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

Stacy J. Park,* Maria T. Wiekowski,† Sergio A. Lira,‡ and Borna Mehrad2*

Neutrophils infiltrate airway walls in patients with allergic airway diseases and in animal models of these illnesses, but their contribution to the pathogenesis of allergic airway disease 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.


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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)−/−-deficient mice (FVB background) were purchased from Charles River Laboratories or The Jackson Laboratory. Generation of transgenic mice with tetracycline-inducible airway-specific promoter, CC10; and a reporter construct, consisting of the tetracycline-responsive DNA element driving the transcription of CC10. 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 μg 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 intranasal challenge with A. fumigatus conidia (107 g/ml) 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 Ag in PBS intra-nasally on days 7, 14, and 21, followed by intratracheal inoculation of 5 × 106 A. fumigatus conidia on day 0. Conidia were prepared by staining with A. 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 using a haemocytometer.

Selective neutropenia was induced using a modification of a previous protocol (30) by i.p. administration of 80 μg of purified RB6-8CS 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/μl) 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 CC10- transgenic mice, 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 littermates CC10-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 (Pean), the indicator of airflow obstruction calculated from the plethysmograph pressure-time waveform (33), was measured continuously and values for each mouse averaged for a 3-min period immediately following exposure to aerosolized methacholine (Sigma-Aldrich). The correlation of Pean 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 107 cells in 100 μl of PBS via a lateral tail vain. In some studies, transferred neutrophils were first labeled with the vital cyanine dye, Cy5.5 (Spirax), or with a murine CD11b-specific Abs (clone 30-F11), anti-CD45R-fluorescein isothiocyanate and -FITC (M1/70), anti-CD11c-biotin (HL3), anti-CD45-PerCP (clone 53-5, 5L), and anti-CD11b-phycoerythrin and -FITC (M1/70, anti-CD11c-biotin (HL3), anti-CD45-PerCP (clone 53-5, 5L), and anti-CD11b-phycoerythrin and -FITC (M1/70, anti-CD11c-biotin (HL3), anti-CD45-PerCP (clone 53-5, 5L), and anti-CD11b-phycoerythrin and -FITC (M1/70, anti-CD11c-biotin (HL3), anti-CD45-PerCP (clone 53-5, 5L), and anti-CD11b-phycoerythrin and -FITC (M1/70, anti-CD11c-biotin (HL3), anti-CD45-PerCP (clone 53-5, 5L), and anti-CD11b-phycoerythrin and -FITC (M1/70, anti-CD11c-biotin (HL3), anti-CD45-PerCP (clone 53-5, 5L), and anti-CD11b-phycoeryth
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. 3a). 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).

**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 model is shown in Fig. 3b. 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 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<sup>+</sup> 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 ± 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.

**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 ± 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 Penh 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 ± 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 ± 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 ×100).
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 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 CXCL-1 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
sustained 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.

Discussion

The infiltration of eosinophils into the airway walls and lumen has long been recognized as a defining feature of asthma, and absence of neutrophils in airway allergy 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 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 allergic responses (Fig. 10).

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).

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 ± 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.

FIGURE 7. Effect of neutrophil depletion in the context of transgenic overexpression of CXCL1 on allergic airway responses to Aspergillus. Data represent mean ± 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.
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, 65).

**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.

**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; \(\ast\), \(p < 0.05\) 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; \(\ast\), \(p < 0.05\) 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; \(\ast\), \(p < 0.05\) comparing MMP-9/− recipients of wild-type cells to MMP-9/− recipients of MMP-9/− cells at that time point; \(\ast\ast\), \(p < 0.05\) comparing MMP-9/− recipients of wild-type cells to MMP-9/− recipients of MMP-9/− cells over time.
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 Th1/Th2 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 be mediate Th-1 pattern inflammation in response to several classes of microbial pathogens (16, 67–69). In contrast, adoptive transfer of either OVA-specific Th1 or Th2 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 inflammation in sputum from subjects with asthma exacerbation. J. Allergy Clin. Immunol. 95: 843–852.


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