Eosinophils contribute to the resolution of lung-allergic responses following repeated allergen challenge

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Background: Eosinophils accumulate at the site of allergic inflammation and are critical effector cells in allergic diseases. Recent studies have also suggested a role for eosinophils in the resolution of inflammation.

Objective: To determine the role of eosinophils in the resolution phase of the response to repeated allergen challenge.

Methods: Eosinophil-deficient (PHIL) and wild-type (WT) littermates were sensitized and challenged to ovalbumin (OVA) 7 or 11 times. Airway inflammation, airway hyperresponsiveness (AHR) to inhaled methacholine, bronchoalveolar lavage (BAL) cytokine levels, and lung histology were monitored. Intracellular cytokine levels in BAL leukocytes were analyzed by flow cytometry. Groups of OVA-sensitized PHIL mice received bone marrow from WT or IL-$10^{-/-}$ donors 30 days before the OVA challenge.

Results: PHIL and WT mice developed similar levels of AHR and numbers of leukocytes and cytokine levels in BAL fluid after OVA sensitization and 7 airway challenges; no eosinophils were detected in the PHIL mice. Unlike WT mice, sensitized PHIL mice maintained AHR, lung inflammation, and increased levels of IL-4, IL-5, and IL-13 in BAL fluid after 11 challenges whereas IL-10 and TGF-β levels were decreased. Restoration of eosinophil numbers after injection of bone marrow from WT but not IL-10–deficient mice restored levels of IL-10 and TGF-β in BAL fluid as well as suppressed AHR and inflammation. Intracellular staining of BAL leukocytes revealed the capacity of eosinophils to produce IL-10.

Conclusions: After repeated allergen challenge, eosinophils appeared not essential for the development of AHR and lung inflammation but contributed to the resolution of AHR and inflammation by producing IL-10. (J Allergy Clin Immunol 2014;■■■:■■■-■■■.)

Key words: *Eosinophils, resolution of inflammation, IL-10*

Asthma is the most common chronic respiratory condition in Western countries. Despite advances in asthma treatment strategies, disease prevalence, severity, and morbidity remain high, particularly among certain ethnic groups.[1] A number of clinical and experimental studies have addressed the underlying mechanisms of the disease to identify novel therapeutic targets. The most widely accepted mechanistic theory is that asthma is a T_H2-type cell-mediated airway inflammatory disease in which production of allergen-specific IgE, accumulation of eosinophils at airway inflammatory sites and in peripheral blood, and increases in IL-4, IL-5 and IL-13 levels have been linked to the pathophysiology of the disease.[2-4]

In this thinking, eosinophils play a central role, identified as major effector cells in large part because of the numbers that are detected in the airways and lung parenchyma and their ability to secrete a wide array of proinflammatory cytokines including IL-2, IL-4, IL-5, IL-10, IL-12, IL-13, IL-16, IL-18, and transforming growth factor (TGF)-α/β, chemokines (RANTES and eotaxin-1), lipid mediators (platelet-activating factor and leukotriene C_4), and 4 cationic proteins: major basic protein, eosinophil cationic protein, eosinophil peroxidase (EPO), and eosinophil-derived neurotoxin.[5-7] Nevertheless, the specific role of eosinophils in asthma has been controversial because dissociations between the number of eosinophils in the airways and lung function have been observed in several clinical and experimental studies. To this point, the early failures of anti–IL-5 to modify lung disease despite significant reductions in airway and peripheral blood eosinophil numbers triggered a reexamination of the role of eosinophils in asthma.[8-15] In animal models of asthma, eosinophils have been intensively investigated in terms of the development of airway inflammation, airway hyperresponsiveness (AHR), and airway remodeling. Initially, studies in mice depleted of eosinophils[16-20] or rendered eosinophil-deficient in the absence of IL-5[21] demonstrated a failure to develop lung-allergic responses. Subsequently, genetically manipulated, eosinophil-deficient (PHIL) mouse strains were generated, including GATA1-deficient[22-25] and an eosinophil-deficient strain created through EPO-diphtheria toxin A targeting (PHIL).[26-28] However, when the role of eosinophils in the development of AHR and airway inflammation was examined in these novel strains, the results were contradictory; GATA1-deficient mice developed AHR similar to wild-type (WT) controls, whereas PHIL mice failed to develop AHR. Some of the discrepancies may have been strain-dependent.[23] Specific depletion of eosinophil

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Abbreviations used
AHR: Airway hyperresponsiveness
BAL: Bronchoalveolar lavage
EPO: Eosinophil peroxidase
MCh: Methacholine
OVA: Ovalbumin
PAS: Periodic acid–Schiff
PHIL: Eosinophil-deficient mouse strain
R_L: Lung resistance
TGF: Transforming growth factor
WT: Wild type

granule-specific proteins had little impact on the development of AHR[29,30] and eosinophils appeared dispensable in the development of airway remodeling and AHR after repeated allergen challenge.[25] Functionally, the role of eosinophils in airway remodeling may be more important than effects on lung function.[22] With increased attention on mechanisms resulting in resolution of inflammation, eosinophil-derived anti-inflammatory mediator generation has been highlighted.[31,32]

In earlier studies, we noted that repeated allergen challenge of sensitized mice was associated with a decline in AHR, even at time points when airway eosinophilia was sustained.[33,34] At these time points, increased levels of IL-10 were detected in bronchoalveolar lavage (BAL) fluid.[34] In the present study, we investigated the role of eosinophils in both the development and resolution phases of allergen-induced airway inflammation and AHR using a repetitive allergen challenge model in both WT and PHIL mice. Under these conditions, a role for eosinophils could not be demonstrated in the development phase but eosinophils were essential to the resolution of AHR and airway inflammation through their ability to produce the anti-inflammatory cytokine IL-10.

METHODS

Animals

EPO-diphtheria toxin A transgenic mice (PHIL, C57BL/6 background)[26] were bred at National Jewish Health. Female PHIL mice were mated with male C57BL/6 mice purchased from Jackson Laboratories (Bar Harbor, Me). The genotypes of PHIL mice and their WT littermates were confirmed by using PCR analysis on tail DNA.[26] IL-10–deficient (IL-$10^{-/-}$) mice (B6.129P2-IL10^{tm1Cgn}/J) were purchased from Jackson Laboratories. All mice were housed under specific pathogen-free conditions and maintained on an ovalbumin (OVA)-free diet at National Jewish Health. All experimental animals used in this study were under a protocol approved by the Institutional Animal Care and Use Committee of National Jewish Health.

Sensitization and repetitive airway challenge to OVA

Sensitization and repetitive airway challenges were carried out as described previously.[34] Briefly, 6-week-old female WT littermates and PHIL mice were sensitized by intraperitoneal injection of 20 μg of OVA (Fisher Scientific, Pittsburgh, Pa) emulsified in 2.25 mg of alum (Imject Alum; Thermo Scientific Pierce Protein Research Products, Rockford, Ill) or saline in a total volume of 100 μL on days 0 and 14. On days 28 to 30, followed by 2 times a week for 2 weeks (total 7 challenges; OVA/OVA-7) or 2 times a week for 4 weeks (total 11 challenges; OVA/OVA-11), mice were challenged with aerosolized OVA (1% w/v in saline for 20 minutes) (Fig 1, *A*). Sham-sensitized but OVA-challenged mice served as controls.

Measurement of airway responsiveness

Airway responsiveness to inhaled aerosolized methacholine (MCh; Sigma-Aldrich, St Louis, Mo) was assessed 48 hours after the last challenge.[35,36] Mice were anesthetized with 200 mg/kg of pentobarbital and ventilated with 160 breaths/min and a tidal volume of 0.15 mL and 2 cm H_2O positive end-expiratory pressure (SN-480-7, SHINANO Manufacturing Co, Ltd, Tokyo, Japan) through an intratracheal tube. Aerosolized MCh (0, 12.5, 25, 50, and 100 mg/mL in saline) was administered to mice for 10 seconds, with a tidal volume of 0.45 mL and frequency of 60 breaths/min, through bypass tubing via an ultrasonic nebulizer (model 5500D, DeVilbiss Healthcare LLC, Somerset, Pa) placed between the expiratory port of the ventilator and the 4-way connector. Airway responsiveness was measured as the change in lung resistance (R_L) after exposure to increased concentrations of aerosolized MCh. R_L was continuously monitored for up to 3 minutes after aerosolized MCh exposure, and maximum values of R_L were taken and expressed as the percent change from baseline following saline aerosol. Baseline values (saline) for R_L were not significantly different among the groups.

Bronchoalveolar lavage

Immediately after the assessment of AHR, lungs were lavaged one time with 1 mL of Hanks' balanced salt solution through the tracheal tube. Recovered BAL fluid supernatants were stored at −80°C. Total leukocyte numbers in BAL fluid were counted using a hemocytometer, and differential cell counts were performed by counting at least 200 cells on HEMA 3^{-}stained (Fisher Scientific Company, Middletown, Va) cytospin slides (Thermo Shandon Cytospin 3 Cytocentrifuge; Thermo Fisher Scientific, Pittsburgh, Pa) using standard hematologic procedures in a blinded fashion.

Measurement of cytokine levels

IL-4, IL-5, IL-10, IL-12p70, IL-13, TGF-β, and IFN-γ levels were measured by ELISA (eBioscience, San Diego, Calif) according to the manufacturer's directions.

Lung histopathology and morphometric analyses

After BAL was recovered, lungs were removed and fixed in 10% (w/v) neutralized buffered formalin (pH 7.4). Lung tissues were embedded in paraffin and 5-μm thick sections were cut. Mucus-containing goblet cells were detected by staining with periodic acid–Schiff (PAS). Histologic analyses were performed in a blinded manner by light microscopy linked to an image capture system (BX51 microscope, DP72 digital camera, and QC-capture image capture software, version 2.68, Quad-Cities Online, Moline, Ill). Quantitative morphometry analyses were performed using Image J 1.47h (the US National Institutes of Health; http://rsb.info.nih.gov/ij/). The number of PAS-positive goblet cells was determined only in cross-sectional areas of the airway wall. Six to 8 different fields per slide in 4 to 6 samples from each group of mice were examined in a blinded manner.

Injection of bone marrow cells

To reconstitute eosinophils in PHIL mice, suspensions of bone marrow cells were obtained from WT or IL-$10^{-/-}$ mice and injected (5×10^6 cells in 200 μL of Hanks' balanced salt solution) via the lateral tail vein on day 21. Thirty days later, mice were challenged to OVA on 3 consecutive days followed by 2 times a week for 2 or 4 weeks (Fig 1, *B*).

Intracellular cytokine staining

Intracellular IL-10 staining of eosinophils from BAL fluid of mice challenged 7 times was carried out. BAL fluid leukocytes were stimulated for 8 hours with phorbol 12-myristate 13-acetate (50 ng/mL) and ionomycin (1 μM) in the presence of brefeldin A (10 μg/mL). After stimulation, FcγII/III receptors were blocked with antimouse CD16/CD32 antibody (clone 2.4G2, BD Biosciences, San Jose, Calif) and stained with anti–CCR3-fluorescein isothiocyanate (FITC) (R&D Systems, Minneapolis, Minn) and anti-Siglec-F