

Coexpression of IL-5 and Eotaxin-2 in Mice Creates an Eosinophil-Dependent Model of Respiratory Inflammation with Characteristics of Severe Asthma¹

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Mouse models of allergen provocation and/or transgenic gene expression have provided significant insights regarding the cellular, molecular, and immune responses linked to the pathologies occurring as a result of allergic respiratory inflammation. Nonetheless, the inability to replicate the eosinophil activities occurring in patients with asthma has limited their usefulness to understand the larger role(s) of eosinophils in disease pathologies. These limitations have led us to develop an allergen-naïve double transgenic mouse model that expresses IL-5 systemically from mature T cells and eotaxin-2 locally from lung epithelial cells. We show that these mice develop several pulmonary pathologies representative of severe asthma, including structural remodeling events such as epithelial desquamation and mucus hypersecretion leading to airway obstruction, subepithelial fibrosis, airway smooth muscle hyperplasia, and pathophysiological changes exemplified by exacerbated methacholine-induced airway hyperresponsiveness. More importantly, and similar to human patients, the pulmonary pathologies observed are accompanied by extensive eosinophil degranulation. Genetic ablation of all eosinophils from this double transgenic model abolished the induced pulmonary pathologies, demonstrating that these pathologies are a consequence of one or more eosinophil effector functions. *The Journal of Immunology*, 2007, 178: 7879–7889.

Mouse models have greatly expanded our understanding of the pathogenesis of the inflammatory conditions associated with asthma, as well as the fundamental immune responses leading to lung dysfunction. However, the lack of extensive eosinophil degranulation relative to human pulmonary patients is a particularly striking difference between asthmatics and the available mouse models (see for example, Refs. 1–4 vs 5). Degranulation in humans (and even some nonmurine animal models (e.g., guinea pigs)) contributes to both airway histopathology/

remodeling (6) and lung dysfunction (7, 8). Specifically, severe asthma patients exhibit evidence of extensive eosinophil degranulation (reviewed in Refs. 9–11) linked with marked methacholine sensitivity and significant airway epithelial/smooth muscle remodeling. In contrast, similar observations are lacking in the mouse as exemplified in studies of knockout animals deficient of the abundant eosinophil secondary granule proteins, major basic protein-1 (MBP-1)³ (12) or eosinophil peroxidase (EPO) (13). Thus, the lack of extensive degranulation in the mouse has limited insights regarding the extent by which eosinophils contribute to allergic pulmonary disease.

The recent definition of specific expression patterns of the eosinophil agonist ligands of CCR3 (i.e., eotaxin-1, -2, and -3 in humans (14–16) and eotaxin-1, -2 in mice (17–19)) suggested that these ligands may have novel consequences on eosinophil activities and, in turn, allergen-induced pulmonary pathologies. In addition, several observations from the literature and our laboratory highlight the importance of these receptor-ligand interactions, as well as IL-5 expression, for eosinophil recruitment and the execution of eosinophil effector functions (EEFs). These observations/data suggest that multiple signals including the coordinate expression of IL-5 and one or more CCR3 ligands may potentially be required for the complement of activities associated with pulmonary eosinophils: 1) activation and degranulation of human eosinophils occur following exposure to both IL-5 and CCR3 ligands

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³ Abbreviations used in this paper: MBP, major basic protein; EPO, eosinophil peroxidase; EEF, eosinophil effector function; BAL, bronchoalveolar lavage; RT, room temperature; PAS, periodic-acid Schiff; AHR, airway hyperresponsiveness.

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(see for example, Ref. 20); these effects were synergistic as exposure to a single stimulus (i.e., IL-5 or CCR3 ligand alone) had little to no consequences. In contrast, mouse eosinophils failed to degranulate in response to IL-5 and CCR3 ligands (our unpublished observations) suggesting additional signals are required. In support of this conclusion, ectopic expression of IL-5 or CCR3 ligands (e.g., eotaxin-1) in mice by themselves failed to induce eosinophil degranulation, requiring additional stimuli such as an overlapping allergen provocation to induce increased levels of eosinophil degranulation (21, 22). 2) Interestingly, assessments of cytokine/chemokine expression in wild-type vs eosinophil-less mice (i.e., *PHIL* (23)) demonstrated that systemic levels of both IL-5 and eotaxin-1 were unexpectedly elevated in the absence of eosinophils (our unpublished data), suggesting that these receptor-ligand interactions are part of feedback loops that appear to be required for eosinophil-mediated activities; 3) CCR3 receptor-ligand interactions mediated by eotaxin-2 are potential regulators of Th2-driven immune responses in the pulmonary microenvironment (e.g., IL-13 expression (24)) and thus may have direct effects on induced pathologies. Collectively, these observations suggested that the coordinate expression of IL-5 and a CCR3 ligand such as eotaxin-2 may, in part, underlie many of the mechanisms leading to the recruitment of eosinophils and the execution of EEFs. Indeed, studies of patients have clearly shown that increased levels of IL-5 and eotaxin-1/2 are necessary for both eosinophil tissue accumulation and the execution of EEFs accompanying disease pathology (25).

The hypothesis that the coexpression of IL-5 and one or more CCR3 ligands is one of the critical events leading to disease pathology suggested the possibility that a transgenic approach dysregulating the expression of IL-5 and eotaxin-1/2 may be sufficient to elicit eosinophil activities that are not necessarily observed in established allergen challenge models. In this report, we demonstrate that systemic overexpression of IL-5 and local expression of eotaxin-2 in the lungs of transgenic mice were together sufficient signals to elicit extensive eosinophil degranulation and induce pulmonary pathologies more representative of the changes displayed by severe asthma patients. Furthermore, through a breeding strategy with an eosinophil-less line of mice (*PHIL* (23)), inflammatory changes in the lung were unambiguously linked to EEFs. These mice thus provide a novel inflammatory model that is singularly dependent on eosinophils, allowing studies to define the potential contribution(s) of this proinflammatory leukocyte to the pathologies observed in asthma patients.

Materials and Methods

Generation of transgenic mice

Transgenic mouse models expressing either mouse or human eotaxin-2 were generated by cloning open reading frames encoding these respective genes (InvivoGen) into a lung-specific shuttle vector downstream of a 2.3-kb *Bam*HI fragment of the rat Clara cell secretory protein CC10 promoter (a gift of J. Gitlin, Washington University School of Medicine, St. Louis, MO) and upstream of a 2.1-kb fragment of the human growth hormone gene (26); the latter segment providing a poly(A) addition signal sequence and necessary exon-intron splicing motifs for efficient expression in transgenic mice (27). The resulting transgenic constructs were excised from plasmid sequences and injected into embryos derived from a cross of F₁(CBA/CaJ × C57BL/6J) females and C57BL/6J males. Transgenic-positive founder animals were identified by PCR using forward primers specific for each eotaxin-2 open reading frame (human eotaxin-2: 5'-CAC CAC CAA GAA GGG CCA GCA GTT-3'; mouse eotaxin-2: 5'-CAC AAT CCC TGC CCC TAC CTC CTT-3') and reverse primers from the human growth hormone gene (5'-ACA GAG GGA GCC GGA GAG CAA GAG-3' and 5'-AGT GCC CCC GTC CCA TCT ACA-3', respectively). The transgenes were identified as a 475-bp amplicon (human eotaxin-2) and a 627-bp amplicon (mouse eotaxin-2). PCRs were assembled in 50-μl volumes using the GeneAmp XL-PCR kit (Applied Biosystems) and a cycling strategy of 94°C for 5 min followed by 30 cycles of 94°C (1 min),

60°C (2 min), 72°C (2 min), and a final extension of 72°C for 7 min. Subsequent generations of transgenic animals were the result of backcrosses (>F₅) onto the inbred strain C57BL/6J. The IL-5 transgenic line, NJ.1638, was generated as described previously (28) and was backcrossed >20 generations onto the C57BL/6J strain.

The studies described in this report using either single or double transgenic mice were performed exclusively with mice hemizygous for one or both transgenes. Transgene-negative littermates and/or C57BL/6J mice purchased from The Jackson Laboratory were used as control animals. The mice were maintained in ventilated microisolator cages housed in the specific pathogen-free animal facility at Mayo Clinic Arizona (Scottsdale, AZ). The sentinel cages within the animal colony surveyed negative for the presence of known mouse pathogens. Protocols and studies involving animals were conducted in accordance with National Institutes of Health and Mayo Foundation institutional guidelines.

Induction of allergic airway inflammation

C57BL/6J mice (8–16 wk of age) were sensitized and challenged with chicken (OVA) as described earlier (13). Mice were assessed for pulmonary cellular infiltrates, eosinophil degranulation, and histopathologies on day 28 (i.e., 2 days following the last OVA challenge) of this protocol.

Preparation and quantification of bronchoalveolar lavage (BAL) fluid cellularity

The number and type of cells recruited to the airways were determined by BAL modifying a protocol previously described (22). BAL fluid was recovered following instillation of 1 ml of 2% FCS in PBS. The BAL fluid was initially centrifuged at low speed (10 min at 400 × g (4°C)) for cell isolation. However, cell-free BAL fluid for assessment of eosinophil peroxidase activity and the presence of free eosinophil granule proteins was prepared by a second high-speed centrifugation (10 min, 10,000 × g) at 4°C that was necessary to clear all remaining cells from the fluid.

Intratracheal instillation of eotaxins

Human and mouse eotaxins (R&D Systems) were reconstituted with sterile saline and stored <1 mo at –80°C before use. Intratracheal instillation of 5 μg (20 μl) of a given eotaxin was performed using slightly anesthetized (brief exposure to 3% isoflurane) animals and BAL fluid was collected from these mice at 0, 6, 12, and 24 h following instillation.

Isolation of mouse eosinophils

Eosinophils were recovered at >98% purity from the IL-5-expressing transgenic line NJ.1638 as previously described (28).

ELISA of BAL eotaxin levels

Human or mouse eotaxin-2 levels in BAL fluid were determined with the human eotaxin-2/CCL24 Immunoassay kit (DCC240; R&D Systems) or the mouse eotaxin-2/CCL24 DuoSet ELISA kit (DY528), respectively. All assays were performed as per the manufacturer's instructions.

Immunohistochemical detection of tissue eosinophils and eosinophil degranulation

Lung tissue eosinophilia was determined by immunohistochemistry using a rat mAb (rat mAb 14.7.4) which recognizes an epitope unique to both mouse and human eosinophil MBP-1 (13).

Colorimetric assessment of eosinophil peroxidase activity

EPO activity in cell-free BAL fluid and cell culture medium was determined using a modification of a method described earlier (13).

Flow cytometric assessments

Eosinophils recovered from the blood of IL-5-transgenic mice or leukocytes from BAL fluid samples (viability of 97% by trypan blue exclusion) were stained with an FITC-conjugated rat monoclonal anti-mouse CCR3 Ab (R&D Systems) and a hamster monoclonal anti-mouse CD69 Ab (BD Pharmingen). Flow cytometry was performed on a FACScan cytofluorometer (BD Biosciences). Data acquisition and analysis were performed using CellQuest software (BD Biosciences).

Determination of eosinophil degranulation using an EPO immunoblot assay

A unique immunoblot assay based on mAbs generated against individual mouse eosinophil granule proteins was developed to detect mouse eosinophil degranulation in biological fluids (e.g., BAL). Nitrocellulose membranes (0.2 μ m, cat. no. 162-0147; Bio-Rad) were pretreated by submersion in PBS for 15 min and blot-dried on filter paper before being placed in a Bio-Dot Microfiltration Apparatus (Bio-Rad). The wells of the membrane were rehydrated with 100 μ l of PBS to ensure uniform binding of the Ag and this rehydrating solution was subsequently removed by applying vacuum. Individual wells were filled with 100 μ l of Ag or standard solution, which were allowed to filter through the membrane by gravity flow. Following binding, each well was vacuum-washed with 100 μ l of PBS and the membrane was removed from the apparatus. The nitrocellulose membranes were blocked with 1% "Blocker Casein" in PBS (Pierce) for 45 min at room temperature (RT) followed by binding of a biotinylated mouse anti-mouse EPO mAb (1 μ g/ml in the blocking solution), and an incubation for 1 h at RT. The Ab-bound membrane was washed three times (5 min each) with (PBS + 0.05 of Tween 20). Streptavidin alkaline phosphatase conjugate (Roche Applied Science) was applied for 1 h at RT followed by three washes (5 min each) with PBST before incubating the membrane for 3 min in the dark with Lumi-Phos WB Chemiluminescent Substrate (alkaline phosphatase; Pierce). Chemiluminescence intensity was detected with CL-XPosure Film (Pierce). Experimental samples were compared with a standard curve of protein lysates derived from known numbers of eosinophils.

Transmission electron microscopy

BAL cells or blood eosinophils were fixed with Trump's fixative (1% glutaraldehyde, 4% formaldehyde, 0.1 M phosphate buffer (pH 7.2)), prepared for electron microscopy, and photographed as described in earlier studies (12, 13).

Assessments of pulmonary histopathology: collagen deposition/fibrosis, epithelial and airway smooth muscle hyperplasia, and goblet cell metaplasia/epithelial cell mucin accumulation

Pulmonary histopathology was assessed in lungs fixed in 10% formalin (inflated with a fixed volume (1 ml) of fixative) before embedding in paraffin. Sections (4 μ m) were stained with either H&E (general histological assessments such as epithelial and airway smooth muscle hypertrophy/hyperplasia), Masson's Trichrome (collagen deposition/fibrosis), and periodic acid-Schiff (PAS; goblet cell metaplasia/airway epithelial cell mucin accumulation). In the case of PAS, staining for goblet cell metaplasia and mucin cell content of the airway epithelium, parasagittal lung sections were counterstained with hematoxylin/methyl green and analyzed by bright-field microscopy. Goblet cell metaplasia/mucin accumulation in the airway epithelium of mice from different groups were based on evaluations previously described (29).

Partial bone marrow engraftment

Partial (~5%) bone marrow chimeras were generated by exposing (75 cGy whole body irradiation) 3-mo-old female triple transgenic mice resulting from a cross of IL-5, eotaxin-2, and *PHIL* single transgenic animals. A total of 1×10^7 bone marrow cells from wild-type male donors were transferred by tail vein injection. Mice were used in experiments following a 60-day recovery period. Donor cell engraftment of ~5% was achieved as determined by real-time PCR quantifying X vs Y chromosome-specific sequences (30).

Assessment of lung function: determination of airway hyperresponsiveness (AHR)

Functional phenotypes of the mice were assessed using techniques previously described (23, 31, 32). Mice were anesthetized (pentobarbital; 90 μ g/g body weight), intubated, and paralyzed with pancuronium bromide (0.5 μ g/g body weight). Ventilation was maintained with a computer controlled ventilator (flexivent-SCIREQ). Pulmonary resistance was determined with the "snapshot" function and peak responses to each dose were used to construct response curves to doubling doses of inhaled methacholine (0.78–50 mg/ml).

Statistical analysis

Data were analyzed and graphed using the GraphPad Prism statistics program (GraphPad Prism Software). Results are presented as means $\pm$ SE. Statistical analysis was performed using *t* tests with differences between means considered significant when *p* < 0.05.

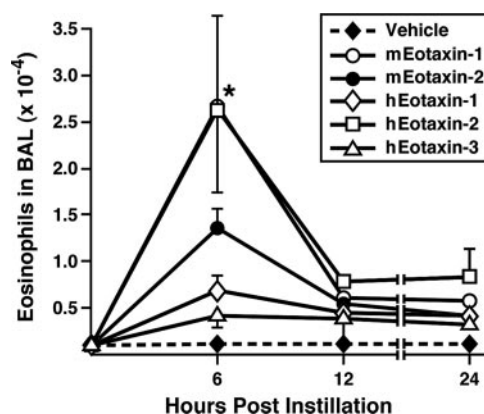


FIGURE 1. IL-5 activities and CCR3 receptor-ligand interactions synergistically mediate eosinophil accumulation and activation in the lung. The known mouse and human eotaxins (5 μ g in 20 μ l of saline) were instilled via the trachea (intratracheal) of allergen naive IL-5-transgenic mice (28). Each CCR3 ligand, regardless of the species of origin, resulted in an increase in BAL eosinophils with kinetic maxima of 6-h postinstillation. The data presented are means (*n* $\geq$ 4) $\pm$ SE.

Results

Exogenous eotaxin introduced into the lungs elicits IL-5-dependent eosinophil recruitment in mice

The ability of focal concentrations of CCR3 ligands in the airway lumen to elicit eosinophil recruitment was examined by direct instillation of human (-1, -2, -3) and mouse (-1, -2) eotaxins (5 μ g) into the lungs of either allergen-naive wild-type mice (C57BL/6J) or transgenic animals constitutively expressing IL-5 from mature T cells (line: NJ.1638 (28)) (Fig. 1). Instillation of individual eotaxins into wild-type mice, regardless of the species of origin, failed to induce the accumulation of eosinophils in the airways (data not shown). In contrast, following intratracheal instillation of each of the human and mouse eotaxins into IL-5-transgenic mice, there was a time-dependent accumulation of eosinophils in the BAL with kinetic maxima at 6-h postinstillation. Human eotaxin-2 and mouse eotaxin-1 displayed almost equivalent effects on eosinophil chemotaxis and, together with mouse eotaxin-2, were the CCR3 ligands with the highest levels of activities.

Constitutive transgenic expression of both eotaxin-2 and IL-5 in mice results in a significant pulmonary eosinophilia

Our objective was to investigate further the synergy between local eotaxin-2 and systemic IL-5 levels. In addition to the previously described transgenic model constitutively expressing IL-5 from mature peripheral T cells (28), we have created transgenic mice expressing either human or mouse eotaxin-2 in the lung epithelium. Several independent transgenic lines of mice expressing either human or mouse eotaxin-2 using the rat *CC10* gene promoter (26) were initially characterized on the basis of induced BAL eotaxin-2 levels which varied greatly between lines (1–95 pg/ml). Surveys of peripheral blood showed no obvious effects on white blood cell counts or composition (data not shown). Transgene-positive founders expressing either human or mouse eotaxin-2 were chosen for further study and each line was backcrossed to C57BL/6J mice for a minimum of five generations. All data reported are derived from mice 2–6 mo of age. The eotaxin-2-transgenic lines had live births and numbers of weaned offspring comparable to wild-type mice and the transgenes were inherited equally among male and female pups, indicating autosomal insertions. The apparent morbidity and life expectancy

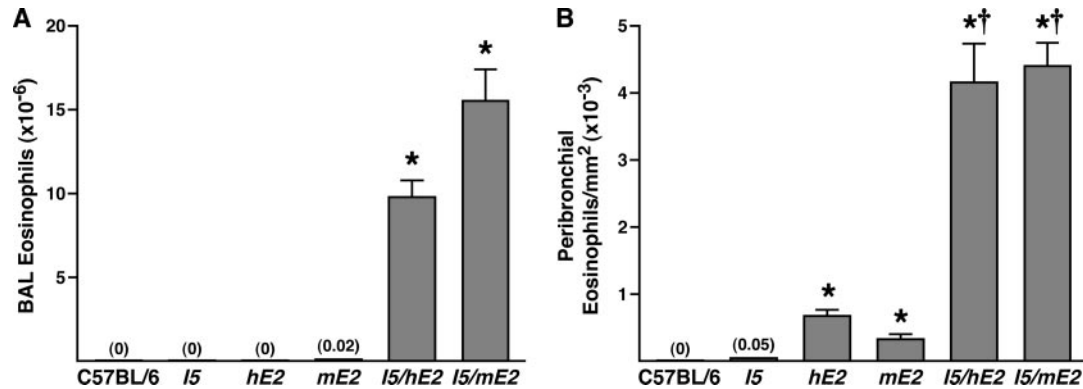


FIGURE 2. Transgenic coexpression of systemic IL-5 and lung-specific eotaxin-2 synergistically leads to an induced pulmonary eosinophilia not evident in either single IL-5- or eotaxin-2-transgenic mice. Airway (A) and peribronchial tissue (B) eosinophil levels were determined via cell counts/differential assessments of cytopsin preparations of leukocytes derived from BAL and immunohistochemistry using a rat mAb (rat mAb 14.7.4) specific for mouse MBP, respectively. The data presented are means ($n \geq 3$) $\pm$ SE. †, Significant difference ($p < 0.05$) compared with C57BL/6J control mice as well as I5, mE2, and hE2 single transgenic animals. *, Significant difference ($p < 0.05$) compared with C57BL/6J control mice.

of these eotaxin-2-expressing founders were also unchanged relative to transgene-negative littermates.

Eosinophil accumulation in the airways (BAL) and peribronchial tissue areas (MBP immunohistochemistry) of eotaxin-2 or IL-5 single transgenic mice was assessed relative to the induced eosinophilia occurring in IL-5/eotaxin-2 (I5/E2) double transgenic animals (Fig. 2). Eosinophils were virtually absent in the BAL from IL-5 single transgenic mice as well as the BAL from either human or mouse eotaxin-2 single transgenic animals (Fig. 2A). In contrast and similar to the intratracheal instillation of eotaxin-2 into IL-5-transgenic mice, I5/E2-transgenic animals displayed a significant BAL eosinophilia, confirming a cooperativity between IL-5 and eotaxin-2 that is capable of producing an airway eosinophilia (19). This induced eosinophilia in double transgenic mice also extended to peribronchial tissue areas of the lung (Fig. 2B). Interestingly, constitutive lung epithelial cell expression of eotaxin-2 in single transgenic animals was alone sufficient to induce a baseline peribronchial tissue eosinophilia that was not observed in either IL-5 single transgenic or C57BL/6J control animals (Fig. 2B).

The eosinophils accumulating in the lungs of mice coexpressing eotaxin-2 and IL-5 display evidence of activation and the release of secondary granule proteins (i.e., degranulation)

Eosinophils recruited to the airways of wild-type mice following an acute OVA provocation (i.e., 2 days following the last of three challenges of OVA sensitized mice) displayed evidence of an activated phenotype as characterized by CD69 expression in a subpopulation of cells (Fig. 3). Our studies (12, 13), as well as those from other laboratories (see for example, Ref. 5), have demonstrated that these eosinophils nonetheless fail to display evidence of extensive degranulation. That is, although expression of CD69 uniquely on eosinophils recruited to the BAL (i.e., peripheral blood eosinophils are CD69⁺ (33)) may represent some form of activation (34, 35), this expression does not correlate with extensive eosinophil degranulation.

Significantly, airway eosinophils from both I5/E2 double transgenic mouse lines contained a subpopulation of presumably activated CD69⁺ cells (Fig. 3). However, unlike eosinophils recruited to the lung in response to allergen, airway eosinophils from the I5/E2 double transgenic mouse lines displayed unambiguous evidence for the release of eosinophil granule proteins (i.e., extensive degranulation). This degranulation was established by four independent methods and occurred in both the lumen and lung tissues of I5/E2 mice.

1) Assessment of EPO activity in cell-free BAL. Little to no EPO activity was detected in cell-free BAL fluid from either saline or OVA-treated wild-type mice as well as IL-5 and eotaxin-2 (mouse or human) single transgenic animals (Fig. 4A). In contrast, significant levels of peroxidase activity were detected in cell-free BAL fluid from I5/mE2 and I5/hE2 animals, demonstrating that the airway eosinophils in these lines of mice are releasing this eosinophil granule protein.

2) Detection of EPO protein levels in cell-free BAL. A sensitive immunoblot assay for the presence of soluble EPO in BAL fluid was developed using a unique anti-mouse EPO-specific mAb (Fig. 4B).

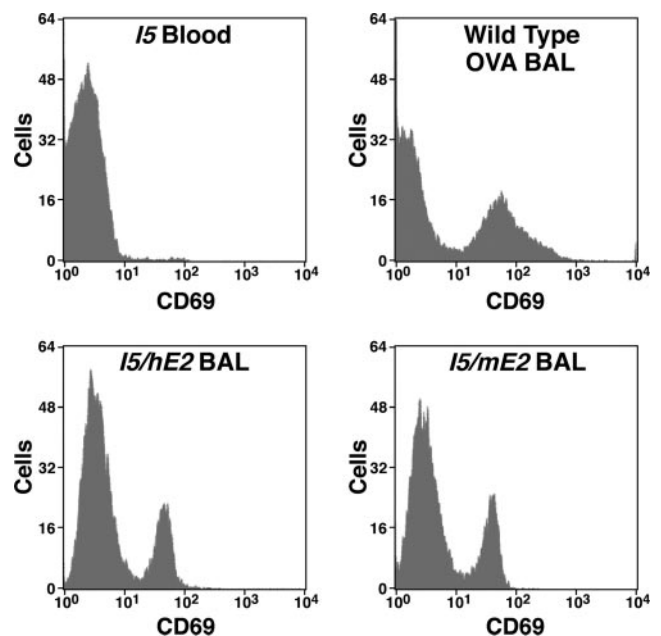


FIGURE 3. Activated (i.e., CD69⁺) eosinophils were present in the airway lumen of allergen-challenged wild-type animals as well as both IL-5/human eotaxin-2 and IL-5/mouse eotaxin-2 double transgenic mice. Representative examples of the CD69 expression status of eosinophils (i.e., CCR3⁺ cells) derived from the BAL of either OVA-treated, wild-type, or allergen naive double transgenic mice are shown. These data demonstrate that a significant fraction of eosinophils from both groups of mice ($n \geq 3$ mice/group) displayed an activated (i.e., CD69⁺) phenotype. Peripheral white blood cells from the parental IL-5-transgenic line served as a negative control, showing that inactive eosinophils were CD69⁺.

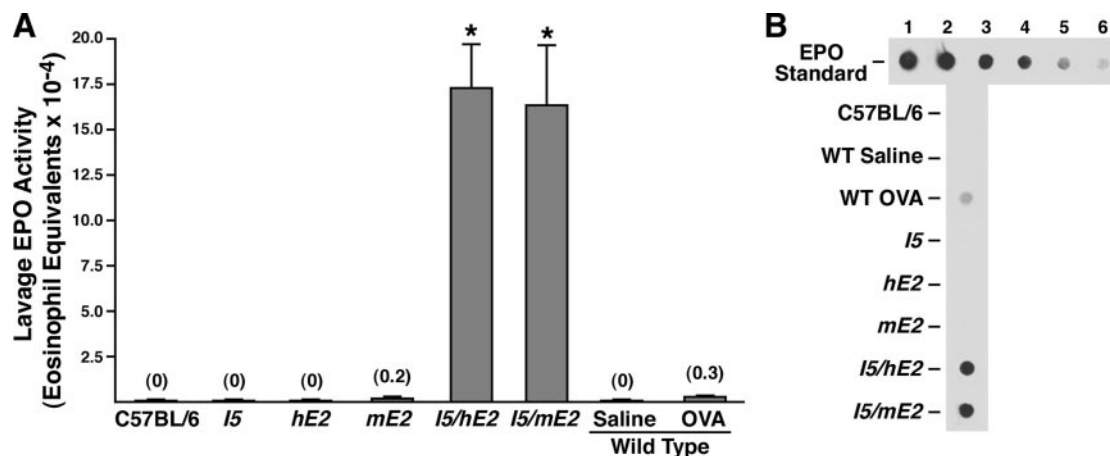


FIGURE 4. Extracellular EPO in the airways of both *I5/hE2* and *I5/mE2* double transgenic mice provides evidence of eosinophil degranulation. **A**, Significant levels of EPO enzymatic activity, expressed in terms of eosinophil equivalents derived from a standard curve, were detected by a colorimetric assay in the cell-free BAL of *I5/E2* double transgenic mice and not in the BALs derived from either IL-5 or eotaxin-2 single transgenic mice. Saline control and OVA-sensitized/aerosol-challenged wild-type mice are shown for comparison. The data presented are means $\pm$ SE ($n = 4$). **B**, A highly specific immunoblot assay of BAL fluid using a biotinylated anti-mouse EPO mAb detected the presence of EPO protein in the cell-free BAL of *I5/E2* double transgenic mice and not in the BALs derived from either IL-5 or eotaxin-2 single transgenic mice. Low levels of soluble EPO were detected in OVA-sensitized/aerosol-challenged wild-type mice. EPO standard samples numbered 1–6 are eosinophil cell lysates blotted as a serial dilution before reaction with biotinylated anti-mouse EPO mAb corresponding to 6.0 , 3.0 , 1.5 , 0.75 , 0.38 , and 0.19×10^4 eosinophil equivalents, respectively.

Similar to the measure of EPO activity, this assay showed significant amounts of EPO protein in the BAL fluid from *I5/mE2* and *I5/hE2* mice and no detectable EPO protein in the BAL of single transgenic animals or saline-treated wild-type controls. However, it is noteworthy that unlike assessments of peroxidase activity, this assay is sensitive enough to detect the low level of eosinophil degranulation that was shown to occur in OVA-treated wild-type mice (36).

3) The identification of degranulating eosinophils by electron microscopy. Transmission electron microscopy revealed that the morphology of secondary granules of BAL-derived eosinophils

from either *I5/mE2* and *I5/hE2* mice was consistent with an activated phenotype (e.g., irregular cell surface processes (37)) accompanied by extensive piecemeal degranulation (Fig. 5). Eosinophils derived from *I5/mE2* and *I5/hE2* mice showed the loss, partial loss, and/or fragmentation of the electron-dense MBP-containing cores in nearly all of the granules and decreases in the opacity of the electron translucent matrix areas of granules (37). Significantly, evidence of degranulation was not limited to a fraction of the BAL eosinophils from *I5/E2* mice but, instead, occurred in all BAL eosinophils examined (>100 eosinophils/mouse).

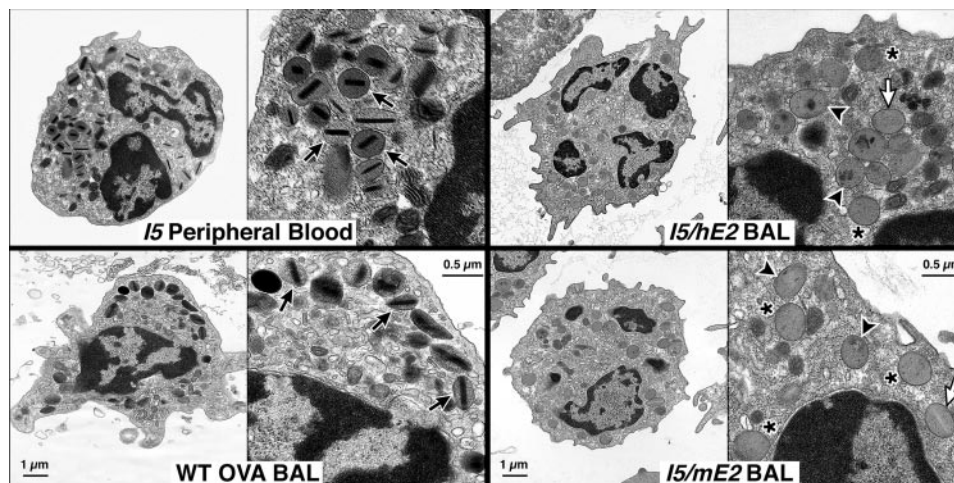


FIGURE 5. Transmission electron microscopy demonstrates that eosinophils from *I5/hE2* and *I5/mE2* double transgenic mice display morphological characteristics of eosinophil activation and evidence of extensive piecemeal degranulation. Low (left panels) and high (right panels) magnification electron microscopic views of eosinophils from the blood of IL-5 single transgenic mice (*I5* peripheral blood), BAL-derived eosinophils from OVA-sensitized/aerosol-challenged wild-type animals (WT OVA BAL), and BAL eosinophils from allergen-naïve double transgenic mice (*I5/hE2* and *I5/mE2*) are shown for morphological comparison. In contrast to peripheral blood eosinophils which display the smooth rounded surface of a resting cell phenotype, BAL eosinophils from OVA-treated wild-type mice and both *I5/hE2* and *I5/mE2* double transgenic animals displayed the irregular cytoplasmic membrane processes characteristic of eosinophil activation. Significantly, although activated BAL eosinophils from OVA-treated wild-type mice displayed virtually no evidence of degranulation, all BAL eosinophils from either *I5/E2* double transgenic line of mice displayed hallmark features of piecemeal degranulation, highlighted by the partial and/or complete loss of electron-dense secondary granule crystalline cores and the loss of electron opacity within the granule matrix. Black arrows denote typical secondary granules with electron dense crystalline cores and electron translucent matrix areas. White arrows indicate secondary granules with “ghost-like” crystalline cores. Arrowheads denote secondary granules containing fragmented crystalline cores. *, Secondary granules that have lost evidence of electron-dense crystalline cores.

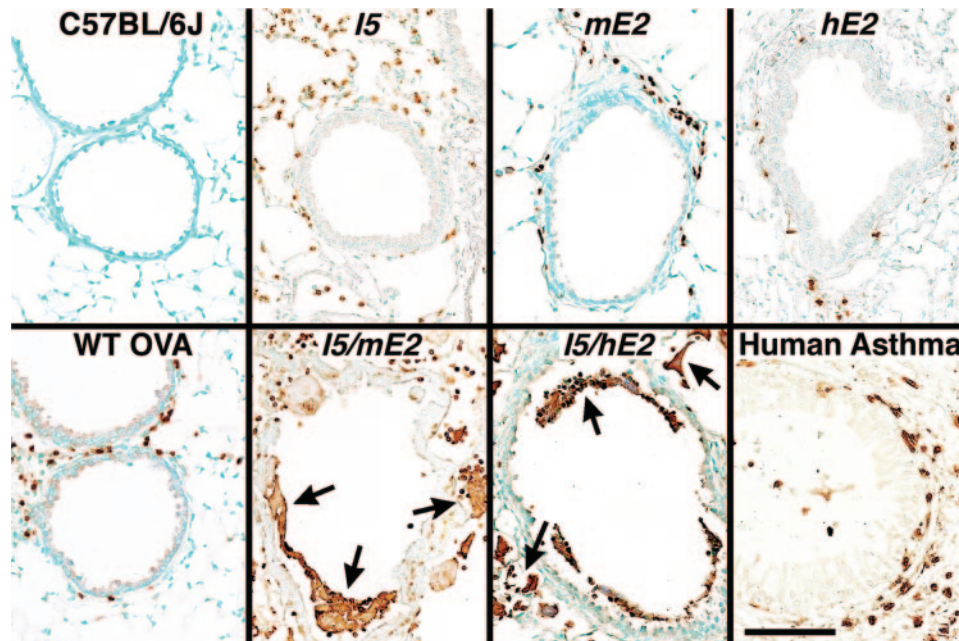


FIGURE 6. Coexpression of T cell-derived IL-5 and airway-derived eotaxin-2 leads to focal concentrations of accumulated eosinophils in the peribronchial areas of the lung that are accompanied by evidence of extensive eosinophil degranulation. Immunohistochemistry using a rat mAb (rat mAb 14.7.4) specific for mouse MBP demonstrated the spatial localization of the pulmonary eosinophils (greyish/black staining cells) in allergen-naïve wild-type (C57BL/6J), IL-5 (*IL-5*), mouse eotaxin-2 (*mE2*), and human eotaxin-2 (*hE2*) animals, as well as OVA-sensitized/aerosol-challenged wild-type (WT OVA) mice. In contrast to the modest accumulation of intact eosinophils observed in the peribronchial regions of OVA-treated, wild-type, and allergen-naïve eotaxin-2 single transgenic mice, MBP immunohistochemical staining of lung sections from either *IL-5/hE2* or *IL-5/mE2* double transgenic mice revealed a profound tissue eosinophilia with localized accumulation in all peribronchial areas. These data also provide evidence that coexpression of IL-5 and eotaxin-2 also induced the extensive release and extracellular deposition of MBP (i.e., eosinophil degranulation) within the lung parenchyma (i.e., brown staining areas denoted by black arrows in both the airway lumen and tissue compartments). A similar pattern of eosinophil accumulation and degranulation was observed in severe asthma patients as revealed in lung sections stained using a species cross-reactive eosinophil-specific rabbit polyclonal anti-mouse MBP antiserum (22). Scale bars, 100 μ m.

4) The detection of granule protein deposition using immunohistochemistry. A rat anti-mouse MBP mAb used for immunohistochemistry revealed that the tissue eosinophilia in both the single and double transgenic mice was spatially complex and unique to each line (Fig. 6). Tissue eosinophils were abundantly present in IL-5-transgenic mice; however, this was limited to the lung vasculature and not other regions of the parenchyma such as the peribronchial or submucosal areas. In contrast, ectopic transgenic expression of mouse or human eotaxin-2 alone in the lungs resulted in a pulmonary tissue eosinophil infiltrate that was both limited exclusively to the peribronchial and submucosal regions and was comparable in magnitude to OVA-treated wild-type mice. No significant differences were observed in the number or specific location of eosinophils accumulating in human vs mouse eotaxin-2 single transgenic mice. More importantly, in each of these single transgenic lines the eosinophils remained intact showing again little evidence of degranulation. However, the lungs of either *IL-5/mE2* or *IL-5/hE2* mice presented a significantly different spatial pattern of eosinophil infiltration that included extensive eosinophil degranulation. The induced eosinophilia and degranulation in these double transgenic mice occurred in the peribronchial/submucosal regions, within the cellular debris found in conducting airways, as well as the spaces associated with the outlying alveoli. Significantly, this pattern shared similarities with the eosinophil accumulation and extracellular matrix MBP deposition observed in asthma patients (Fig. 6).

Coexpression of IL-5 and eotaxin-2 induces pulmonary histopathologies that are eosinophil dependent

A more detailed assessment of the consequences of the extensive eosinophil degranulation occurring in *IL-5/hE2* mice showed that

this phenomenon was accompanied by significant pulmonary remodeling events that are generally not evident in many of the currently available mouse models. For example, *IL-5/hE2* mice displayed structural remodeling changes that included airway epithelial hypertrophy and epithelial desquamation, which together with evidence of cellular debris accumulation, result in significant morphologic airway obstruction (Fig. 7). The small airways of *IL-5/hE2* mice also displayed evidence of scar tissue (i.e., extracellular matrix protein deposition) and airway smooth muscle hyperplasia resulting in remodeling characteristic of constrictive bronchiolitis in humans (38). Moreover, subepithelial fibrosis (i.e., deposition of extracellular matrix proteins such as collagen) as well as the induction of goblet cell metaplasia/airway epithelial mucin accumulation (*GM/MA*) were also observed in this model. The induced pathologies occurring in *IL-5/hE2* mice were evident in all animals examined and in some cases, such as *GM/MA*, the induced airway pathology was equivalent (i.e., within a factor of two) of that occurring in an established acute model of allergen provocation (Fig. 8). However, in many other cases (e.g., airway hypertrophy and collagen deposition) the induced changes in *IL-5/hE2* mice were more substantial than the induced pathologies occurring in any of the established acute and/or chronic models of allergen provocation; in some cases, the pathologies (e.g., significant airway smooth muscle hyperplasia and morphologic airway obstruction) were unique to the *IL-5/hE2* model.

The remodeling events occurring in double transgenic mice were not simply a consequence of IL-5 and/or eotaxin-2 overexpression, but instead, uniquely required the presence of eosinophils. An eosinophil-less version of *IL-5/hE2* mice was generated

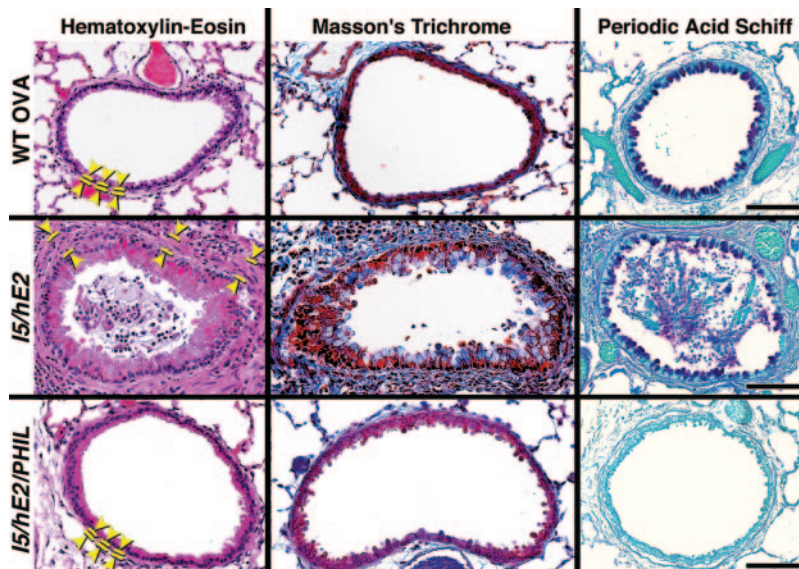


FIGURE 7. Pulmonary histopathologies in allergen-naïve *15/hE2* mice are eosinophil dependent. Representative lung sections stained to highlight specific histopathological changes occurring in wild-type mice subjected to an acute OVA sensitization/aerosol challenge protocol (WT OVA) are shown in comparison to similarly stained lung sections derived from allergen-naïve double transgenic mice expressing both IL-5 and human eotaxin-2 (*15/hE2*) and eosinophil-less triple transgenic mice (*15/hE2/PHIL*) resulting from a cross of *15/hE2* animals with the eosinophil-deficient transgenic line of mice (*PHIL*, Ref. 23). H&E staining demonstrates the thickening/hyperplasia of the airway epithelium as well as an underlying increase in extracellular matrix deposition and an apparent expansion of bronchial airway smooth muscle (regions between yellow lines with arrows). Masson's trichrome staining shows extensive peribronchial collagen deposition (blue staining material) reflective of extensive subepithelial fibrosis. PAS staining (dark purple staining) of *15/hE2* mice also demonstrates the appearance of bronchioles that display *GM/MA* and mucus plugging. The observed pathologies occur in all *15/hE2* mice and are more extensive than those observed in OVA-sensitized/aerosol-challenged animals (i.e., they are more representative of the changes occurring in severe asthma patients (11)). Significantly, the observed hallmark histopathologies in the *15/hE2* model are eosinophil dependent as the lung sections from eosinophil-less *15/hE2/PHIL* triple transgenic mice revealed the complete absence of each of the noted lung changes. Scale bar, 100 μ m.

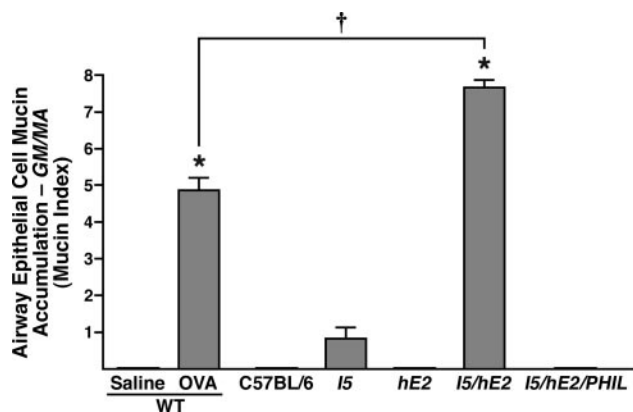


FIGURE 8. The induced goblet cell metaplasia/epithelial cell mucin accumulation (*GM/MA*) in allergen-naïve *15/hE2* double transgenic mice was comparable to the level observed in C57BL/6J mice subjected to an acute OVA-sensitized/aerosol challenge protocol. Quantitative assessments of *GM/MA* (assessed from PAS-stained sections as described in the *Materials and Methods*) showed that unlike allergen-naïve C57BL/6J or single transgenic *15* or *hE2* mice, *15/hE2* double transgenic animals displayed this airway histopathology at levels comparable to C57BL/6J mice following an acute OVA sensitization/aerosol challenge protocol. Mucin content indices are displayed as means $\pm$ SE (three to five animals per group). All evaluations of histopathology were performed in duplicate as independent observer-blinded assessments. †, $p < 0.05$. *, Significantly different ($p < 0.05$) from saline-treated control wild-type mice (Sal/wild type), allergen-naïve C57BL/6J mice (C57BL/6), single IL-5 transgenic (*15*) and human eotaxin 2 (*hE2*) mice, as well as eosinophil-less triple transgenic animals (*15/hE2/PHIL*).

from a breeding program with *PHIL*, a transgenic line of mice congenitally deficient of eosinophils (23). The unique value of this triple transgenic model (*15/hE2/PHIL*) lies in the specificity of the eosinophil ablation induced in *PHIL*-transgenic animals. That is, a detailed assessment of *PHIL* mice previously demonstrated that only the eosinophil lineage was affected in these animals without the induction of artifactual effects on immune responses associated with other inflammatory pathways such as responses to endotoxin (23). Significantly, each of the induced pulmonary histopathologies described in the parental double transgenic model were absent in *15/hE2/PHIL* mice (Figs. 7 and 8), demonstrating an unambiguous linkage between the induced pathologies occurring in this model and the presence of eosinophils. That is, in addition to the lack of overt pulmonary pathologies, staining with eosinophil-specific anti-MBP Ab showed that triple transgenic animals (*15/hE2/PHIL*) were devoid of eosinophils (Fig. 9A) but yet maintained expression of both IL-5 and human eotaxin-2 (data not shown).

Reconstitution of the pulmonary eosinophilia in 15/hE2/PHIL mice restores the induced histopathologies found in 15/hE2 animals

Hemopoietic engraftment of minimally irradiated (75 cGy) *15/hE2/PHIL* mice with marrow from wild-type donors provided sufficient numbers of hemopoietic stem cells ($\sim 5\%$) to reconstitute eosinophilopoiesis in triple transgenic mice. Assessments of the lungs of these engrafted mice revealed that reconstitution of eosinophilopoiesis in *15/hE2/PHIL* mice was sufficient to restore pulmonary eosinophil levels similar to those observed in the parental *15/hE2* line of mice (Fig. 9B). Significantly, this restoration was also accompanied by eosinophil degranulation and the development of lung remodeling events such as *GM/MA*.

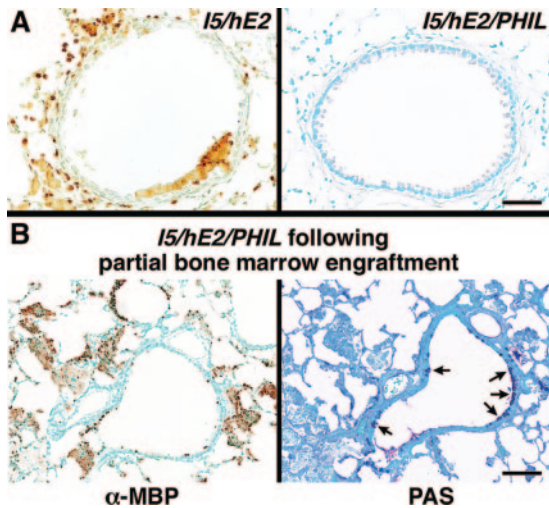


FIGURE 9. The pulmonary histopathologies associated with *15/hE2* mice are uniquely a function of the pulmonary eosinophilia associated with this double transgenic model. *A*, Immunohistochemistry of representative lung sections using a rat mAb (rat mAb 14.7.4) specific for mouse MBP revealed that unlike the complex pattern of eosinophil accumulation in the lungs of *15/hE2* mice, eosinophils were absent from the lungs of *15/hE2/PHIL* animals. Scale bar, 50 μ m. *B*, Partial bone marrow engraftment of 4-mo-old *15/hE2/PHIL*-recipient mice, with as little as 5% wild-type marrow, is nonetheless sufficient to restore the pulmonary eosinophilia and extensive eosinophil degranulation in the lungs (anti-MBP, brown staining) occurring in the parental *15/hE2* model. This restored eosinophilia was also accompanied by the appearance of histopathologies (e.g., *GM/MA* (PAS, purple staining (arrows))). Scale bars, 100 μ m.

15/hE2 mice display a significant increase in baseline airway resistance and a profound response to methacholine challenge (i.e., AHR) both of which were dependent on the presence of eosinophils

The morphologic airway obstruction demonstrated in histopathology of *15/hE2* mice was confirmed functionally in assessments of baseline lung mechanics of these animals. Specifically and in contrast to established mouse models of allergen challenge where baseline airway resistance is unchanged relative to allergen naive animals (see for example, Ref. 31), *15/hE2* mice displayed a $>50\%$ increase in baseline airway resistance relative to age- and sex-matched control (i.e., C57BL/6J) animals (1.56 ± 0.15 vs 1.02 ± 0.08 cm H₂O s/ml, respectively). Equally important was the observation that this increase was dependent on the presence of eosinophils. In contrast to *15/hE2* mice, triple transgenic animals devoid of eosinophils (*15/hE2/PHIL*) did not display a significant increase in baseline airway resistance relative to C57BL/6J mice (1.09 ± 0.05 vs 1.02 ± 0.08 cm H₂O s/ml, respectively).

Assessments of AHR following aerosolized methacholine challenge also demonstrated that unlike an acute OVA provocation protocol in wild-type mice, which typically induces an increase in AHR of several-fold relative to allergen-naive animals (39), methacholine challenge of *15/hE2* mice provoked an exaggerated response that resulted in respiratory distress even at remarkably low concentrations of aerosolized methacholine. Specifically, whereas it was possible to expose OVA-treated wild-type animals to aerosolized methacholine derived from solutions of a wide range of concentrations (0–50 mg/ml) without life-threatening consequences (Fig. 10), methacholine exposure of *15/hE2* mice was limited to concentrations of 12.5 mg/ml or less. Beyond this dose, all of the animals either suffered from severe respiratory distress and/or in some cases death (8 from a cohort of 25 mice

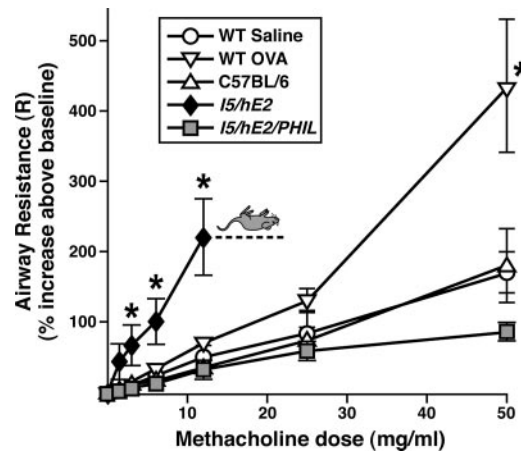


FIGURE 10. *15/hE2* mice display an extreme methacholine sensitivity that is dependent on eosinophils. Aerosolized methacholine-induced airway resistance (R) was assessed using an invasive ventilator-based technique (flexiVent, SCIREQ). Unlike C57BL/6J wild-type controls, as well as saline vs OVA-treated wild-type animals, *15/hE2* mice responded to remarkably low doses of aerosolized methacholine (maxima of ~ 12 mg/ml) before displaying overt respiratory distress that often resulted in the death of some subject animals. The entirety of the heightened methacholine sensitivity in *15/hE2* mice was dependent on the presence of eosinophils as methacholine-induced responses were abolished in eosinophil-less *15/hE2/PHIL* triple transgenic mice, returning to levels observed in C57BL/6J control animals; *, $p < 0.05$.

(i.e., 32%). Indeed, all of the *15/hE2* mice displayed heightened sensitivities to methacholine and degrees of induced respiratory distress even below the 12.5 mg/ml dose, difficulties not observed in OVA-treated wild-type mice. These methacholine-induced responses were restricted to the double transgenic animals as either single *15*- or *hE2*-transgenic mice displayed dose-response curves equivalent to allergen-naive C57BL/6J control animals (data not shown). Significantly, the extreme sensitivity to methacholine in the *15/hE2* model was abolished in eosinophil-less triple (*15/hE2/PHIL*) transgenic mice with the methacholine dose responses of these animals returning to levels displayed by allergen-naive C57BL/6J controls. These data demonstrate that the induced pulmonary eosinophilia of *15/hE2* mice was exclusively responsible for the observed AHR occurring in the double transgenic model.

Discussion

The *15/hE2*-transgenic line of mice provides a unique opportunity to determine the role(s) eosinophils have in remodeling and lung dysfunction. Several characteristics of this model are worth noting: 1) in contrast to other established models of pulmonary inflammation, *15/hE2* transgenic mice display extensive airway and tissue eosinophil degranulation; 2) *15/hE2*-transgenic mice develop a spectrum of pulmonary pathologies, including the accumulation of cellular debris in the airways leading to morphologic and functional obstruction and AHR characterized by extreme methacholine sensitivity; 3) unlike allergen provocation models that are dependent on complex immune responses to allergen that are both multiple and overlapping in character, the remodeling and lung dysfunction occurring in the double transgenic model is singularly dependent on the presence of eosinophils; 4) *15/hE2*-transgenic mice were created on an inbred strain background (C57BL/6J) previously little used for remodeling studies (i.e., such studies have been almost exclusively performed on mice of the BALB/cJ background).

In many respects, differences between humans and mice regarding allergic respiratory inflammation are likely to be a result of

evolutionary selective pressures that promote species differences in both basic physiology/biochemistry and immune responses. The execution of EEFs in the lungs of human vs mouse models appears to be a well-documented example of a species-specific difference. For example, eosinophil degranulation in humans occurs in the airway lumen and surrounding tissue by both cytolysis and piecemeal release (2, 3). In contrast, most reports suggest that mouse eosinophils display little to no degranulation (see for example, Refs. 5 and 40) and in the few studies describing this phenomenon, eosinophil degranulation was limited to cells in the airway lumen and to piecemeal release (36, 41). Nonetheless, the apparent lack of extensive degranulation of mouse eosinophils may also, in part, reflect inadequacies of existing models for such studies. That is, although the eosinophil degranulation occurring in the lungs of allergen-challenged mice is small relative to that observed in asthma patients, it does occur.

Four independent assessments of degranulation were performed using *I5/hE2* mice, including assays that were both novel (e.g., immunoblot assay for soluble granule proteins) and established (e.g., immunohistochemical detection of granule proteins and BAL EPO activity), as well as an assay that is generally accepted as the "gold standard" for detecting this phenomenon (i.e., electron microscopy). Interestingly, the extensive eosinophil degranulation detected in the airways and lung tissue of double transgenic mice occurs among all eosinophils accumulating in the lungs of *I5/hE2* mice. Moreover, this extensive degranulation was accompanied by the appearance of CD69⁺ BAL-derived eosinophils and the appearance of irregular cell surface processes on these eosinophils (i.e., markers of activation). However, each of these markers also appear on eosinophils from the BAL of allergen-challenged wild-type mice that display only very low levels of degranulation, suggesting that, as in humans, levels of eosinophil "priming" and "activation" exist in the mouse that likely stratify eosinophils into subpopulations.

It is also noteworthy that the induced effects of IL-5 and eotaxin-2 coexpression were independent of the species origin of eotaxin-2 (i.e., both mouse and human eotaxin-2 induced similar effects), implying the conservation of CCR3 receptor-ligand interactions between humans and mice. Paradoxically, although eotaxin-1 and eotaxin-2 have each been proposed to bind CCR3 receptors, the importance of eotaxin-2 appears to be unique relative to other CCR3 receptor ligands. That is, in an earlier study coexpressing IL-5 and eotaxin-1 in mice an extensive pulmonary eosinophilia was observed; however, eosinophil degranulation was limited to low levels (i.e., comparable to levels in allergen-challenged wild-type mice) and was reported only in the airway lumen of these mice (21). The recent observations that the spatial and temporal expression of eotaxin-2 was unique relative to eotaxin-1 in the mouse suggest that expression of eotaxin-2 may have a novel role in these models (18). Nonetheless, mechanistic studies are required to understand the relative differences between these CCR3 ligands, the potential importance of the levels and/or kinetics of eotaxin-1/-2 expression, or why in one species (humans) expression is sufficient to elicit eosinophil degranulation and in another species (mouse) it is not.

The eosinophil degranulation occurring in *I5/hE2* mice is unique to an in vivo setting and cannot be replicated by ex vivo exposure of purified mouse eosinophils to eotaxin-2 and IL-5. Specifically, the ability of eotaxin-2 and IL-5 to mediate eosinophil activation and degranulation was tested ex vivo by culturing peripheral blood eosinophils (isolated from *I5* mice) in the presence of these eosinophil agonists, assessing for both CD69 expression (i.e., activation) and release of one or more eosinophil secondary granule proteins (i.e., degranulation). Surprisingly, whereas a nonspecific

stimuli, such as exposure to phorbol 12-myristate 13-acetate, was able to both activate peripheral blood eosinophils and induce degranulation, we were unable to induce either CD69 surface expression or the release of EPO through cytokine/chemokine coculture with purified eosinophils (data not shown). This limitation suggests the requirement of either additional costimulatory receptor-ligand interactions (e.g., *c-kit*/stem cell factor (42) or adenosine/adenosine receptors (43)), additional cell-cell interactions (e.g., T cell-mediated activation of eosinophils (29)), or multiple eosinophil-extracellular-matrix interactions (e.g., VLA4- and/or ICAM-mediated adhesion (44)) to elicit eosinophil degranulation in the mouse. In any case, the importance/necessity of multiple concurrent interactions to achieve eosinophil degranulation appears to be restricted to the mouse as costimulation ex vivo of human eosinophils with IL-5 and a CCR3 ligand alone is sufficient to elicit degranulation (20).

The linkage of the pulmonary pathologies in *I5/hE2* mice with eosinophil accumulation and degranulation shares a remarkable similarity to the pattern demonstrated in many patients with pulmonary obstructive disease; this similarity is unique among existing mouse models. Specifically, the lung changes occurring in allergen provocation mouse models, which fail to display significant eosinophil degranulation, are often small in character and unlike the more severe pathologies associated with human obstructive diseases (11). In contrast, the pathologies of *I5/hE2* mice include the appearance of cellular debris and mucus in the lumen that result in airway obstruction and lung remodeling events resulting in easily quantifiable changes that have characteristics of fixed airway disease. Significantly, this morphologic airway obstruction had functional consequences on pulmonary mechanics similar to those observed with severe asthma patients (e.g., an increase in baseline airway resistance). That is, unlike established models of acute allergen provocation, *I5/hE2* mice displayed increases in baseline airway resistance (i.e., relative to controls) reflective of the airway obstruction associated with significant lung remodeling. Moreover and again similar to patients with severe asthma, *I5/hE2* mice also displayed an extreme sensitivity to methacholine challenge resulting in respiratory distress and even death of some subject animals. It is noteworthy that all of the pathologies occurring in *I5/hE2* mice were not only dependent on the presence of eosinophils (i.e., histopathologies and lung dysfunction were absent in *I5/hE2/PHIL* mice) but also occurred independent of allergen and thus may provide insights regarding the large population of asthmatics in which atopy does not appear to be a significant risk factor (45).

The eosinophil dependence of the pulmonary pathologies occurring in the *I5/hE2* model suggests a causative link exists between these pathologies and one or more EEFs. In the context of allergen provocation models, several potential mechanisms have been repeatedly hypothesized to underlie many of these pathologies in both patients as well as mouse models, including IL-13 expression (46), TGF- β activities in the lung (47), the production of small molecule mediators of inflammation such as cysteinyl (cys-) leukotrienes (48) or leukotriene B₄ (49), and the release of other tissue-damaging molecules (e.g., cationic granule proteins (1, 50)). However, for many of these mechanisms, their relative importance and relevant cellular source(s) in the lung have remained debatable and/or unclear. The availability of a transgenic model of pulmonary inflammation representative of many characteristics of human obstructive diseases that is also singularly dependent on eosinophil-mediated activities provides a "road map" leading to the definition of the contributory roles of specific EEFs in allergen-mediated remodeling and lung dysfunction. That is, a reductionist approach taken with our transgenic model will define

unambiguously the significance of particular EEFs without the complicating presence of multiple and often overlapping immune responses associated with allergen provocation (e.g., allergen-specific T cell-mediated activities). In turn, the simplicity of this approach will likely also lead to a greater understanding of the role(s) of these granulocytes with an expectation of valid extrapolation to obstructive pulmonary disease in patients.

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