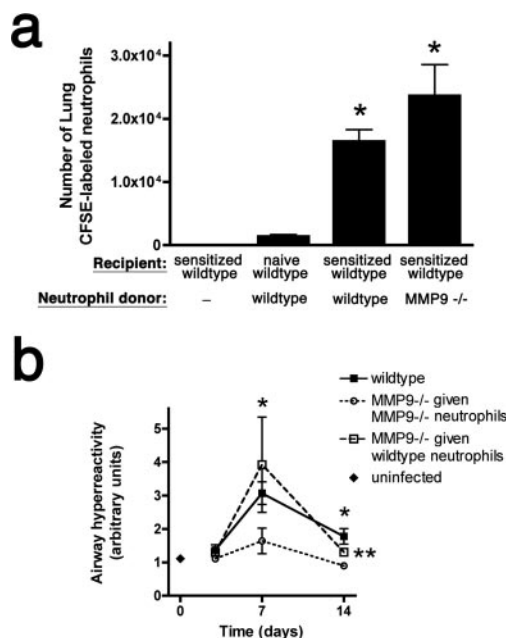


FIGURE 10. Role of neutrophil-derived MMP-9 in allergic airway response to *Aspergillus*. *a*, CFSE-labeled neutrophils from wild-type or MMP-9-deficient donors were administered i.v. to naive or sensitized mice just before challenge with *A. fumigatus* conidia and their numbers quantified in the lungs after 1 day. Data shown mean $\pm$ SEM; $n = 4$ mice in each group; *, $p < 0.05$ as compared with naive recipients of wild-type neutrophils. *b*, Neutrophils from wild-type or MMP-9^{-/-} donors were administered to sensitized MMP-9^{-/-} recipients every other day after challenge with *A. fumigatus* conidia. “Uninfected” indicates sensitized wild-type mice before intratracheal administration of *A. fumigatus* conidia on day 0; “wild-type” indicates sensitized wild-type mice, challenged with conidia but not given any cells. Data represent mean $\pm$ SEM; $n = 4$ mice in each group; *, $p < 0.05$ comparing MMP-9^{-/-} recipients of wild-type cells to MMP-9^{-/-} recipients of MMP-9^{-/-} cells at that time point; **, $p < 0.05$ comparing MMP-9^{-/-} recipients of wild-type cells to MMP-9^{-/-} recipients of MMP-9^{-/-} cells over time.

65), and their common receptor in the mouse, CXCR2, is necessary for development of the allergy phenotype (66).

An unexpected finding in this study was that the number of lung neutrophils affected the severity of airway hyperresponsiveness and remodeling without any appreciable effect on the balance of lung Th-1/Th-2 cytokines or serum IgE levels. In addition, this effect was independent of the accumulation of eosinophils in the lungs observed in this model (27). This was surprising, because recruited neutrophils have been shown to mediate Th-1 pattern inflammation in response to several classes of microbial pathogens (16, 67–69). In contrast, adoptive transfer of either OVA-specific Th-1 or Th-2 T cells to naive mice has been shown to result in enhanced pulmonary expression of ELR⁺ CXC chemokines and lung neutrophil influx upon exposure of the recipients to OVA (15). Taken together with our findings, this suggests that, at least in the context of airway allergy, recruited neutrophils may represent a common effector mechanism of airway inflammation in both Th-1- and Th-2-acquired immunity.

Our data also indicated a role for neutrophil-derived MMP-9 in neutrophil-mediated airway allergic responses. The association of neutrophil presence and MMP-9 activity is recognized in human airway allergy (25, 70, 71) and MMP-9 deficiency affects airway responses in mouse models of airway allergy (44–47, 72, 73). Importantly, while our results underline the relevance of neutrophil-derived MMP-9 in airway inflammation, they do not preclude a role for other cellular sources of this enzyme in the context of allergy; indeed, our results indicate that MMP-9 activity in the lungs is substantially reduced, but not completely ablated, in the absence of neutrophils.

These data provide evidence for neutrophil infiltration and MMP-9 activity as critical steps in the development of airway allergy. These results have several significant implications: first, the events downstream of MMP-9, such as modulation of local CC chemokine gradients (46, 72) and facilitating IL13-mediated airway remodeling (47, 73), may, at least in part, be dependent on neutrophil presence. In addition, our data provide a rationale for the study of events proximal to neutrophil recruitment to the airway (such as local production of IL-17 and ELR⁺ CXC chemokines) as potential therapeutic targets in airway allergy.

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Disclosures

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