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

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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-naive 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)† (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

Received for publication February 5, 2007. Accepted for publication April 4, 2007.

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1 This work was supported by the Mayo Foundation and grants from the National Institutes of Health HL05228 and K26-R019709 (to J.J.L.) HL058723 (to N.A.L.), and National Center for Research Resources-Centers of Biomedical Research Excellence P20RR15557, P01-HL67004, and HL-EB67273 (to C.C.I.). Additional support was provided by American Heart Association Grants 045580Z (to J.J.L.) and 0555639Z (to N.A.L.) and Respiratory Postdoctoral Training Grant HL07897 (to J.J.L.) HL058723 (to N.A.L.), and National Center for Research Resources-Centers of Biomedical Research Excellence P20RR15557, P01-HL67004, and HL-EB67273 (to C.C.I.). Additional support was provided by American Heart Association Grants 045580Z (to J.J.L.) and 0555639Z (to N.A.L.) and Respiratory Postdoctoral Training Grant HL07897 (to S.I.O. and E.A.J.).

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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.
cycling strategy of 94°C for 5 min followed by 30 cycles of 94°C (1 min),

volumes using the GeneAmp XL-PCR kit (Applied Biosystems) and a

genes (InvivoGen) into a lung-specific shuttle vector downstream of a

Transgenic mouse models expressing either mouse or human eotaxin-2

of this conclusion, ectopic expression of IL-5 or CCR3 ligands

are part of feedback loops that appear to be required for eosino-

phils-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 expres-

sion (24)) and thus may have direct effects on induced pathologies.

Collectively, these observations suggested that the coordinate exp-

ression 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 ex-

ecution 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 pa-

thology suggested the possibility that a transgenic approach
disregulating the expression of IL-5 and eotaxin-1/-2 may be suffi-
cient to elicit eosinophil activities that are not necessarily observed
in established allergen challenge models. In this report, we de-

monstrate that systemic overexpression of IL-5 and local expression
of eotaxin-2 in the lungs of transgenic mice were together suffi-
cient signals to elicit extensive eosinophil degranulation and in-
duce 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 BamHI fragment of the rat Clara cell secretory protein CC10 pro-
moter (29). Transgenic founders 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 GTA GTT-3′; mouse eotaxin-2: 5′-CAC
AAT CCC TGC CCC TAC CTT CTT-3′) and reverse primers from the

human growth hormone gene (5′-ACA GAG GGC GGA GAG CAA
GAG-3′ and 5′-AGT GCC CCC TCA 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

and a final extension of 72°C for 7 min. Subsequent generations of transgenic animals were the result of back-
crosses (>F2) 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 trans-
genic 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 con-
ducted 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 pulmo-
nary 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 re-
covered 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 per-

cent 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

Human or mouse eotaxin-2 levels in BAL fluid were determined using the
human 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 deter-

mined using a modification of a method described earlier (13).

Flow cytometric assessments

Eosinophils recovered from the blood of IL-5-transgenic mice or leuko-
cytes 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 cytofluorom-
eter (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 μm, 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 μl 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 μl 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 μl 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 μg/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 Lumiphos 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 μm) 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, paragastigal 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 × 10^6 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 μg/g body weight), intubated, and paralyzed with pancuronium bromide (0.5 μg/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 ± SE. Statistical analysis was performed using t tests with differences between means considered significant when p < 0.05.

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 μg) 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 CCl10 gene promoter (26) were initially characterized on the basis of induced BAL eosinophil-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
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<sup>+</sup> (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<sup>+</sup> 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.
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).

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

\[\text{FIGURE 5.} \quad \text{Transmission electron microscopy demonstrates that eosinophils from} \ I5/hE2 \text{and} \ I5/mE2 \text{double transgenic mice display morphological characteristics of eosinophil activation and evidence of extensive piecemeal degranulation. Low (left panels) and high (right panels) magnification electron microscopic views of eosinophils from the blood of IL-5 single transgenic mice (I5 peripheral blood), BAL-derived eosinophils from OVA-sensitized/aerosol-challenged wild-type animals (WT OVA BAL), and BAL eosinophils from allergen-naive double transgenic mice (I5/hE2 and I5/mE2) are shown for morphological comparison. In contrast to peripheral blood eosinophils which display the smooth rounded surface of a resting cell phenotype, BAL eosinophils from OVA-treated wild-type mice and both} \ I5/hE2 \text{and} \ I5/mE2 \text{double transgenic animals displayed the irregular cytoplasmic membrane processes characteristic of eosinophil activation. Significantly, although activated BAL eosinophils from OVA-treated wild-type mice displayed virtually no evidence of degranulation, all BAL eosinophils from either} \ I5/E2 \text{double transgenic line of mice displayed hallmark features of piecemeal degranulation, highlighted by the partial and/or complete loss of electron-dense secondary granule crystalline cores and the loss of electron opacity within the granule matrix. Black arrows denote typical secondary granules with electron dense crystalline cores and electron translucent matrix areas. White arrows indicate secondary granules with “ghost-like” crystalline cores. Arrowheads denote secondary granules containing fragmented crystalline cores. * Secondary granules that have lost evidence of electron-dense crystalline cores.}\]
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 I5/mE2 or I5/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 I5/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, I5/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 I5/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 I5/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 I5/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 I5/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 I5/hE2 mice was generated...
from a breeding program with PHIL, a transgenic line of mice congenitally deficient of eosinophils (23). The unique value of this triple transgenic model (I5/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 I5/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 (I5/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 I5/hE2/PHIL mice restores the induced histopathologies found in I5/hE2 animals

Hemopoietic engraftment of minimally irradiated (75 cGy) I5/hE2/PHIL mice with marrow from wild-type donors provided sufficient numbers of hemopoietic stem cells (~5%) to reconstitute eosinophiloipoiesis in triple transgenic mice. Assessments of the lungs of these engrafted mice revealed that reconstitution of eosinophilopoiesis in I5/hE2/PHIL mice was sufficient to restore pulmonary eosinophil levels similar to those observed in the parental I5/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 7.** Pulmonary histopathologies in allergen-naive I5/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-naive double transgenic mice expressing both IL-5 and human eotaxin-2 (I5/hE2) and eosinophil-less triple transgenic mice (I5/hE2/PHIL) resulting from a cross of I5/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 I5/hE2 mice also demonstrates the appearance of bronchioles that display GM/MA and mucus plugging. The observed pathologies occur in all I5/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 I5/hE2 model are eosinophil dependent as the lung sections from eosinophil-less I5/hE2/PHIL triple transgenic mice revealed the complete absence of each of the noted lung changes. Scale bar, 100 μm.

**FIGURE 8.** The induced goblet cell metaplasia/epithelial cell mucin accumulation (GM/MA) in allergen-naive I5/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-naive C57BL/6J or single transgenic I5 or hE2 mice, I5/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 ± 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-naive C57BL/6J mice (C57BL/6), single IL-5 transgenic (I5) and human eotaxin 2 (hE2) mice, as well as eosinophil-less triple transgenic animals (I5/hE2/PHIL).

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I5/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 I5/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), I5/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 I5/hE2 mice, triple transgenic animals devoid of eosinophils (I5/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 I5/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 I5/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 (i.e., 32%)). Indeed, all of the I5/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 I5- 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 I5/hE2 model was abolished in eosinophil-less I5/hE2/PHIL triple transgenic mice, returning to levels observed in C57BL/6J control animals; *, p < 0.05.

Discussion

The I5/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, I5/hE2 transgenic mice display extensive airway and tissue eosinophil degranulation; 2) I5/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) I5/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 cytolytic 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 culture 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 increased 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.

Acknowledgments

We thank the invaluable contribution of numerous individuals not listed as authors, including the tireless efforts of the Mayo Clinic Arizona Core facilities (Laboratory Animal Resources and Histology Core: Dr. Ron Marler; Immunology Core: Tammy Brehm-Gibson; Medical Graphic Arts: Marv Ruona; Research Library Services: Joseph Esposito). In addition, we express our gratitude to the Lee Laboratories administrative staff (Linda Mardel and Margaret (Peg) McGarry) without whom we could not function as an integrated group.

Disclosures

The authors have no financial conflict of interest.

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