

T_H2-Mediated Pulmonary Inflammation Leads to the Differential Expression of Ribonuclease Genes by Alveolar Macrophages

Stephania A. Cormier, Shubing Yuan, Jeffrey R. Crosby, Cheryl A. Protheroe, Dawn M. Dimina, Edith M. Hines, Nancy A. Lee, and James J. Lee

Divisions of Hematology/Oncology and Pulmonary Medicine, Department of Biochemistry and Molecular Biology, Mayo Clinic Scottsdale, S.C. Johnson Medical Research Center, Scottsdale, Arizona

The eosinophil-associated ribonuclease (Ear) family in the mouse consists of thirteen genes, eleven of which encode RNases that have physical/functional properties similar to the human Ears, eosinophil-derived neurotoxin and eosinophil cationic protein. The expression of Ear genes in the mouse is confined to sites of known eosinophilopoiesis, with the exception of the lung. Two Ear genes, Ear1 and Ear2, are predominantly expressed in the lungs of naive mice. Total Ear gene expression and RNase activity in bronchoalveolar lavage fluid increases significantly upon the induction of pulmonary inflammation using an ovalbumin (OVA) model of allergic sensitization and challenge. Interestingly, pulmonary Ear11 transcripts, which are absent in naive mice, accumulate as a consequence of OVA-mediated T_H2 inflammation in the lung. The induction of Ear11 expression is dependent on the presence of T cells, in particular, CD4⁺ T lymphocytes. This effect is likely the result of the elaboration of T_H2 cytokine levels, because pulmonary instillation of interleukin-4 or interleukin-13 induces the accumulation of Ear11 transcripts in naive animals. This study demonstrates that despite an allergen-mediated pulmonary eosinophilia and earlier studies showing that Ears are constituents of eosinophil secondary granules, alveolar macrophages are a significant source of these RNases in lungs of OVA-treated mice.

The onset and progression of allergic asthma is accompanied by a complex series of overlapping, and often concurrent, inflammatory responses in the lung that are orchestrated by CD4⁺ T_H2 lymphocytes (1–5) and the expression of T_H2 proinflammatory cytokines (e.g., interleukin [IL]-4, -5, and -13 [6–8]). This inflammation is characterized by cellular infiltrates that are, in part, thought to be the underlying cause(s) of the accompanying airway obstruction and lung dysfunction. In particular, the differential recruitment of eosinophils to the airway mucosa and lumen are common features of allergic respiratory disease, occurring in > 75% of reported cases (9). Considerable evidence has amassed in patients, as well as animal models, suggesting that eosinophils and CD4⁺ T lymphocytes play an integral role as the

major effector cells responsible for the pathologies associated with allergic inflammatory diseases (10, 11). However, the mechanisms by which these individual effector cells contribute to the etiology of asthma has remained unresolved despite direct attempts to determine factors responsible for observed pulmonary pathologies. The current paradigm suggests that CD4⁺ T lymphocytes produce a T_H2 type pattern of cytokines (e.g., increased production of IL-4, IL-5, and IL-13) and elicit chemokine expression from endothelial/epithelial cells, inducing the recruitment of proinflammatory leukocytes such as eosinophils. In turn, eosinophils release additional mediators, including secondary granule proteins, that are believed to lead to the pathologic changes occurring in the lung.

Several lines of evidence indicate that eosinophils are also part of innate immune responses that target specific pathogens before, and/or concurrently with, B and T cell-mediated adaptive immune responses. For example, eosinophils are capable of phagocytosing and killing specific bacterial cell types (12, 13), and many studies have suggested that a major homeostatic function of the eosinophil is to defend against large nonphagocytosable targets that often escape the specific killing mechanisms associated with B and T cells (e.g., helminthic larvae and protozoan parasites [14–17]). Although several independent eosinophil effector functions are likely to contribute to allergen-mediated changes in the lung (e.g., release of leukotrienes [18], secretion of immunoregulatory cytokines [19], generation of reactive oxygen species [20, 21]), the release of a diverse group of proteins sequestered in eosinophil secondary granules (i.e., degranulation) has been highlighted as an important effector function.

One of the groups of proteins identified in the secondary granule is the eosinophil-associated RNases (Ears) (22–25). In particular, studies of the human Ears, eosinophil-derived neurotoxin (EDN), and eosinophil cationic protein (ECP), showed that they potentiate innate mechanisms capable of targeting specific pathogens. Micromolar concentrations of ECP *in vitro* damage and kill a wide variety of parasites, bacteria, and mammalian cells (26, 27). In addition, both ECP and EDN appear to possess noncytotoxic cell agonist activities critical to immunomodulation during allergic inflammation (28). Recent evidence suggests that these human Ears also possess antiviral activities directed against single-stranded RNA viruses such as respiratory syncytial virus (RSV) (29–31), and thus may be key components of innate defenses against viral infection. Collectively, these studies suggest that eosinophil recruitment to the lung in response to inflammatory stimuli may, in part, be a con-

(Received in original form April 18, 2002 and in revised form July 11, 2002)

Address correspondence to: Dr. James (Jamie) J. Lee, Division of Pulmonary Medicine, Department of Biochemistry and Molecular Biology, S.C. Johnson Medical Research Center, Mayo Clinic Scottsdale, 13400 East Shea Boulevard, Scottsdale, AZ 85259. E-mail: jlee@mayo.edu

Abbreviations: bronchoalveolar lavage, BAL; BAL fluid, BALF; eosinophil-associated ribonuclease, Ear; eosinophil cationic protein, ECP; eosinophil-derived neurotoxin, EDN; glyceraldehyde 3-phosphate dehydrogenase, GAPDH; major basic protein, MBP; ovalbumin, OVA; respiratory syncytial virus, RSV; ribonuclease, RNase; saline, SAL.

Am. J. Respir. Cell Mol. Biol. Vol. 27, pp. 678–687, 2002

DOI: 10.1165/rcmb.4882

Internet address: www.atsjournals.org

sequence of innate defense mechanisms that potentially provide a molecular/cellular link between respiratory viral infection, pulmonary immune dysfunction, and the onset/progression of asthma.

Our previous studies in the mouse demonstrated that the Ear gene family is comparatively large relative to humans, and includes eleven genes as well as two pseudogenes (23, 25). Moreover, the presence of Ear transcripts in the lungs of naive animals suggested that the expression of these genes may not necessarily be restricted to eosinophils (25, 32). In this study, we demonstrate that the alveolar macrophage is another cellular source of Ears in the lungs of mice. The data show that ovalbumin (OVA) sensitization/challenge results in a significant increase in pulmonary Ear expression, including the *de novo* appearance of transcripts from a unique Ear gene, Ear11. *In situ* hybridization of lung sections confirmed that alveolar macrophages were the cellular source of this allergen-induced expression. Thus, two potential sources of Ears are available to account for the increased RNase activity observed in bronchoalveolar lavage (BAL) fluid (BALF) from allergen-treated mice: eosinophils recruited to the lung (sequestered, preformed Ear proteins representing several [5–6] genes) and resident alveolar macrophages (expression and secretion of Ear1, Ear2, and Ear11). This macrophage-associated increase in Ear expression following OVA sensitization/challenge, as well as the appearance of Ear11 transcripts, was dependent on the presence of CD4⁺ T cells. Interestingly, this increased Ear expression was independent of IL-5, but occurred in naive mice administered IL-4 or IL-13, suggesting that alveolar macrophage-derived Ears, and not eosinophil-derived Ears, may have a pulmonary effector function(s) at both homeostatic baseline and during T_H2-mediated inflammatory responses in the lung.

Materials and Methods

Mice

Gene knockout mice were obtained from the Jackson Laboratories (Bar Harbor, ME) where they have been bred and maintained on a C57BL/6J genetic background. The mice used in this study were lacking either T cells ($\alpha\beta/\gamma\delta$) (C57BL/6J-Tcr β^{tm1Mom} Tcr δ^{tm1Mom} [33]), CD4⁺ cells (C57BL/6J-Cd4 ^{tm1Kw} [34]), CD8⁺ cells (C57BL/6-Cd8a ^{tm1Mak} [35]), or IL-5 (C57BL/6-IL-5 ^{tm1Kopf} [36]). Control C57BL/6J mice were obtained from Jackson Labs, and all procedures were conducted on mice 8–12 wk of age maintained in ventilated microisolator cages housed in a specific pathogen-free animal facility. Sentinel mice within this animal colony were negative for antibodies to viral and other known mouse pathogens. Protocols and studies involving animals were conducted in accordance with National Institutes of Health and Mayo Clinic Foundation guidelines.

Sensitization and Induction of Allergic Airway Response with OVA

Mice were sensitized and challenged with chicken OVA grade IV (Sigma, St. Louis, MO) as previously described (37). Briefly, mice were sensitized by an intraperitoneal injection (100 μ l) of 20 μ g OVA emulsified in 2 mg Imject Alum (Al[OH]₃/Mg[OH]₂; Pierce, Rockford, IL) on Days 0 and 14. Mice were subsequently challenged with an aerosol generated from a 1% (wt/vol) OVA solution

in saline for 20 min on Days 24, 25, and 26 using an ultrasonic nebulizer (DeVilbiss, Somerset, PA). All assays were performed on Day 28. Control saline-treated animals were injected intraperitoneally with saline on Days 0 and 14 and challenged with nebulized saline on Days 24, 25, and 26 as described above.

IL-4/IL-13 Instillation

Lyophilized mouse recombinant IL-13 was a kind gift of Dr. Debra Donaldson and Dr. Joseph Sypek (Genetics Institute, Boston, MA) and recombinant IL-4 was purchased from Roche (Indianapolis, IN). Both were dissolved in PBS containing 0.1% bovine serum albumin (vehicle) at a concentration of 10 μ g/ μ l, and individual aliquots were stored at –80°C. Before use, aliquots were thawed on ice and diluted to 0.25 μ g/ μ l with vehicle. Experimental groups of mice were lightly anesthetized with an intramuscular injection of a ketamine/xylazine cocktail (40 μ g ketamine and 6 μ g xylazine in saline per gram mouse weight), and 5 μ g of recombinant IL-4/IL-13 (total volume = 20 μ l), or vehicle alone, were administered by intratracheal instillation on three consecutive days. Assessments of eosinophils in BALFs, pulmonary RNA isolation for RT-PCR analyses, and lung tissue isolation from all groups of mice were performed on Day 28 as described below.

Ear Gene Expression

Total RNA isolation. Total lung RNA was isolated using Qiagen (Valencia, CA) Mini/Midi RNAeasy Kits as per the manufacturer's instructions.

Northern blot analysis. RNA hybridization was performed as previously described (25). In summary, 15 μ g of total RNA was size fractionated on a 1.2% formaldehyde-agarose gel and transferred to Genescreen (+) (NEN-Dupont, Boston, MA). The RNA blot was prehybridized in ULTRAhyb (0.4 M [Na⁺]; Ambion, Austin, TX) for 2 h at 42°C and subsequently hybridized at 42°C overnight (> 14 h) in a buffer consisting of ULTRAhyb containing 1 $\times$ 10⁶ cpm of ³²P-labeled Ear2 probe per milliliter of hybridization solution. The hybridized membrane was sequentially washed at room temperature with 1 $\times$ SSC/0.2% SDS (2 $\times$ 5 min) and 0.1 $\times$ SSC/0.2% SDS (2 $\times$ 5 min) at 50°C. The reduced criteria of the hybridization and washing conditions were selected on the basis of permitting the Ear2 probe to cross-react with other members of the Ear gene subgroup (23). Hybridization was visualized by autoradiography with Kodak BioMax MR X-ray film.

RT-PCR of total lung RNA. Samples were prepared for RT-PCR analyses of gene expression as previously described (25). Single-stranded cDNA was synthesized by reverse transcription using an oligo dT_{12–18} primer and 200 ng of total RNA. The reaction conditions for PCR included 2 μ l of template cDNA (synthesized from 200 ng of total RNA or 2 ng of control plasmid), 0.2 mM of each dNTP (dATP, dGTP, dCTP, and dTTP), 0.2 μ M of each primer, 4% dimethylsulfoxide (except mouse glyceraldehyde 3-phosphate dehydrogenase (GAPDH) reactions which did not contain DMSO), 1 $\times$ PCR reaction buffer containing 1.5 mM MgCl₂ (Boehringer Mannheim, Indianapolis, IN), and 0.65 U of Taq polymerase (Boehringer Mannheim). PCR assays were performed using a Gene Amp 9700 System (Perkin-Elmer, Foster City, CA). The specific primers used were as follows: (1) mouse glyceraldehyde 3-phosphate dehydrogenase (GAPDH): forward, 5'-GACT GTGGATGGCCCCCTCTGGA-3' and reverse, 5'-CACCTGTG TCTGTAGCCGTATTC-3'; (2) Ear1/2/3/8/9/10 primers: forward, 5'-GCCATCATGCTGGGACA-3' and reverse, 5'-GTGGAGTT CTGGGGTTACA-3'; (3) Ear5/11 primers: forward, 5'-TTGC CTCATGCCAGCCATTG-3' and reverse, 5'-TGACTGGCCGG AGTTGTGAG-3'. The cycling reactions were essentially the same for each primer set and included 25 cycles of the following: 94°C,

30 s; 60°C (GAPDH), 65°C (Ear1/2/3/8/9/10), or 63°C (Ear 11/5) for 1 min, and 72°C for 30 s. These 25 cycles were followed by an extension period at 72°C for 5 min. The following controls were included with every reaction: GAPDH-PCR reactions using RNA (not reverse transcribed) to demonstrate the absence of contaminating DNA and PCR using Ear2 and Ear11 cDNA plasmid clones to show specificity of the primers in each RT-PCR.

Isolation of cDNA Clones and Sequence Analysis

Total RNA was isolated from lung tissue and used to make cDNA as described above. The cDNA was amplified using primers (forward primer: 5'-CGACTTGTCTCCTGCTG-3' and reverse primer: 5'-TGTCCCATCCAAGTGAAC-3') capable of amplifying all Ear genes (25). Double stranded cDNA products were cloned into the pcDNA2 vector (Invitrogen, Carlsbad, CA) and sequencing reactions of plasmid templates were performed using cycle-sequencing reactions and analyzed using an Applied Biosystem model 373A automated DNA sequencer (Perkin-Elmer).

BAL and RNase Activity Assay

Lungs were lavaged two times with 0.5 ml PBS (i.e., total volume = 1 ml). The recovered BALFs were pooled and centrifuged at 4°C (500 × g; 10 min) generating a BAL cell pellet and a cell-free supernatant (BALF). The amount of protein present in the BALF was determined using the BCA Protein Assay Reagent (Pierce, Rockford, IL). The BALF was then stored at -80°C. RNase activity in the BALF was assessed using the RNaseAlert QC System (Ambion). A reaction mixture containing 400 ng total BAL-derived protein, RNaseAlert buffer, and 200 nM fluorescent RNA substrate, in a total reaction volume of 500 µl, was equilibrated at 37°C for 10 min. The samples were excited at 490 nm and the emission at 520 nm was recorded using a fluorescent spectrophotometer (F-4500; Hitachi, San Jose, CA). The RNase activity of each sample was determined from a standard curve of the RNase activity generated with RNase A (Cat. #2271; Ambion). This assay is linear over a range of 0–250 pg for up to 2 h. Individual BAL samples were assayed in triplicate and a minimum of 5 animals/group were lavaged to obtain the data presented.

Immunohistochemistry and *In Situ* Hybridization

The lungs of sacrificed mice were gravity-inflated (i.e., constant pressure) with 0.5 ml of 4% paraformaldehyde, fixed overnight at 4°C, dehydrated, and embedded in paraffin. Sections (4 µm) were processed for either confocal microscopy using antibodies specific for eosinophil secondary granule proteins (i.e., major basic protein [MBP] and Ears) or *in situ* hybridization using antisense radiolabeled RNA probes for Ear2 and Ear11.

Eosinophil granule protein immunofluorescence. Immunofluorescence was performed using rabbit polyclonal antisera against mouse MBP and a rat monoclonal antibody against mouse Ears (MT3 25.1.1). Antigen-antibody complexes were detected as previously described (37, 38). Briefly, the sections were deparaffinized, hydrated, and blocked with PBS containing 1.0% normal goat serum for 30 min prior to incubation with primary antibody for MBP and Ears (each diluted 1:3000) for 60 min. Antibody-bound slides were washed in PBS and incubated (30 min) with FITC-conjugated goat anti-rabbit antibody (1:100 dilution) and Alexa 568-conjugated goat anti-rat antibody (1:680 dilution). Slides were analyzed using the Zeiss Laser Scanning Confocal Microscope (LSM 510; Zeiss, Thornwood, NY).

In situ hybridization with Ear subgroup specific probes. *In situ* hybridization was performed as described by Lee and colleagues

(39). Briefly, lung sections were hybridized with ³⁵S-labeled antisense RNA probes derived from cDNA clones representing the Ear2 and Ear11 genes. The conditions of hybridization (50°C/50% formamide/0.75M [Na⁺]) and the final wash (65°C/0.015M [Na⁺]) were selected as a sufficiently high criteria to prevent cross-hybridization of the Ear probes (23). Hybridized and washed slides were dipped in emulsion (Kodak NBT-2), exposed for 4 d at 4°C, developed in Kodak D-19 developer, and counterstained with hematoxylin and eosin (H&E; Shandon-Lipshaw, Runcorn, UK) before analysis with a Zeiss Laser Scanning Confocal Microscope (LSM 510) with the use of reflected-light.

Alveolar Macrophage Isolation and Culturing Conditions

Lungs were lavaged two times with 0.5 ml PBS (total volume = 1 ml) and the recovered fluid was centrifuged at 4°C (500 × g; 10 min), generating a BAL cell pellet and a cell-free supernatant. The cell pellets were resuspended in RPMI 1640 supplemented with 2% heat-inactivated FCS, L-glutamine (2 mM), penicillin (100 U/ml), and streptomycin (100 µg/ml) (all supplements were from Gibco, Carlsbad, CA). Differential cell counts were performed by counting at least 400 cells on cytocentrifuged preparations (Cytospin 2; Shandon-Lipshaw), stained with the Diff-Quik Stain Set (Dade Behring; Newark, DE). The cells were plated in 6 well dishes, allowed to adhere to the dishes for 2 h, and the nonadherent cells were removed. The adherent cells (> 99% alveolar macrophages) were removed using a cell scraper and total RNA was isolated as described above.

Statistical Analysis

Data presented are the means (± SE). Statistical analysis was performed on parametric data using Student *t* tests with differences between means considered significant when *P* < 0.05.

Results

OVA Sensitization and Challenge Results in the Increased Expression of Ear Genes in the Lung, Including the *De Novo* Appearance of Ear11

The level of Ear expression in the lungs of saline and OVA-treated mice was assessed by northern blot (Figure 1A) using an Ear2 cDNA sequence at sufficiently low hybridization/wash criteria as to allow cross-reactivity with other members of the Ear subgroup of genes (> 70% nucleotide identity). Significant steady-state levels of Ear transcripts were detected in both saline and OVA-treated lungs; however, OVA sensitization/challenge results in a substantial increase in Ear transcript accumulation relative to saline-treated mice.

The Ear transcripts present in the lungs of saline and OVA-treated mice were reverse transcribed, cloned into a plasmid vector, and 40 randomly selected clones from each set (i.e., saline- and OVA-treated groups) were chosen for sequence analysis. Sequencing data revealed that the Ears are differentially expressed in response to OVA sensitization/challenge. Pulmonary Ear transcripts in saline-treated mice were limited to Ear1 and Ear2, representing 13% and 87% of the Ear transcripts, respectively. In contrast, OVA sensitization/challenge led to a change in Ear transcript distribution with the loss of transcripts representing Ear1 and the *de novo* appearance of transcripts from a third Ear genes, Ear11. Thus, only transcripts from Ear2 and Ear11 gene were detected in the lungs of OVA-treated mice, representing 58% and 42%,

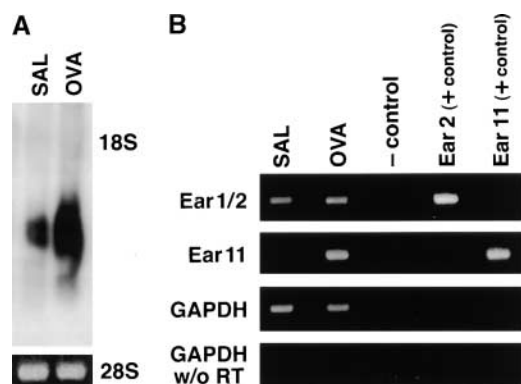


Figure 1. Ear expression increases in response to sensitization and challenge with OVA. (A) Northern blot of total RNA (15 µg) from SAL- or OVA-treated lungs using a ^{32} P-labeled cDNA probe for Ear2. The criteria of the hybridization and washing conditions allowed cross-reactivity of this probe with the other Ear gene family members. (B) Assessment of Ear gene expression in the lungs of SAL- and OVA-treated mice using RT-PCR and primer sets specific for the Ear1/2/3/8/9/10 or Ear5/11 subfamilies. Control reactions using Ear2 and Ear11 cDNA clones as template are included to verify the specificity of the primer sets and a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primer set is included as a control for the reverse transcription reactions.

respectively. It is noteworthy that these sequencing data demonstrate that in addition to the appearance of Ear11 transcripts, OVA sensitization/challenge likely leads to the enhanced accumulation of pulmonary Ear2 transcripts. That is, despite a nominal drop in Ear2 relative transcript prevalence (i.e., 87% versus 58%), the 5- to 10-fold increase in absolute Ear transcript accumulation observed in Figure 1A shows that the OVA treatment led to a significant increase in Ear2 transcript accumulation in the lung. Consistent with the sequencing of these Ear RT-PCR amplicons, the Ear1/2/3/8/9/10 subgroup primer set detects the presence of transcripts in the lungs of both saline- and OVA-treated mice, whereas primers specific for the Ear5/11 subgroup detects Ear11 transcripts only following OVA sensitization/challenge (Figure 1B). Significantly, these data show that even at the sensitivity afforded by PCR, Ear11 transcripts were not detected in saline-treated mice.

BALF was isolated and the amount of RNase activity was assayed to determine if the increase in pulmonary Ear transcript levels in response to OVA was accompanied by a concomitant increase in airway RNase activity. The data demonstrate that considerable RNase activity is present in the airway lumen at homeostasis, consistent with the constitutive expression of Ear1 and Ear2 (Figure 2). Furthermore, this baseline RNase activity increases significantly in response to OVA sensitization/challenge, paralleling the OVA-induced increase in lung-associated Ear gene expression and the appearance of Ear11.

Eosinophils and Macrophages are Potential Sources of Ears in the Lungs of OVA-Treated Mice

We used immunofluorescence and confocal microscopy to identify potential cellular sources of Ears in the lung (Figure

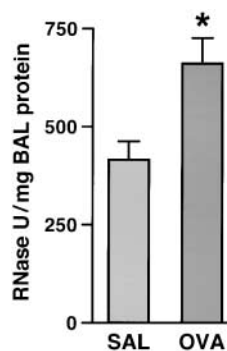


Figure 2. Total BAL RNase activity increases significantly in response to OVA sensitization and challenge. Data are expressed as the means $\pm$ SEM ($n = 5$ mice/group). * $P < 0.05$.

3). Cells staining positive for Ears are not abundant in the peribronchial/perivascular regions of the lungs from saline-treated mice. In contrast, OVA sensitization/challenge results in a significant increase in the number of cells staining positive for Ear proteins in these regions. This pattern (i.e., low numbers in the lungs of naive mice that increase significantly following OVA sensitization/challenge) is consistent with the distribution of eosinophils (a known source of Ear proteins). Indeed, all of the cells staining positive for Ears from either saline- or OVA-treated groups were identified as eosinophils (i.e., the MBP⁺ cells), demonstrating that significant levels of Ears are sequestered only in pulmonary eosinophils.

The presence of a secretory signal peptide in the Ears (23) suggested that other cells in the lung may produce Ears that are secreted and thereby escape immunohistochemical detection with anti-Ear antibodies. To identify such sources, *in situ* hybridization with probes specific for either Ear1/Ear2 or Ear11 (i.e., the only identified Ears expressed in the lung) was employed (Figure 4). *In situ* hybridization using an Ear1/Ear2-specific probe demonstrated the presence of numerous positive staining cells in the lungs of both saline- and OVA-treated mice. In contrast, Ear11 transcripts were not observed in the lungs of saline-treated mice and were detected only after OVA challenge. Higher magnification and differential staining of these sections (*insets* of Figure 4) demonstrated that all of the cells hybridizing to the Ear1/Ear2/Ear11 probes were alveolar macrophages. Surprisingly, none of the eosinophils present in the lung exhibited evidence of hybridization to any of the Ear probes used. It is also noteworthy that no differences were evident in the number of Ear1/Ear2-positive macrophages present in either the saline- or OVA-treated mouse lungs. In addition, unlike Ear1/Ear2 expression, which occurred in most, and perhaps all, alveolar macrophages, Ear11 transcripts appeared to accumulate only in a subset of macrophages.

Pulmonary Ear11 Gene Expression Is Dependent on the Inflammatory Responses Mediated by CD4⁺ T Cells

The expression of Ear1/Ear2 and Ear11 genes was assayed in the presence or absence of T cells ($\alpha\beta^{-/-}$, $\gamma\delta^{-/-}$), CD4⁺ cells, or CD8⁺ cells (Figure 5). Ear1/Ear2 transcripts were present in the lungs of both saline- and OVA-treated mice regardless of the T cell deficiencies. Sequence analysis of

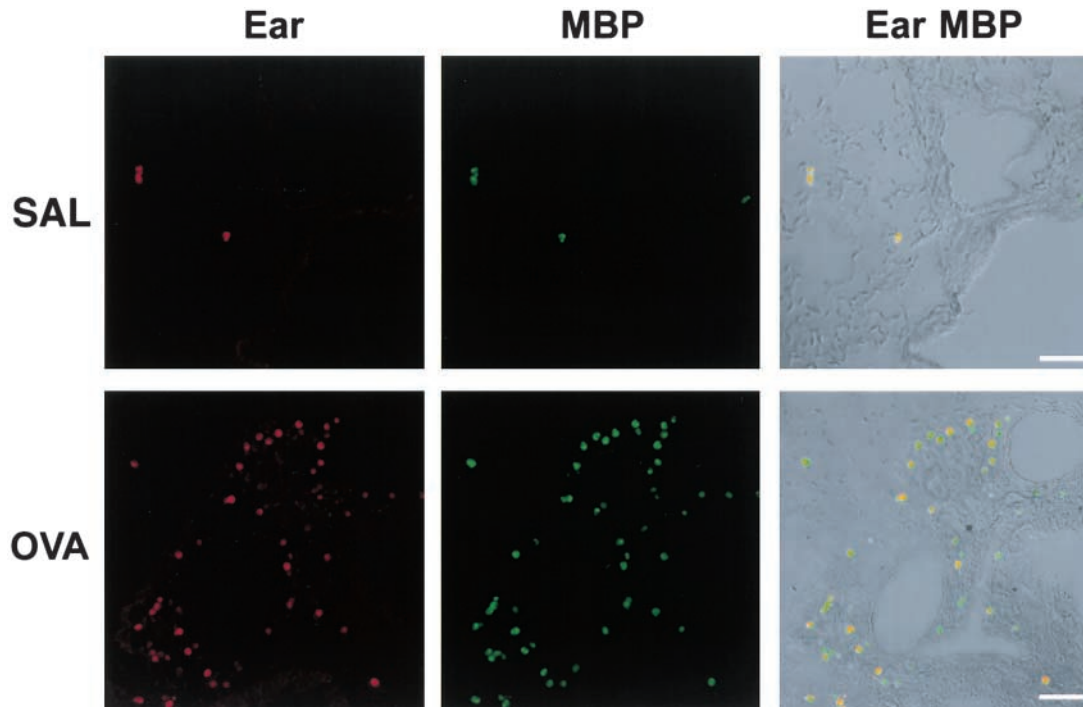


Figure 3. Ears remain sequestered in the resident pulmonary eosinophils of SAL- and OVA-sensitized/challenged mice. Confocal photomicrograph of representative lung sections of SAL- and OVA-treated mice that were subjected to immunofluorescent staining with an MBP-specific monoclonal antibody (green) and an Ear-specific monoclonal antibody (red). The overlay of the two staining patterns appears as yellow. Scale bar: 50 μ m.

cloned RT-PCR amplicons demonstrated transcript distribution patterns equivalent to those observed in the lungs of wild-type mice (data not shown). In contrast, the OVA-mediated induction of Ear11 transcript accumulation in the lung was not detected in mice deficient of either all T cells ($\alpha\beta^{-/-}$, $\gamma\delta^{-/-}$) or CD4⁺ cells (i.e., in addition to wild-type mice, Ear11 transcripts were present only in OVA-treated CD8^{-/-} animals).

Induction of Ear11 in the Lung Is Mediated by the T_H2 Cytokines IL-4 and/or IL-13, but Not IL-5

The dependence of Ear11 expression in the lung on the presence of T cells, particularly CD4⁺ cells, suggested that this expression was tightly linked to T_H2-associated inflammatory signals and perhaps T_H2 cytokines themselves. Consistent with this hypothesis, intratracheal administration of either IL-4 or IL-13 into naive mice resulted in the induction of the Ear11 gene independent of OVA sensitization/challenge (Figure 6A). Interestingly, RT-PCR of RNA isolated from the lungs of OVA-sensitized/challenged IL-5^{-/-} mice demonstrated that OVA-induced expression of Ear11 is not dependent on IL-5 and occurs in the absence of this cytokine (Figure 6B).

IL-13 Elicits *De Novo* Expression of Ear11 in Alveolar Macrophages of Naive Mice

Administration (intratracheally) of either IL-4 or IL-13 was necessary and sufficient for the induction of Ear11 gene

expression in naive mice. *In situ* hybridization of lung sections following instillation of either cytokine showed that, similar to OVA sensitization/challenge, alveolar macrophages were the cellular source of the induced Ear11 transcript accumulation in the lung (data not shown). These data were confirmed using RT-PCR of total RNA isolated from purified alveolar macrophages from these mice (Figure 7A). As observed in saline- and OVA-treated mice, Ear1 and Ear2 transcripts were detected in alveolar macrophages recovered from both IL-13- and vehicle (0.1% BSA in PBS)-treated mice. Ear11 transcript accumulation, however, occurred only in macrophages isolated from the lungs of mice administered intratracheal IL-13. Moreover, the induction of Ear expression was also accompanied by an increase in RNase activity in the lungs of IL-13-treated mice (Figure 7B). Significantly, the magnitude of the increase (1.7-fold) in RNase activity (relative to vehicle/saline controls) was comparable to the increase observed in the BALF of OVA-treated wild-type mice (Figure 2 versus Figure 7B).

Discussion

The identification of a conserved subfamily of RNases (i.e., Ears) that were abundant components of the proteins stored in mouse eosinophil secondary granules had led to the hypothesis that these proteins were eosinophil-specific mediators of effector function requiring RNase activity(ies) (23, 25). In particular, the concomitant pulmonary eosinophilia

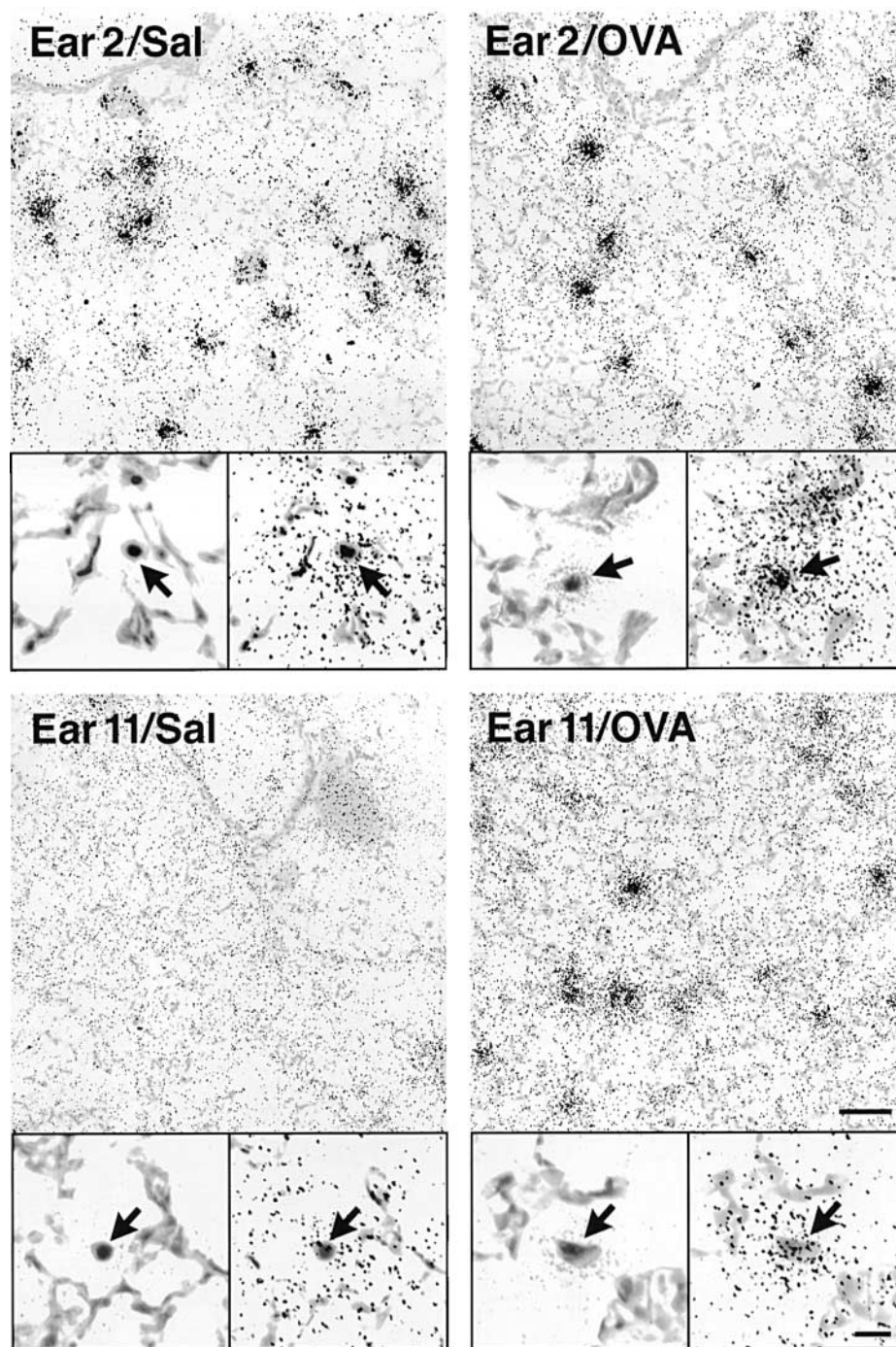


Figure 4. Alveolar macrophages are the source of Ear transcripts at both homeostatic baseline and following OVA sensitization/challenge. Lung sections from SAL- and OVA-treated mice were subjected to *in situ* hybridization using ^{35}S -labeled probes for Ear2 and Ear11 (counterstained with hematoxylin/eosin). This figure shows representative parasagittal sections that include conducting airways and alveolar spaces. The *insert photographs* in each panel are representative examples of the positively staining macrophages identified in these tissues. Lung sections from SAL- versus OVA-treated mice demonstrate that Ear1/Ear2 transcripts are present in the resident macrophages of both naive (i.e., SAL) and OVA-treated mice. In comparison, the data show that Ear 11 transcripts are also restricted to alveolar macrophages; however, these transcripts were present only in OVA-treated mice. Scale bar: 50 μm . *Inset:* Arrows denote alveolar macrophages hybridizing to Ear probes. Scale bar: 10 μm .

occurring following allergen provocation suggested that Ears may have a role(s) in the onset/progression of allergic respiratory inflammation. Moreover, the recent demonstration of the viricidal activities associated with the human Ears, EDN and ECP, further suggested that eosinophil recruitment to the lung may represent a host defense mechanism against single-stranded RNA viruses such as RSV (29, 40).

The concurrent increases in BAL RNase activity and eosinophil recruitment following OVA sensitization/chal-

lenge in mice provide a significant correlative relationship in support of pulmonary eosinophils (i.e., Ears sequestered in secondary granules) as the source of airway RNase activity. However, several observations suggest that alveolar macrophages are also a significant source of Ears in the lung: (i) RT-PCR analyses demonstrated that the Ear gene expression in the lungs of naive mice (25) correlating with the presence of airway RNase activity occurred despite an exceedingly small resident eosinophil population in the lungs of these mice; (ii) *in situ* hybridization using Ear gene

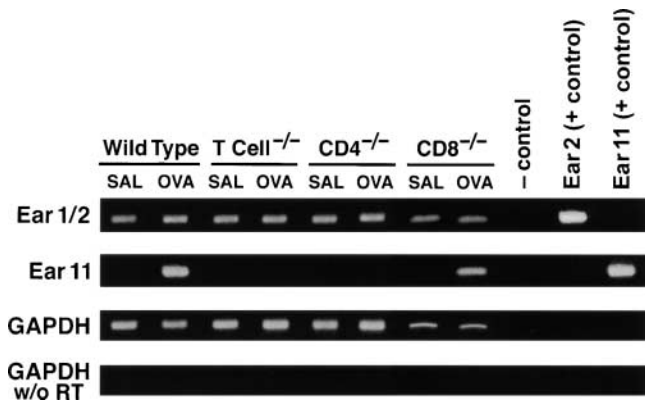


Figure 5. CD4⁺ T lymphocytes are required for OVA-induced Ear11 gene expression in the lung. Assessment of Ear gene expression in the lungs of SAL- and OVA-treated wild-type, T cell- ($\alpha\beta^{-/-}/\gamma\delta^{-/-}$), CD4⁺ cell-, or CD8⁺ cell-deficient mice were performed using RT-PCR and primer sets specific for the Ear1/Ear2 or Ear11 subfamilies. Control reactions using Ear2 and Ear11 cDNA clones as template are included to verify the specificity of the primer sets and a GAPDH primer set is included as a control for the reverse transcription reactions.

probes demonstrated constitutive expression of several Ear genes in resident alveolar macrophages, and not eosinophils, of naive mice. Moreover, allergen provocation led to the enhanced accumulation of Ear2 transcripts and the *de novo* appearance of Ear11 transcripts uniquely in alveolar macrophages of OVA-sensitized mice; (iii) Ear transcripts were identified in alveolar macrophages isolated from mice, including the constitutive presence of Ear1/Ear2 transcripts and the OVA-induced appearance of the Ear11 transcripts; (iv) the expression of Ear genes, including the *de novo* appearance of Ear11 following OVA sensitization/challenge, occurred in the absence of IL-5 and the associated absence of an allergen-induced pulmonary eosinophilia; (v) studies of gene knockout mice deficient of several abundant eosinophil secondary granule proteins (e.g., MBP [41] or EPO [41]), as well as immunohistochemistry studies using Ear and MBP-specific antibodies, demonstrated that a significant amount of eosinophil degranulation does not occur as a consequence of OVA provocation; thus, although eosinophils may sequester significant amounts of several Ear proteins, these proteins are not necessarily released following allergen provocation; and (vi) repeated attempts to detect Ear11 transcripts or protein in purified mouse eosinophils have been futile (unpublished data). Collectively, these data indicate that although eosinophils are capable of expressing Ear genes, and even storing Ear proteins in their secondary granules, the eosinophil does not seem capable of releasing these proteins into the airway lumen (i.e., degranulation). Instead, these data provide a significant and definitive body of evidence that alveolar macrophages express Ear genes and implicates the alveolar macrophage as an alternative, and likely significant, source of pulmonary Ear expression in the mouse.

The appearance and accumulation of Ear11 transcripts in alveolar macrophages from naive mice administered either

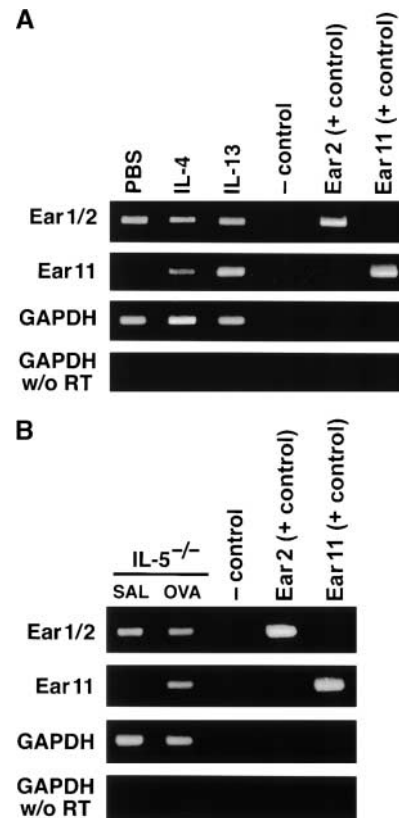


Figure 6. Pulmonary expression of the Ear11 gene in the lung is mediated by the T_H2 cytokines IL-4 and IL-13, but not IL-5. (A) Assessments of Ear gene expression in the lungs of naive mice following intratracheal administration of IL-4 or IL-13 were performed using RT-PCR and primer sets specific for Ear1/Ear2 or Ear11. (B) RT-PCR reactions of total RNA isolated from the lungs of SAL- versus OVA-treated IL-5^{-/-} mice demonstrate that the expression of Ear1/Ear2, as well as the OVA-induced expression of Ear11, is not dependent on the presence of IL-5. Control reactions using Ear2 and Ear11 cDNA clones as template are included to verify the specificity of the primer sets and a GAPDH primer set is included as a control for the reverse transcription reactions.

IL-4 or IL-13 suggests that allergen-induced Ear11 expression is mediated by one or more receptors recognizing these cytokines, including the IL-4/IL-13 common receptor IL-4R α / γ_c (42) as well as potentially unique IL-13 specific receptors (43, 44). The presence of IL-4R α receptors on macrophages is consistent with this hypothesis (42, 45, 46) and suggests that in addition to previously demonstrated changes in T_H2-activated inflammatory macrophages, including an increased capacity for endocytic clearance and changes in cytokine expression profiles (45), IL-4/IL-13 elicits *de novo* changes in gene expression that lead to the release of Ear proteins into the airway lumen. Furthermore, it appeared that only a subset of alveolar macrophages expressed Ear11 in response to IL-4/IL-13, demonstrating that IL-4/IL-13 mediate pulmonary changes that are not ubiquitous. The random spatial distribution of Ear11-expressing macrophages within the lung suggests that additional

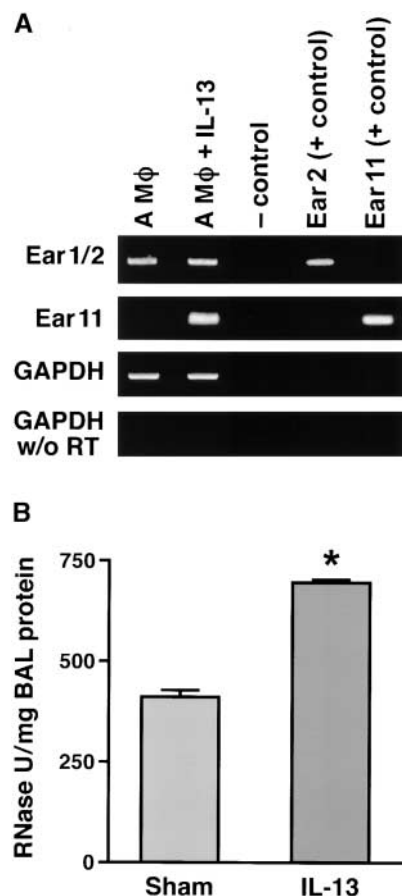


Figure 7. Expression of Ear11 occurs in alveolar macrophages recovered from naive mice following intratracheal instillation of IL-13. (A) RT-PCR reactions of total RNA isolated from alveolar macrophages of vehicle- or IL-13-treated mice using primer sets specific for Ear1/Ear2 or Ear11. Ear2, Ear11, and GAPDH controls are also shown. (B) Airway lumen RNase activity increases significantly in response to IL-13 instillation. Data are expressed as the means $\pm$ SEM ($n = 5$ mice/group). * $P < 0.05$.

immune microenvironmental cues are likely required for expression of Ear11.

The expression of Ear1/Ear2 in apparently all alveolar macrophages suggests that these RNases are a part of a mechanism(s) by which lung homeostasis is maintained. The enhanced accumulation of Ear2 transcripts and the *de novo* appearance of mRNAs representing Ear11 following allergen provocation may be necessary to regain lung homeostasis. Alternatively, the increased presence of Ears, including the appearance of a unique RNase (i.e., Ear11) may be serving a novel and possibly unknown function in the lung. A “housekeeping” explanation for Ear expression is summarized by a scavenger hypothesis: *Ears are expressed by alveolar macrophages as a clearance pathway degrading total RNA released from dying epithelial cells or from microorganisms attempting to colonize the lung.* In this hypothesis, nominal, yet constitutive, expression of Ear1/Ear2 is necessary to remove ever-present cellular debris that would otherwise accumulate and block the airway lumen. In addition,

the inflammation and epithelial cell death associated with pulmonary allergen challenge would necessitate the expansion of this pathway (i.e., the OVA-induced increase of Ear2 transcript accumulation and the appearance of Ear11 transcripts). An extrapolation of this hypothesis is that any destructive pulmonary provocation would necessitate increased Ear gene expression. However, the induction of pulmonary Ear expression appears limited to allergen challenge and does not occur following exposure to LPS (data not shown), suggesting the unlikelihood of a nonspecific clearance mechanism.

The recent demonstration that the human Ear genes, ECP and EDN, are capable of killing single-stranded RNA viruses (e.g., RSV) *in situ* (29, 30, 40) provides a provocative rationale for the expression of Ears in the airway lumen: alveolar macrophage-derived Ear expression is an innate host defensive mechanism against respiratory viral infection. Thus, the constitutive, yet regulated, expression of Ear1/Ear2 provides an organ-specific innate defense against low-level viral exposure. In this model, IL-4/IL-13-mediated expression of Ear11 during allergen challenge may occur as a consequence of eliciting immune pathways that are common with acquired host defense mechanisms against certain viral infections. A logical extension of this hypothesis is that viral infection would also induce pulmonary Ear gene expression and, indeed, our preliminary experiments using a viral model in the mouse demonstrates that RSV infection leads to the *de novo* expression of Ear11 by alveolar macrophages (S. A. Cormier and coworkers, unpublished results). It is noteworthy that the “scavenger hypothesis” and the “antiviral model” are not mutually exclusive and that antiviral functions of Ears may be a selectively advantageous adaptation of an airway clearance mechanism that had originally evolved to restore pulmonary homeostasis following viral infections.

The evolution of a non-eosinophil source of Ears in the lungs of mice (i.e., alveolar macrophages) is not necessarily a common mammalian lung-specific defense and, instead, may be a rodent-specific mechanism that has occurred as a consequence of differences in eosinophil effector functions between mammalian species. For example, in humans, eosinophil degranulation is associated with pulmonary provocation (47–49), thereby providing a strategically important delivery mechanism for large quantities of Ear proteins to the airway lumen. In the mouse, however, overt degranulation of eosinophils in the lung following provocation is not observed (37, 41, 49, 50). Thus, if the antiviral and/or clearance functions provided by airway Ears is selectively advantageous in both species, the mouse may have evolved an alternative strategy to provide these proteins in light of adaptive pressures associated with changing eosinophil effector functions. The evolutionary origin(s) of leukocyte-derived Ear gene expression itself remain(s) equally unclear. The induced expression of specific RNases in human (51, 52) and mouse macrophages (51, 52), as well as eosinophils from both species (23, 25, 29, 40, 53, 54), suggests either that expression evolved independently in multiple leukocytes or occurred early in evolution (i.e., predating the origin of leukocytic subtypes) and has since been retained in multiple cell types. Irrespective of defining these origins,

the retention of these specific RNases in macrophages and eosinophils of divergent mammalian species suggests that there are selective advantages to effector functions mediated by both cell types. The issue that remains is whether the RNase-mediated effector functions in each leukocyte are common, potentially redundant, activities or whether they represent unique leukocyte-specific mechanisms that each contribute to the maintenance of pulmonary homeostasis.

Acknowledgments: The authors wish to thank Dr. Thomas Colby (Mayo Clinic Scottsdale) for his help with pulmonary cell identification and lung histology. They also thank Linda Mardel and Jennifer Ford for assistance in manuscript preparation. They acknowledge the wonderful support of the Graphics Core Facilities (Marv Ruona) and the Histology Core (Lisa Barbarisi) Facilities of the Mayo Clinic Scottsdale. Special thanks also go to Dr. Michael McGarry for critical reading and helpful discussions of this manuscript. The authors would also like to thank the extraordinary efforts of J.M.J. in the development of this paper. This work was supported by the Mayo Foundation and the National Institutes of Health (F32 AR08545 to S.A.C., F32 HL10176 to J.R.C., and R01 HL58723 to N.A.L. and supporting D.D.).

References

- Kon, O. M., and A. B. Kay. 1999. T cells and chronic asthma. *Int. Arch. Allergy Immunol.* 118:133-135.
- Gavett, S. H., X. Chen, F. Finkelman, and M. Wills-Karp. 1994. Depletion of murine CD4⁺ T lymphocytes prevents antigen-induced airway hyper-reactivity and pulmonary eosinophilia. *Am. J. Respir. Cell Mol. Biol.* 10:587-593.
- Walker, C., E. Bode, L. Boer, T. T. Hansel, K. Blaser, and J. Virchow, Jr. 1992. Allergic and nonallergic asthmatics have distinct patterns of T-cell activation and cytokine production in peripheral blood and bronchoalveolar lavage. *Am. Rev. Respir. Dis.* 146:109-115.
- Robinson, D., Q. Hamid, A. Bentley, S. Ying, A. B. Kay, and S. R. Durham. 1993. Activation of CD4⁺ T cells, increased TH2-type cytokine mRNA expression, and eosinophil recruitment in bronchoalveolar lavage after allergen inhalation challenge in patients with atopic asthma. *J. Allergy Clin. Immunol.* 92:313-324.
- Kay, A. B. 1997. T cells as orchestrators of the asthmatic response. *Ciba Found. Symp.* 206:56-67.
- Till, S., S. Durham, R. Dickason, D. Huston, J. Bungre, S. Walker, D. Robinson, A. B. Kay, and C. Corrigan. 1997. IL-13 production by allergen-stimulated T cells is increased in allergic disease and associated with IL-5 but not IFN- γ expression. *Immunology* 91:53-57.
- Bochner, B. S., B. J. Udem, and L. M. Lichtenstein. 1994. Immunological aspects of allergic asthma. *Annu. Rev. Immunol.* 12:295-335. (Review)
- Wills-Karp, M., J. Luyimbazi, X. Xu, B. Schofield, T. Y. Neben, C. L. Karp, and D. D. Donaldson. 1998. Interleukin-13: central mediator of allergic asthma. *Science* 282:2258-2261. (see comments)
- Tomassini, M., A. Tscipoulos, P. C. Tai, V. Gruart, A. B. Tonnel, L. Prin, A. Capron, and M. Capron. 1991. Release of granule proteins by eosinophils from allergic and nonallergic patients with eosinophilia on immunoglobulin-dependent activation. *J. Allergy Clin. Immunol.* 88:365-375.
- Wardlaw, A. J. 1994. Eosinophils in the 1990s: new perspectives on their role in health and disease. *Postgrad. Med. J.* 70:536-552. (Review)
- Corrigan, C. J., and A. B. Kay. 1992. T cells and eosinophils in the pathogenesis of asthma. *Immunol. Today* 13:501-507. (Review)
- Cline, M. J., J. Hanifin, and R. I. Lehrer. 1968. Phagocytosis by human eosinophils. *Blood* 32:922-934.
- Yazdanbakhsh, M., C. M. Eckmann, A. A. Bot, and D. Roos. 1986. Bactericidal action of eosinophils from normal human blood. *Infect. Immun.* 53:192-198.
- Butterworth, A. E. 1984. Cell-mediated damage to helminths. *Adv. Parasitol.* 23:143-235. (Review)
- Butterworth, A. E., R. F. Sturrock, V. Houba, A. A. Mahmoud, A. Sher, and P. H. Rees. 1975. Eosinophils as mediators of antibody-dependent damage to schistosomula. *Nature* 256:727-729.
- Anwar, A. R., S. R. Smithers, and A. B. Kay. 1979. Killing of schistosomula of *Schistosoma mansoni* coated with antibody and/or complement by human leukocytes in vitro: requirement for complement in preferential killing by eosinophils. *J. Immunol.* 122:628-637.
- Abu-Ghazaleh, R. I., G. J. Gleich, and F. G. Prendergast. 1992. Interaction of eosinophil granule major basic protein with synthetic lipid bilayers: a mechanism for toxicity. *J. Membr. Biol.* 128:153-164.
- Henderson, W. R., Jr., D. B. Lewis, R. K. Albert, Y. Zhang, W. J. Lamm, G. K. Chiang, F. Jones, P. Eriksen, Y. T. Tien, M. Jonas, and E. Y. Chi. 1996. The importance of leukotrienes in airway inflammation in a mouse model of asthma. *J. Exp. Med.* 184:1483-1494.
- Woerly, G., N. Roger, S. Loiseau, and M. Capron. 1999. Expression of Th1 and Th2 immunoregulatory cytokines by human eosinophils. *Int. Arch. Allergy Immunol.* 118:95-97.
- Wu, W., M. K. Samoszuk, S. A. Comhair, M. J. Thomassen, C. F. Farver, R. A. Dweik, M. S. Kavuru, S. C. Erzurum, and S. L. Hazen. 2000. Eosinophils generate brominating oxidants in allergen-induced asthma. *J. Clin. Invest.* 105:1455-1463.
- Iijima, H., A. Duget, Q. Hamid, and D. H. Eidelman. 2000. Nitrotyrosine formation and eosinophils in a murine model of allergic asthma. *Am. J. Respir. Crit. Care Med.* 161:A834. (Abstr.)
- Gleich, G. J., D. A. Loegering, M. P. Bell, J. L. Checkel, S. J. Ackerman, and D. J. McKean. 1986. Biochemical and functional similarities between human eosinophil-derived neurotoxin and eosinophil cationic protein: homology with ribonuclease. *Proc. Natl. Acad. Sci. USA* 83:3146-3150.
- Larson, K. A., E. V. Olson, B. J. Madden, G. J. Gleich, N. A. Lee, and J. J. Lee. 1996. Two highly homologous ribonuclease genes expressed in mouse eosinophils identify a larger subgroup of the mammalian ribonuclease superfamily. *Proc. Natl. Acad. Sci. USA* 93:12370-12375.
- Zhang, J., K. D. Dyer, and H. F. Rosenberg. 2000. Evolution of the rodent eosinophil-associated RNase gene family by rapid gene sorting and positive selection. *Proc. Natl. Acad. Sci. USA* 97:4701-4706.
- Cormier, S. A., K. A. Larson, S. Yuan, T. L. Mitchell, K. Lindenberg, P. Carrigan, N. A. Lee, and J. J. Lee. 2001. Mouse Eosinophil-associated ribonucleases: a unique subfamily expressed during hematopoiesis. *Mamm. Genome* 12:352-361.
- Gleich, G. J., C. R. Adolphson, and K. M. Leiferman. 1993. The biology of the eosinophilic leukocyte. *Annu. Rev. Med.* 44:85-101. (Review)
- Abu-Ghazaleh, R. I., H. Kita, and G. J. Gleich. 1992. Eosinophil activation and function in health and disease. *Immunol. Ser.* 57:137-167. (Review)
- Venge, P. 1993. Human eosinophil granule proteins: structure, function and release. In H. Smith and R. M. Cook, editors. *Immunopharmacology of Eosinophils, The Handbook of Immunopharmacology*. Academic Press, Epsom, Surrey, UK. 43-55.
- Domachowski, J. B., K. D. Dyer, C. A. Bonville, and H. F. Rosenberg. 1998. Recombinant human eosinophil-derived neurotoxin/RNase 2 functions as an effective antiviral agent against respiratory syncytial virus. *J. Infect. Dis.* 177:1458-1464.
- Domachowski, J. B., C. A. Bonville, K. D. Dyer, and H. F. Rosenberg. 1998. Evolution of antiviral activity in the ribonuclease A gene superfamily: evidence for a specific interaction between eosinophil-derived neurotoxin (EDN/RNase 2) and respiratory syncytial virus. *Nucleic Acids Res.* 26:5327-5332.
- Olaszewska-Pazdrak, B., K. Pazdrak, P. L. Ogra, and R. P. Garofalo. 1998. Respiratory syncytial virus-infected pulmonary epithelial cells induce eosinophil degranulation by a CD18-mediated mechanism. *J. Immunol.* 160:4889-4895.
- McDevitt, A. L., M. S. Deming, H. F. Rosenberg, and K. D. Dyer. 2001. Gene structure and enzymatic activity of mouse eosinophil-associated ribonuclease 2. *Gene* 267:23-30.
- Mombaerts, P., A. R. Clarke, M. A. Rudnicki, J. Iacomini, S. Itoharu, J. J. Lafaille, L. Wang, Y. Ichikawa, R. Jaenisch, M. L. Hooper, and S. Tonegawa. 1992. Mutations in T-cell antigen receptor genes alpha and beta block thymocyte development at different stages. *Nature* 360:225-231. [Published erratum appears in *Nature* 360(6403):491]
- McCarrick, J. W., J. R. Parnes, R. H. Seong, D. Solter, and B. B. Knowles. 1993. Positive-negative selection gene targeting with the diphtheria toxin A-chain gene in mouse embryonic stem cells. *Transgenic Res.* 2:183-190.
- Fung-Leung, W. P., M. W. Schilham, A. Rahemtulla, T. M. Kundig, M. Vollenweider, J. Potter, W. van Ewijk, and T. W. Mak. 1991. CD8 is needed for development of cytotoxic T cells but not helper T cells. *Cell* 65:443-449.
- Kopf, M., F. Brombacher, P. D. Hodgkin, A. J. Ramsay, E. A. Milbourne, W. J. Dai, K. S. Ovington, C. A. Behm, G. Kohler, I. G. Young, and K. I. Matthaei. 1996. IL-5-deficient mice have a developmental defect in CD5⁺ B-1 cells and lack eosinophilia but have normal antibody and cytotoxic T cell responses. *Immunity* 4:15-24.
- Denzler, K. L., S. C. Farmer, J. R. Crosby, M. T. Borchers, G. Cieslewicz, K. A. Larson, S. Cormier-Regard, N. A. Lee, and J. J. Lee. 2000. Eosinophil major basic protein-1 does not contribute to allergen-induced airway pathologies in mouse models of asthma. *J. Immunol.* 165:5509-5517.
- Lee, J. J., M. P. McGarry, S. C. Farmer, K. L. Denzler, K. A. Larson, T. Carrigan, I. E. Brenneise, M. A. Horton, A. Haczku, E. W. Gelfand, G. D. Leikauf, and N. A. Lee. 1997. Interleukin-5 expression in the lung epithelium of transgenic mice leads to pulmonary changes pathognomonic of asthma. *J. Exp. Med.* 185:2143-2156.
- Lee, J. J., G. Radice, C. P. Perkins, and F. D. Costantini. 1992. Identification and characterization of a novel, evolutionarily conserved gene disrupted by the murine H β 58 embryonic lethal transgene insertion. *Development* 115:277-288.
- Domachowski, J. B., K. D. Dyer, A. G. Adams, T. L. Leto, and H. F. Rosenberg. 1998. Eosinophil cationic protein/RNase 3 is another RNase

- A-family ribonuclease with direct antiviral activity. *Nucleic Acids Res.* 26:3358–3363.
41. Denzler, K. L., M. T. Borchers, J. R. Crosby, G. Cieslewicz, E. M. Hines, J. P. Justice, S. A. Cormier, K. A. Lindenberg, W. Song, W. Wu, S. L. Hazen, G. J. Gleich, J. J. Lee, and N. A. Lee. 2001. Extensive eosinophil degranulation and peroxidase-mediated oxidation of airway proteins do not occur in a mouse ovalbumin-challenge model of pulmonary inflammation. *J. Immunol.* 167:1672–1682.
 42. Zurawski, G., and J. E. Devries. 1994. Interleukin-13, an Interleukin 4-like cytokine that acts on monocytes and B-cells, but not on T-cells. *Immunol. Today* 15:19–26. (Review)
 43. Schnyder, B., S. Lugli, N. Feng, H. Etter, R. A. Lutz, B. Ryffel, K. Sugamura, H. Wunderli-Allenspach, and R. Moser. 1996. Interleukin-4 (IL-4) and IL-13 bind to a shared heterodimeric complex on endothelial cells mediating vascular cell adhesion molecule-1 induction in the absence of the common gamma chain. *Blood* 87:4286–4295.
 44. Mattes, J., M. Yang, A. Siqueira, K. Clark, J. MacKenzie, A. N. McKenzie, D. C. Webb, K. I. Matthei, and P. S. Foster. 2001. IL-13 induces airways hyperreactivity independently of the IL-4R alpha chain in the allergic lung. *J. Immunol.* 167:1683–1692.
 45. Stein, M., S. Keshav, N. Harris, and S. Gordon. 1992. Interleukin 4 potently enhances murine macrophage mannose receptor activity: a marker of alternative immunologic macrophage activation. *J. Exp. Med.* 176:287–292.
 46. Feldman, G. M., and D. S. Finbloom. 1990. Induction and regulation of IL-4 receptor expression on murine macrophage cell lines and bone marrow-derived macrophages by IFN-gamma. *J. Immunol.* 145:854–859.
 47. Collins, D. S., R. Dupuis, G. J. Gleich, K. R. Bartemes, Y. Y. Koh, M. Pollice, K. H. Albertine, J. E. Fish, and S. P. Peters. 1993. Immunoglobulin E-mediated increase in vascular permeability correlates with eosinophilic inflammation. *Am. Rev. Respir. Dis.* 147:677–683.
 48. Metzger, W. J., D. Zavala, H. B. Richerson, P. Moseley, P. Iwamoto, M. Monick, K. Sjoerdsma, and G. W. Hunninghake. 1987. Local allergen challenge and bronchoalveolar lavage of allergic asthmatic lungs. Description of the model and local airway inflammation. *Am. Rev. Respir. Dis.* 135:433–440.
 49. Persson, C. G., and J. S. Erjefält. 1999. Degranulation in eosinophils in human, but not in mouse, airways. *Allergy* 54:1230–1232.
 50. Stelts, D., R. W. Egan, A. Falcone, C. G. Garlisi, G. J. Gleich, W. Kreutner, T. T. Kung, D. K. Nahrebne, R. W. Chapman, and M. Minnicozzi. 1998. Eosinophils retain their granule major basic protein in a murine model of allergic pulmonary inflammation. *Am. J. Respir. Cell Mol. Biol.* 18:9463–9470.
 51. Egesten, A., K. D. Dyer, D. Batten, J. B. Domachowske, and H. F. Rosenberg. 1997. Ribonucleases and host defense: identification, localization and gene expression in adherent monocytes in vitro. *Biochim. Biophys. Acta* 1358:255–260.
 52. Rosenberg, H. F., and K. D. Dyer. 1996. Molecular cloning and characterization of a novel human ribonuclease (RNase k6): increasing diversity in the enlarging ribonuclease gene family. *Nucleic Acids Res.* 24:3507–3513.
 53. Rosenberg, H. F. 1995. Recombinant human eosinophil cationic protein - ribonuclease activity is not essential for cytotoxicity. *J. Biol. Chem.* 270:7876–7881.
 54. Domachowske, J. B., and H. F. Rosenberg. 1997. Eosinophils inhibit retroviral transduction of human target cells by a ribonuclease-dependent mechanism. *J. Leukoc. Biol.* 62:363–368.