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Am J Physiol Gastrointest Liver Physiol 302:G645-G654, 2012. First published 29 December 2011;
doi: 10.1152/ajpgi.00223.2011

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Significance of para-esophageal lymph nodes in food or aeroallergen-induced iNKT cell-mediated experimental eosinophilic esophagitis

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Submitted 7 June 2011; accepted in final form 28 December 2011

Rajavelu P, Rayapudi M, Moffitt M, Mishra A, Mishra A. Significance of para-esophageal lymph nodes in food or aeroallergen-induced iNKT cell-mediated experimental eosinophilic esophagitis. *Am J Physiol Gastrointest Liver Physiol* 302: G645–G654, 2012. First published December 29, 2011; doi:10.1152/ajpgi.00223.2011.—Eosinophilic esophagitis (EoE) is a recently recognized inflammatory disorder driven by food hypersensitivity; however, the specific foods and mechanisms involved are unclear. In patients with EoE, we have found that hypersensitivities to corn and peanuts are the most common. Accordingly, we sensitized and exposed mice either intranasally or intragastrically with corn or peanut extract or saline. Esophageal eosinophilia, the genes of eosinophil-directed cytokines, and allergen-induced antibodies were examined in mice challenged with corn or peanut extract or saline. A high number of esophageal lamina propria eosinophils as well as eosinophilic microabscesses, intraepithelial eosinophils, extracellular eosinophilic granules, thickened and disrupted epithelial mucosa, and mast cell hyperplasia were observed in the esophagus of peanut or corn allergen-challenged mice. Mechanistic analysis indicated that para-esophageal lymph nodes might be critical in the trafficking of eosinophils to the esophagus and in EoE association to airway eosinophilia. Furthermore, experimentation with gene-targeted mice revealed that peanut allergen-induced EoE was dependent on eotaxin and invariant natural killer T (iNKT) cells, as CD1d and eotaxin-1/2 gene-deficient mice were protected from disease induction. Thus we provide evidence that para-esophageal lymph nodes are involved in food- or aeroallergen-induced eosinophilia and patchy EoE pathogenesis, likely a mechanism dependent on eotaxins and iNKT cells.

corn; eotaxin; invariant natural killer T cell; L α mutant mice; peanut

EOSINOPHILIC ESOPHAGITIS (EoE) is a recently recognized esophageal disorder of children and adults characterized by symptoms such as nausea, vomiting, abdominal pain, dysphagia, and food impaction that occur in conjunction with esophageal eosinophilia (24); however, the etiology of EoE is not clearly understood. Several clinical reports suggest that food allergy may be the cause of EoE pathogenesis (13, 35, 36). Most patients with EoE respond to an elemental diet with resolution

of symptoms and normalization of biopsies (39); however, reintroduction of foods often causes esophageal eosinophilia to return (40). The most common food allergies identified were to eggs, soy, chicken, beef, wheat, rice, barley, peas, corn, and peanuts (39, 40). Clinical reports indicate that peanuts or other nuts account for ~80% of fatal food hypersensitivity reactions (5, 38), and the combined skin prick and patch-test-positive dataset of the Cincinnati Center for Eosinophilic Disorders (CCED) indicates that patients with EoE most commonly have pea, peanut, and corn sensitivities (data not yet published). In humans, elimination and reintroduction diets demonstrate the causative nature of foods in EoE, as does intragastric ovalbumin (OVA) inducing EoE in mice (14, 40); however, the role and mechanism of food allergen-induced EoE is not yet clearly understood.

EoE is an allergen-induced Th2 cytokine-mediated disease (26, 28); however, it is not yet well understood which subset of T cells is critical in the initiation and progression of disease pathogenesis. Previously, we reported that T cell-deficient mice are protected from the induction of EoE but that CD4 and CD8 gene-deficient mice induce EoE following allergen challenge (29). Recently, we showed that IL-15 has a significant role in promoting EoE in mice (46). Induced levels of IL-15 are reported in the blood and esophagus of food allergic patients, including those with EoE (33, 46). Notably, IL-15 is a growth and survival factor for invariant natural killer T (iNKT) cells (46), and more recently, we found that a high number of the T cells accumulated in the esophageal mucosa of human EoE are iNKT cells (P. Rajavelu and A. Mishra unpublished data). Therefore, this study is focused to understand the mechanistic aspects of corn- and peanut-induced EoE pathogenesis, including the role of iNKT cells and the significance of local esophageal lymph nodes. Herein, we report that iNKT cell-mediated responses and local esophageal lymph nodes are mechanistically critical in associating airway eosinophilia to EoE pathogenesis.

MATERIALS AND METHODS

Mice. Specific pathogen-free BALB/c and L α mutant mice were obtained from Jackson Laboratory (Bar Harbor, ME). CD1d-deficient (BALB/c) mice (44) were procured from Jochen Mattner, of the Division of Immunobiology, Cincinnati Children's Hospital Medical Center. Eotaxin-1/2 gene-deficient mice were generated in our laboratory (31). All of the experiments were performed on age- and

* P. Rajavelu and M. Rayapudi contributed equally to this work.

** M. Moffitt and Akanksha Mishra are NIH ARRA summer students and initial study contributors.

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sex-matched mice 6–8 wk of age. The mice were maintained in a pathogen-free barrier facility, and animals were handled according to institutional animal care and use committee (IACUC) guidelines with all protocols approved by the IACUC.

Experimental EoE induction. A mouse model of corn or peanut allergen-induced EoE was established using the following protocol. Mice were lightly anesthetized with isoflurane (Iso-Flo; Abbott Laboratories, North Chicago, IL) and sensitized with 200 µg of corn or peanut extract (Greer Laboratories, Lenoir, NC) with 1 mg of alum on day 0 and day 14 by intraperitoneal (IP) injection. On day 21, mice were divided into four groups. Two groups were treated intranasally with 100 µg (50 µl) of corn or peanut extract (Greer Laboratories) or 50 µl of normal saline alone on days 21, 23, and 25 using a micropipette and were euthanized 20–24 h after the last allergen or saline challenge. The other two groups were treated orally or intragastrically with 100 µg (100 µl) purified corn or peanut extract (Greer Laboratories) or 100 µl of normal saline alone on days 21, 23, 25, 27, 29, 31, 33, and 35 and were euthanized on day 36 20–24 h after the last allergen or saline challenge. To avoid high allergen burden in the stomach and reflux, we administered a low dose of peanut extract compared with a number of previously published reports. LPS concentration in peanut and corn extract was measured using Lonza LAL QCL-1000 (cat. no. 50–647U; Lonza, Walkersville, MD) product following the manufacturer's provided protocol. The LPS contamination range for peanut and corn allergen extract was between 0.9 and 1.4 ng/ml. This concentration indicates that mice were administered ~0.09–0.14 ng of LPS per challenge. This low amount of LPS will not affect our present hypothesis because LPS mostly induces Th1 responses, not Th2 responses (8).

Conjugation of *Aspergillus* allergen to Alexafluor 488 dye. The conjugation of Alexafluor 488 dye and *Aspergillus* antigen was performed per the manufacturer's protocol. Alexafluor488-conjugated antigen (100 µg in 25 µl) or 25 µl saline were given intratracheally to the mice per our earlier reported protocol (29). Mice were euthanized 8 h after saline or Alexafluor488-conjugated allergen administration. The lung, mediastinal lymph node, and esophagus were surgically removed, and their cells were isolated per the protocol described earlier (45). Flow cytometric (FCM) analysis was performed to detect the Alexafluor488-conjugated antigen in the cells isolated from these organs.

Eosinophil analysis in the esophagus. The 5-µm, esophageal paraffin tissue sections were immunostained with antiserum against mouse eosinophil major basic protein (anti-MBP) as previously described (23, 27). In brief, endogenous peroxide in the tissue was quenched with 0.3% hydrogen peroxide in methanol followed by nonspecific protein blocking with normal goat serum. Tissue sections were then incubated with rat anti-MBP (1:2,000) overnight at 4°C, followed by incubations with a 1:200 dilution of biotinylated anti-rat IgG secondary antibody and then avidin-peroxidase complex (Vector Laboratories, Burlingame, CA) for 30 min each. These slides were further developed with nickel diaminobenzidine-cobalt chloride solution to form a black precipitate and counterstained with hematoxylin. Negative controls included replacing the primary antibody with normal rat serum.

Bronchoalveolar lavage fluid collection and analysis. Mice were euthanized by CO₂ inhalation. Immediately thereafter, a midline neck incision was made, and the trachea was cannulated. The lungs were lavaged two times with 1.0 ml of PBS containing 1% FCS and 0.5 mM EDTA. The recovered bronchoalveolar lavage fluid (BALF) was centrifuged at 400 g for 5 min at 4°C and resuspended in PBS containing 1% FCS and 0.5 mM EDTA. Total cell numbers were counted with a hemacytometer. Cytospin preparations of 5 × 10⁴ cells were stained with Giemsa-Diff-Quick (Dade Diagnostics, Aguada, PR), and differential cell counts were determined. The BALF eosinophil counts were expressed as an indication of lung eosinophilia.

Mast cell analysis. The 5-µm esophageal paraffin tissue sections were deparaffinized, stained with hexazonized new fuchsin (Sigma

Aldrich, St. Louis, MO) with 4% sodium nitrate in naphthol-AS-D chloroacetate (Sigma-Aldrich) and PBS solution for 30 min, and counterstained with hematoxylin. The histological analysis was performed using light microscopy.

Quantification of esophageal eosinophils and mast cells. Esophageal eosinophils and mast cells were quantified by counting the anti-MBP-positive and toluidine blue-positive cells respectively via digital morphometry using the Metamorph Imaging System (Universal Imaging Corp) and were expressed as eosinophils/mm² or mast cells/mm² as described previously (26, 28).

ELISA analysis of serum IgE. Total serum IgE levels were measured using a BD OptEIA ELISA set (BD Biosciences, San Diego, CA) per the manufacturer's protocol. Briefly, after 10% FBS blocked nonspecific protein binding, each mouse serum sample or purified mouse IgE sample was applied to an anti-mouse IgE monoclonal antibody-coated, 96-well ELISA plate (Immuron; DYNEX Technologies, Chantilly, VA). The plate was incubated for 2 h at room temperature and washed with 0.05% Tween-20 in PBS. Biotinylated anti-mouse IgE monoclonal antibody was applied to each well, followed by avidin-horseradish peroxidase conjugate reagent. Finally, tetramethylbenzidine substrate solution (BD Biosciences Pharmingen) was added to each well, and the ELISA plate reader was used to read the developed color. The IgE concentration of each sample was calculated by using a standard curve.

Real-time PCR analysis. The RNA samples (1 µg) were subjected to reverse transcription using iScript reverse transcriptase (Bio-Rad, Hercules, CA) according to the manufacturer's instructions. Messenger RNA (mRNA) levels of the eosinophil active cytokines (IL-5 and IL-13) and chemokines (eotaxin-1 and eotaxin-2) were quantified by real-time PCR using the LightCycler instrument and LightCycler FastStart DNA master SYBR green I as a ready-to-use reaction mix (Roche, Indianapolis, IN). SYBR green 1 mixture provided a single-peak dissociation curve. Results were normalized to β-actin amplified from the same cDNA mix and expressed as fold induction compared with the controls. cDNA were amplified using the primers listed in Table 1.

Statistical analysis. The nonparametric Mann-Whitney *U*-test was employed for comparison of data between two groups. Parametric data were compared using *t*-tests or ANOVA. Values are reported as means ± SD; *P* values <0.05 were considered statistically significant.

RESULTS

Characterization and significance of food allergen-induced model of EoE. A highly significant percent (40–50%) of patients with EoE show sensitivity to peanut and corn allergens (unpublished data of Cincinnati Center for Eosinophilic Disorder, Cincinnati Children's Hospital Medical Center); therefore, we tested the hypothesis that peanut and corn allergens promote experimental EoE. Mice were sensitized and challenged with peanut or corn extract per the schematic oral, intragastric, and intranasal allergen challenge protocol shown (Fig. 1, A and B). The corn- or peanut-sensitized mice chal-

Table 1. List of primer sequences

mIL-5	5'-TCCCATGAGCACAGTGGTGAAG 5'-CACAGTACCCCGACAGTTT
mIL-13	5'-CATGGCGCTCTGGGTGACTG 5'-CGGCCAGGTCCCACTCCATA
mEotaxin-1	5'-GGCTCACCCAGGCTCCATCC 5'-TTTTGGTCCAGGTGCTTTGTGG
mEotaxin-2	5'-CTCCTTCTCCTGGTAGCCTGC 5'-GTGATGAAGATGACCCCTGCCTT
mβ-Actin	5'-CGATGCCCTGAGGCTCTTTCC 5'-CATCCTGTCAGCAATGCCTGGG

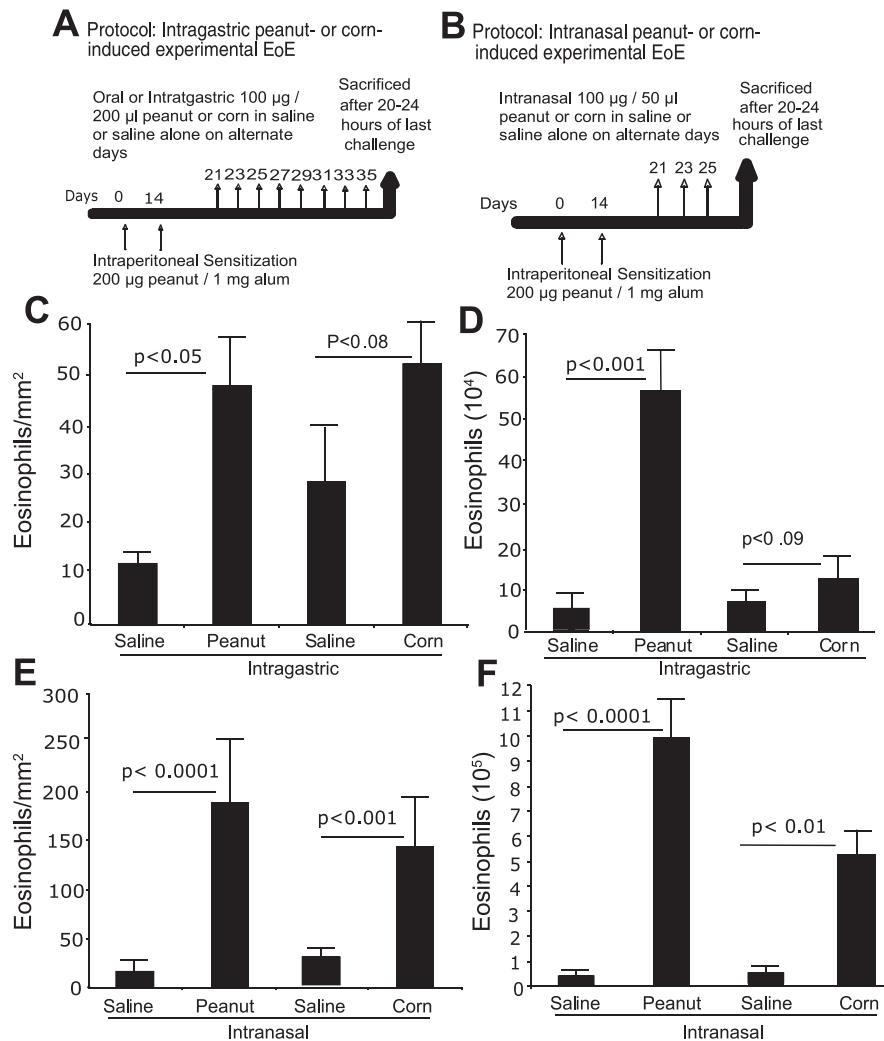


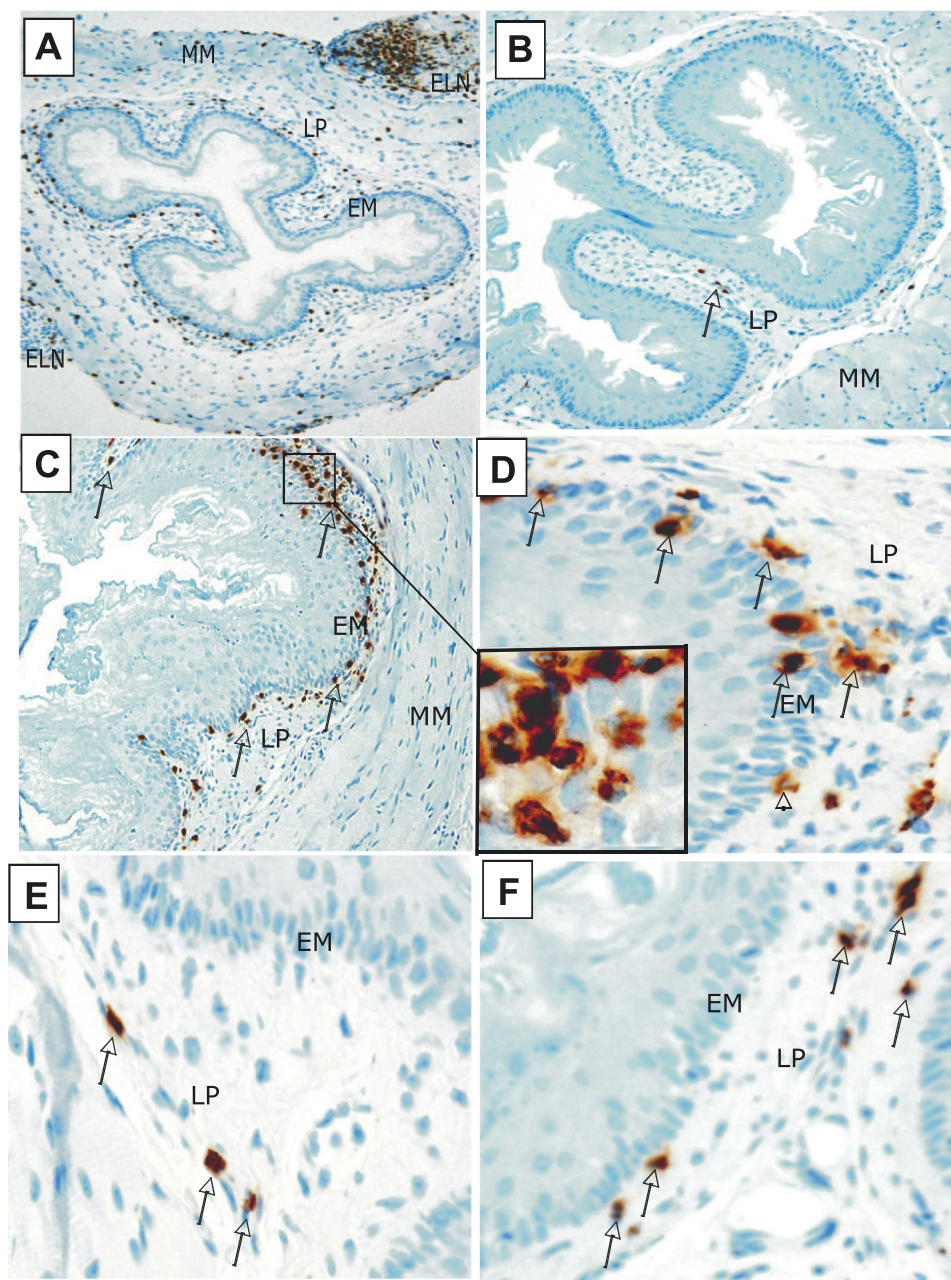
Fig. 1. Esophageal eosinophil quantification following intranasal or intragastric corn or peanut allergen exposure. Mice sensitized intraperitoneally with 200 μ g peanut or corn allergen with 1 mg alum and challenged orally or intragastrically or intranasally with 100 μ g of corn or peanut allergen were analyzed for the number of eosinophils in the lung and esophagus following the protocol shown (A, B). EoE, Eosinophilic esophagitis. The number of eosinophils in the esophagus (C, E) and bronchoalveolar lavage fluid (D, F) were analyzed from mice euthanized 20–24 h after the last saline or allergen challenge. Data are expressed as means $\pm$ SD, $n = 12$ mice/group.

lenged with the respective oral allergen did not show any eosinophil accumulation in the esophagus (data not shown). However, a significant increase in esophageal eosinophils was observed in mice sensitized and challenged intragastrically with peanut allergen, but not with corn allergen, compared with their respective saline-challenged mice (Fig. 1C). Notably, significant induction of esophageal eosinophilia in intragastrically challenged, allergen-sensitized mice but not in orally challenged, allergen-sensitized mice indicates that esophageal allergen exposure is not required for the induction of EoE and that some alternative mechanism is operational in promoting eosinophils in the esophagus. Interestingly, induced airway eosinophilia was observed in the intragastrically peanut allergen-sensitized and -challenged mice but not in mice sensitized and challenged intragastrically with corn (Fig. 1D) or orally with peanut or corn (data not shown). Orally administered allergen may lead to the development of tolerance and, therefore, not promote any eosinophilia in mice. These observations encouraged us to test the hypothesis that food allergen-induced EoE may be dependent on the magnitude of airway eosinophilia. This hypothesis would also explain the clinical observation that a number of food allergen-sensitive patients with EoE have coexisting asthma. Accordingly, to test this hypothesis and understand the mechanism of food allergen-induced

EoE, we induced airway eosinophilia by intranasal challenge with corn or peanut allergen of allergen-sensitized mice. Of note, intranasal delivery of both corn and peanut allergen promoted a significantly higher level of esophageal and airway eosinophilia (Fig. 1, E and F).

Para-esophageal lymph nodes have a critical role in inducing food allergen-induced EoE. We also hypothesized that para-esophageal lymph nodes may have a critical role in the trafficking of airway eosinophils to the esophagus. Para-esophageal lymph nodes are anatomically attached to the trachea. Therefore, we examined local esophageal lymph nodes for eosinophil accumulation in allergen-sensitized mice intranasally challenged with peanut or corn allergen. In these mice, local lymph nodes accumulated a large number of eosinophils along with a high number of eosinophils in the muscularis mucosa, lamina propria, and esophageal epithelial mucosa, a characteristic feature of EoE (Fig. 2A). Anatomical localization of esophageal eosinophils in these mice revealed that eosinophils were accumulated in all the regions of the esophagus including the epithelial and muscularis mucosa. Herein, we show eosinophil localization only in the peanut allergen-challenged mice because the response to peanut or corn allergen in terms of anatomical esophageal eosinophil localization was comparable. A small number of lamina propria eosinophils

Fig. 2. Eosinophil analysis in the esophageal lymph node and esophageal mucosa following saline or peanut allergen-induced experimental eosinophilic esophagitis (EoE). Immunohistochemical identification (by anti-major basic protein, MBP, staining) of eosinophils in the esophageal lymph nodes (ELN), along with muscularis mucosa (MM), lamina propria (LP), and epithelial mucosa (EM) of intranasally peanut allergen-challenged mice are shown (A, original magnification $\times 100$). Eosinophils in the esophagus of intranasally saline-challenged (B, original magnification $\times 100$) and peanut allergen-challenged Balb/c mice (C, original magnification $\times 100$ and D, original magnification $\times 400$). Eosinophils (arrows) are located in the LP of intranasally saline-challenged mice (B) and the LP and EM of intranasally peanut allergen-challenged mice (C and D). Higher-power magnification photomicrographs reveal eosinophil microabscesses in the basal epithelial layer and extracellular anti-MBP staining (D, inset of the area designated by the arrowhead, original magnification $\times 1,000$). Intragastrically saline- (E, original magnification $\times 400$) and peanut allergen- (F, original magnification $\times 400$) challenged mice had eosinophils in the LP.



were detected in saline-challenged mice (Fig. 2B) compared with a large number of intraepithelial and lamina propria eosinophils along with disrupted esophageal epithelial cell integrity following food allergen-induced EoE (Fig. 2, C and D). Interestingly, eosinophilic microabscesses and extracellular MBP immunoreactivity were detected, suggesting release of eosinophilic granules into the esophageal mucosa (Fig. 2D, inset). Mice challenged intragastrically with saline or peanut allergen showed eosinophils in the lamina propria; however, the magnitude of eosinophils in the esophagus of peanut allergen-challenged mice was significantly higher (Fig. 2, E and F). Furthermore, we did not observe any eosinophilia in the lower gastrointestinal segments of mice challenged intranasally with peanut or corn allergen, whereas mice challenged intragastrically with peanut or corn allergen showed a signifi-

cant increase in eosinophils in the small intestine (data not shown).

Antigen trafficking after intratracheal delivery of Alexafluor488-conjugated allergen. We next tested the hypothesis that antigen translocation from the target organ to the inflamed organ plays a critical role in the induction of EoE. To examine antigen trafficking, we conjugated the *Aspergillus* allergen with the fluorescent dye Alexafluor 488 (Molecular Probes, Eugene, OR). Our FCM data analysis shows a shift in the mean fluorescence of cells isolated from the lung, mediastinal lymph node, and esophagus of mice challenged with Alexafluor488-conjugated allergen compared with cells isolated from mice challenged with saline (Fig. 3, A–C). To further determine the role of the mediastinal lymph node in EoE pathogenesis, we intranasally challenged wild-type and *Lta* gene-mutated

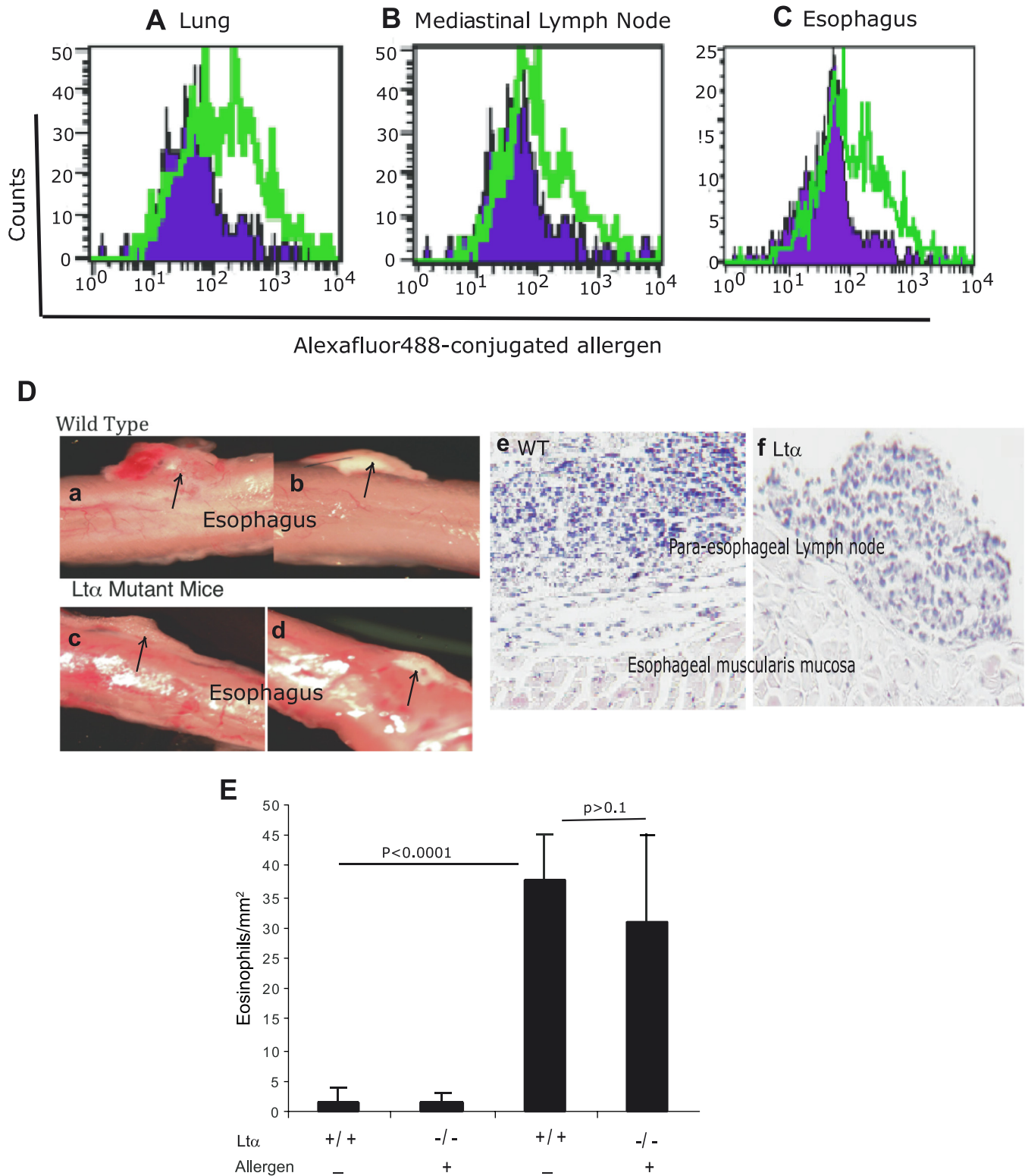


Fig. 3. Antigen detection in the lung, mediastinal lymph node, and esophagus following intratracheal allergen challenge and the role of para-esophageal lymph nodes in EoE. A representative fluorescence-activated cell sorting (FACS) histogram demonstrates the presence of Alexafluor-conjugated *Aspergillus* allergen in the lung, mediastinal lymph node, and esophagus (A–C). Antigen-positive cells from the populations isolated from each of these organs were analyzed on FACS; forward and side scatter are shown for the lung (A), mediastinal lymph node (B) and esophagus (C). The mean fluorescence shift 3 h after the last intratracheal saline or Alexafluor-conjugated allergen challenge is shown. Alexafluor-conjugated *Aspergillus* antigen-challenged mice showed higher mean fluorescence compared with saline-challenged mice. The filled histograms represent saline-challenged mice cells from the isolated cells of each respective organ. The dissecting microscopic photograph of para-esophageal lymph node in wild-type (WT) and Lt α mutant mice are shown and marked by arrows (D, a–f) and a microscopic photomicrograph of para-esophageal lymph node in WT and Lt α mutant mice (D, e–f) are shown (original magnification $\times 200$). A comparable esophageal eosinophilia in wild-type and Lt α gene-mutated mice are shown following allergen challenge (E). Data are shown as means $\pm$ SD, $n = 10$.

mice with allergen. The $L\alpha$ gene-mutated mice lack all major lymph nodes including the mediastinal lymph node (21); however, they have para-esophageal lymph nodes. The dissecting microscopic picture of para-esophageal lymph node in allergen-challenged wild-type (Fig. 3, *Da* and *Db*) and $L\alpha$ gene-mutated mice (Fig. 3, *Dc* and *Dd*) and photomicrograph of para-esophageal lymph node of wild-type (Fig. 3*De*) and $L\alpha$ (Fig. 3*Df*) are shown. Interestingly, both allergen-challenged wild-type and $L\alpha$ gene-mutated mice developed comparable esophageal eosinophilia (Fig. 3*E*).

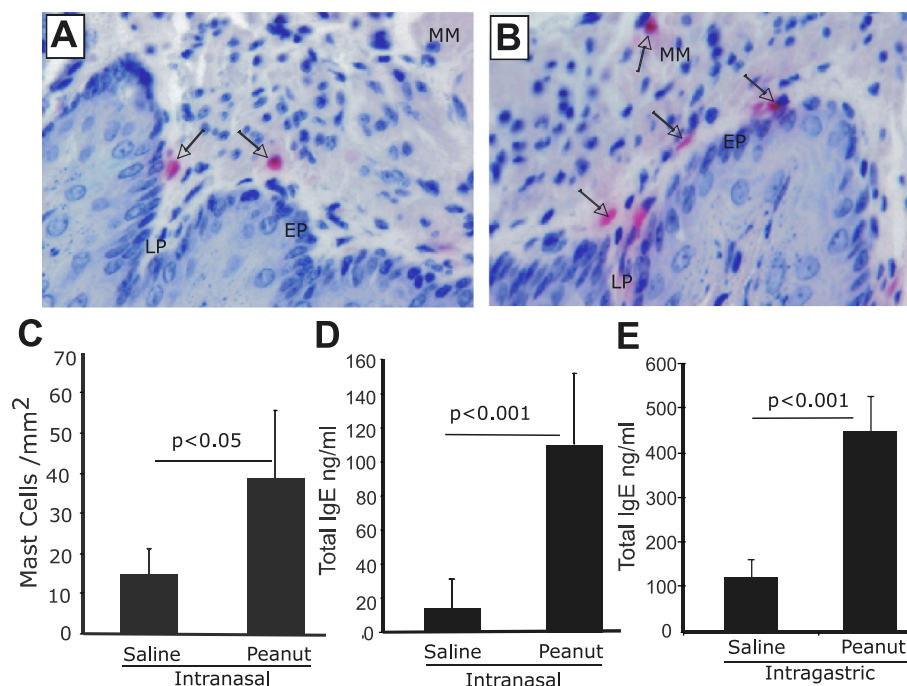
Mast cells are increased in a peanut allergen-induced mouse model of EoE. Because both intranasal and intragastric delivery of peanut allergen promotes esophageal eosinophilia, we investigated mast cell accumulation in the esophagus following peanut allergen challenge. We examined the number of mast cells in esophageal tissue sections of saline- or peanut allergen-challenged mice by performing chloroacetate esterase staining. The histopathological analysis identified a number of mast cells in the epithelial mucosa, lamina propria, and muscularis mucosa of intranasally peanut allergen-challenged mice; however, saline-challenged mice showed mast cells only in the lamina propria (Fig. 4, *A* and *B*). An approximately two-fold increase in mast cells was observed in intranasally peanut allergen-challenged compared with saline-challenged mice (Fig. 4*C*), whereas a comparable number of mast cells were found in intragastrically peanut allergen- or saline-challenged mice (data not shown). The esophageal mast cell numbers of intranasally or intragastrically peanut allergen-challenged mice were $38.6 \pm 14.2/\text{mm}^2$ and $16.69 \pm 8.1/\text{mm}^2$, respectively, compared with $14.6 \pm 6.2/\text{mm}^2$ and $16.1 \pm 6.1/\text{mm}^2$ in their respective saline-challenged mice (means $\pm$ SD, $n = 14$ mice/group). Allergen-induced IgE has a critical role in mast cell-mediated allergic responses; we therefore examined the levels of total IgE in intranasally and intragastrically peanut allergen-challenged mice. The analysis indicated a significant increase in the levels of total IgE in intranasally (Fig. 4*D*) or

intragastrically (Fig. 4*E*) peanut allergen-challenged mice compared with their respective saline-challenged mice. The induction of total IgE in intragastrically challenged mice is higher than that of intranasally challenged mice, indicating that the intragastric route of allergen challenge induces a more systemic response in B cells than the intranasal route.

Eosinophil active cytokine and chemokine transcripts are induced in the esophagus of peanut allergen-induced EoE. We next tested whether peanut extract exposure in peanut/alum-sensitized mice induces eosinophil active cytokine expression in the esophagus. Accordingly, we performed quantitative real-time PCR analysis for IL-5, IL-13, IL-15, eotaxin-1, eotaxin-2, and β -actin mRNA expression in the esophagus of mice challenged intranasally with saline or peanut allergen. The mice challenged intranasally with peanut allergen showed mRNA expression increases of ~ 15 -fold for IL-5, ~ 4 -fold for IL-13, ~ 3 fold in IL-15, 20-fold for eotaxin-1, and ~ 10 -fold for eotaxin-2 relative to saline-challenged mice following β -actin mRNA normalization (Fig. 5, *A–E*). Mice challenged intragastrically with peanut allergen show an increase of esophageal IL-5, IL-13 and IL-15 transcript expression, but that increase is not statistically significant (data not shown).

Eotaxin-1/-2-deficient mice are protected from peanut allergen-induced EoE. Because transcript levels of eotaxin-1 and eotaxin-2 are induced in the esophagus following peanut allergen exposure in mice, we tested the hypothesis that the eosinophil active chemokines eotaxin-1 and eotaxin-2 are critical in the induction of EoE. Accordingly, we induced EoE in eotaxin-1 and -2 double gene-deficient (eotaxin-1/-2) mice following the protocol shown (Fig. 1, *A* and *B*). The esophagus of wild-type mice challenged intragastrically or intranasally with peanut allergen showed a significant increase in the number of eosinophils compared with their respective saline-challenged wild-type mice, whereas saline- and peanut allergen-challenged eotaxin-1/-2 double gene-deficient mice showed comparable levels of esophageal eosinophilia (Fig. 6, *A* and *B*). The

Fig. 4. Mast cell accumulation in the esophagus following peanut exposure. Peanut/alum-sensitized mice exposed intranasally to saline or peanut allergen were analyzed for esophageal mast cells by chloroacetate esterase staining. The esophageal mast cells (arrows) of mice challenged intranasally with saline (*A*) or peanut allergen (*B*) are shown 18–20 h after the last saline or allergen exposure. Original magnifications of photomicrograph are $\times 125$. The number of mast cells was quantified by morphometric analysis following digital microscopy of the esophageal sections of saline- or peanut allergen-challenged mice (*C*). The levels of total serum IgE following intranasally (*D*) or intragastrically (*E*) delivered saline or peanut allergen challenge are shown. Data are obtained 18–20 h after the last treatment. Data are expressed as means $\pm$ SD, $n = 14$ mice/group.



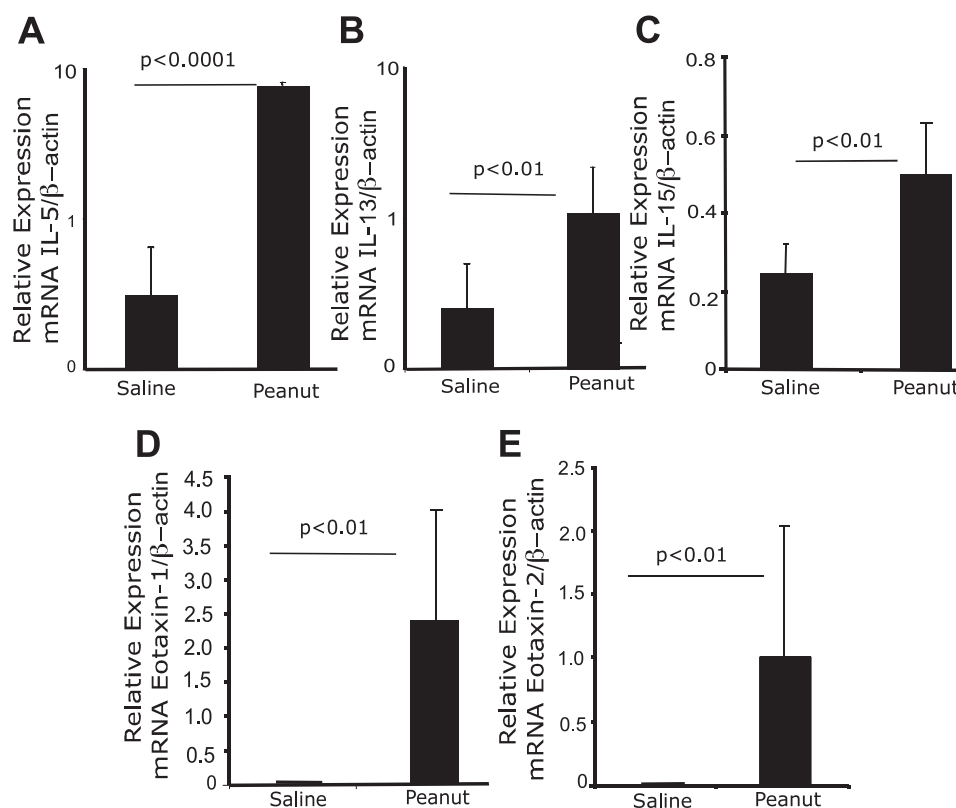


Fig. 5. Peanut allergen-induced mRNA expression of eosinophil active cytokines in the esophagus. The quantitative real-time PCR analyses of the mRNA levels of the eosinophil active cytokines IL-5 (A), IL-13 (B), and IL-15 (C) following saline or peanut allergen exposure in mice are shown. The mRNA levels of the eosinophil active chemokines eotaxin-1 (D) and eotaxin-2 (E) following saline or peanut allergen exposure in mice are shown. Data are expressed as means $\pm$ SD, $n = 10$ mice/group.

number of eosinophils in the esophagus of intranasally and intragastrically peanut allergen-challenged wild-type mice was $167.4 \pm 54.6/\text{mm}^2$ and $36.4 \pm 9.4/\text{mm}^2$, respectively, compared with $22.3 \pm 17.9/\text{mm}^2$ and $8.1 \pm 2.9/\text{mm}^2$ of their respective saline-challenged mice (means $\pm$ SD, $n = 12$ mice/group). However, the eosinophils in the esophagus of intranasally and intragastrically peanut allergen-challenged eotaxin-1/-2 double gene-deficient mice were $8.6 \pm 6.3/\text{mm}^2$ and $0.8 \pm 0.6/\text{mm}^2$, respectively, compared with $6.3 \pm 2.7/\text{mm}^2$ and $0.5 \pm 0.5/\text{mm}^2$ of their respective saline-challenged mice (means $\pm$ SD, $n = 11$ mice/group).

iNKT cells are critical in promoting peanut allergen-induced EoE. We recently showed that IL-15, which is a growth and survival factor for iNKT cells, has a critical role in aeroallergen-induced EoE (46). Therefore, we tested whether iNKT cells are critical in peanut allergen-induced EoE. Accordingly, we induced EoE in wild-type and CD1d gene-deficient mice (44) following the protocol shown (Fig. 1, A and B). The esophagus of intranasally and intragastrically peanut allergen-challenged wild-type mice showed a significantly increased number of eosinophils compared with saline-challenged wild-type mice, whereas saline- and peanut allergen-challenged CD1d gene-deficient mice showed comparable levels of esophageal eosinophilia (Fig. 7, A and B). The number of esophageal eosinophils in intranasally and intragastrically peanut allergen-challenged wild-type mice were $186 \pm 85.8/\text{mm}^2$ and $38.9 \pm 8.2/\text{mm}^2$, respectively, compared with $21.7 \pm 8.3/\text{mm}^2$ and $10.8 \pm 1.7/\text{mm}^2$ of their respective saline-challenged mice (means $\pm$ SD, $n = 12$ mice/group). However, the number of esophageal eosinophils in intranasally and intragastrically peanut allergen-challenged CD1d gene-deficient mice were $29.8 \pm 28.7/\text{mm}^2$ and $10.9 \pm 4.7/\text{mm}^2$, respectively,

compared with $48.7 \pm 9.8/\text{mm}^2$ and $10.3 \pm 4.4/\text{mm}^2$ of their respective saline-challenged mice (means $\pm$ SD, $n = 12$ mice/group). A partial reduction of airway eosinophilia was observed in both intranasally and intragastrically peanut allergen-challenged mice (data not shown).

DISCUSSION

Eosinophil infiltration into the esophagus is commonly observed in patients with diverse diseases including gastroesophageal reflux, drug reactions, eosinophilic gastroenteritis, and primary EoE (3, 7, 11, 37, 42). In patients with EoE, the esophageal eosinophilic infiltration can be ameliorated by a food allergen elimination diet (2, 20), suggesting that sensitized individuals may develop esophageal eosinophilic inflammation in response to food allergens. Although some direct and indirect evidence indicates that food allergens induce EoE in human and experimental murine models (14, 40), the mechanisms are not yet clearly understood. We now demonstrate that intranasal exposure of sensitized mice to peanut and corn extract induced eosinophilia and mastocytosis in the esophagus. The accumulation of eosinophils (especially intraepithelial eosinophils) and presence of extracellular granules and eosinophilic microabscesses mimicked the pathophysiological changes observed in individuals with EoE (10, 24). Herein, we show that peanut or corn allergen challenge of sensitized mice is sufficient to elicit eosinophil accumulation in the esophagus, including accumulation of eosinophils in the epithelial mucosa. Interestingly, these findings are in accordance with the peanut and corn hypersensitivities observed in the compiled EoE patient combined skin prick and patch test-positive dataset of the CCED, shown in Supplemental Fig. S1 (supplemental

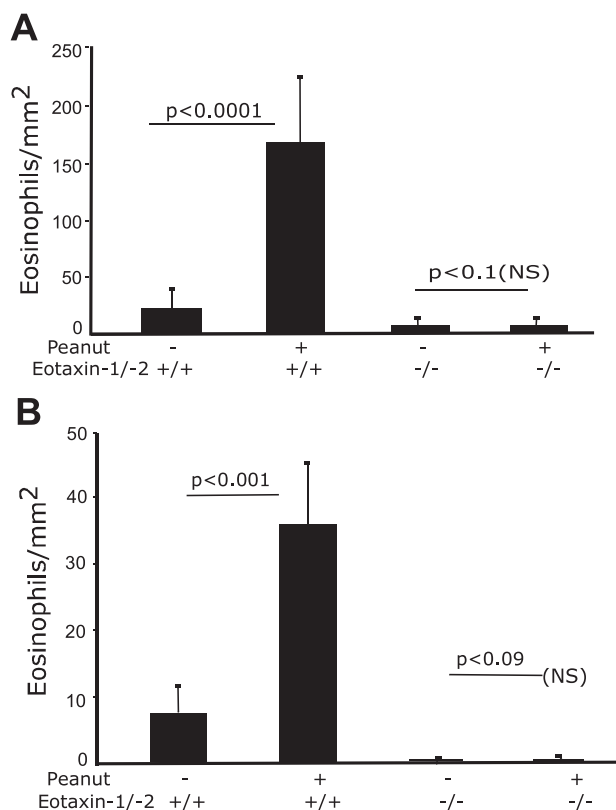


Fig. 6. Peanut extract-induced EoE in eotaxin-1/-2 gene-deficient mice. The levels of eosinophils in the esophagus of wild-type and eotaxin-1/-2 double gene-deficient mice were analyzed following peanut extract exposure. Wild-type (+/+) and eotaxin-1/-2 double-deficient (-/-) mice were sensitized and challenged with intranasally (A) or intragastrically (B) delivered saline (-) or peanut extract (+), and the number of eosinophils in the esophagus was determined by anti-MBP staining. The data are expressed as mean $\pm$ SD, $n = 12$ mice/group. NS, not significant.

material for this article is available online at the *American Journal of Physiology Gastrointestinal and Liver Physiology* website). However, our findings indicate that peanuts are a more potent inducer of EoE, as both intranasal and intragastric delivery of peanut allergen to sensitized mice induces a higher number of airway and esophageal eosinophils than corn allergen. Interestingly, no gastrointestinal eosinophilic inflammation was observed in mice intranasally challenged with peanut allergen; however, intragastrically peanut and/or corn allergen-challenged mice showed a significant increase of eosinophils in the small intestine, indicating that allergen-sensitized mice challenged with peanut or corn extract may promote eosinophilic gastrointestinal disorders.

The present study uncovers several principles concerning food allergen-induced EoE. First, we demonstrate the ability of peanut and corn allergens to induce esophageal eosinophilia. Second, the magnitude of eosinophils induced in the esophagus following peanut or corn allergen exposure is much higher than that of the previously reported aeroallergen (25) or insect allergen (32) exposure. Third, we show that allergen trafficking occurs from the target organs to the inflamed site. Fourth, we show that local esophageal lymph nodes might be a critical link in promoting esophageal and airway eosinophilia. Interestingly, intranasal challenge with peanut allergen promotes a higher number of airway and esophageal eosinophils compared

with intragastric challenge, providing a rationale to use an intranasal food allergen challenge model to study the mechanism of EoE pathogenesis. These studies showed that the magnitude of airway eosinophilia impacts the pathogenesis of EoE in mice and is in accordance with reports that showed a number of food allergen-sensitive patients with EoE have coexisting asthma or other eosinophilic allergic diseases (1). Both intranasal and intragastric routes of food allergen delivery have physiological relevance. It has been shown that respiratory tract sensitization absolutely adds to the food allergy burden and that peanut allergen cross-reacts with airborne allergens (grass) in humans. There is also evidence that intranasally delivered antigens tend to get swallowed (<http://www.niaid.nih.gov/topics/entericdiseases/Documents/intranasal.pdf>) (25); therefore, intranasally delivered food antigen has a physiological correlate and is relevant to use for examining the mechanism of EoE pathogenesis. Additionally, the presence of eosinophils in local esophageal lymph nodes highlights the significance of this lymphatic tissue and in the adjoining esophageal mucosa might be the cause of the patchy nature of EoE in humans. Furthermore, these novel data provide physical evidence linking airway eosinophilia to EoE, at least in an experimental murine model. A similar localization of esophageal eosinophilia was observed in both corn and peanut aller-

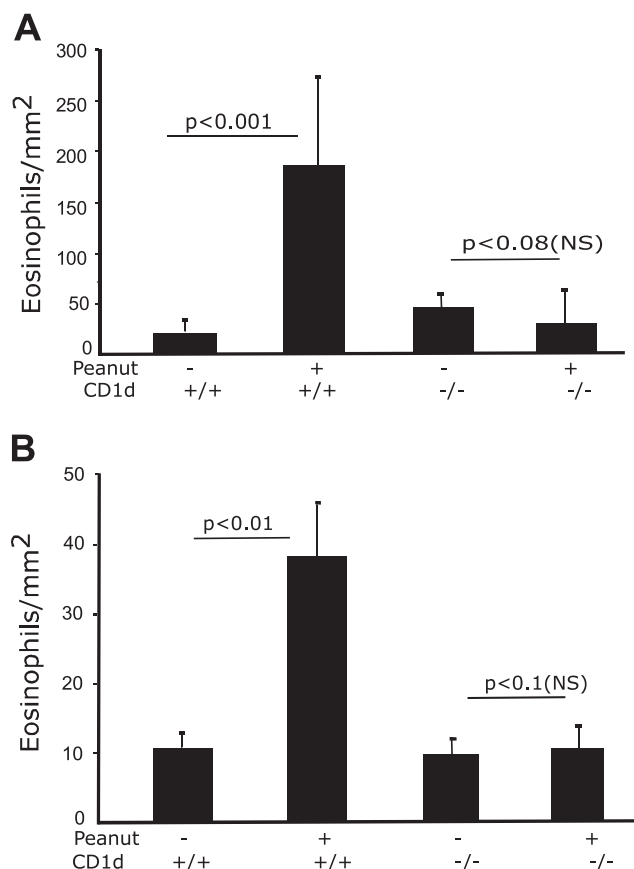


Fig. 7. Peanut extract-induced EoE in CD1d gene-deficient mice. The levels of eosinophils in the esophagus of wild-type and CD1d gene-deficient mice were analyzed following peanut extract exposure. Wild-type (+/+) and CD1d-null (-/-) mice were sensitized and challenged with intranasally (A) or intragastrically (B) delivered saline (-) or peanut extract (+), and the number of eosinophils in the esophagus was determined by anti-MBP staining. The data are expressed as means $\pm$ SD, $n = 12$ mice/group. NS, not significant.

gen-challenged mice. Lastly, we show that peanut challenge in sensitized mice increases the number of mast cells in the esophageal mucosa and of IgE in the blood. Notably, both the peanut and corn allergen-induced pathological characteristics observed in mice are in accordance with human EoE pathogenesis (9, 18, 41). Additionally, eosinophil active cytokines (IL-5 and IL-13) and chemokines eotaxin-1 and eotaxin-2 are induced in the esophagus of both intranasally and intragastrically peanut allergen-challenged mice. However, the levels induced via intragastrically delivered peanut allergen did not reach significance. The lack of strong induction of these cytokines and chemokines may be the reason that intragastrically delivered peanut allergen did not induce robust esophageal eosinophilia.

Furthermore, because aeroallergen or insect allergen-induced EoE was previously shown to be dependent on the eosinophil active chemokines eotaxin-1 and -2 (25, 32), we tested the role of eotaxins in peanut-induced EoE pathogenesis. Our experiments indicate that mice genetically deficient in eotaxin-1/-2 genes are protected from the induction of peanut-induced EoE. These data are in accordance with the earlier reported studies that eotaxins play a significant role in the induction of EoE pathogenesis (25, 32). As it was not clear which T cell subtypes initiate the disease, we tested the role of iNKT cells in peanut allergen-induced EoE and found that iNKT cells are critical in peanut allergen-induced EoE. The CD1d gene-deficient mice (iNKT cell deficient) are protected from the induction of EoE following peanut allergen sensitization and challenge. It has been shown that iNKT cells function at the junction between innate and adaptive immunity and express a conserved invariant T cell receptor that has a potent immunoregulatory function (19, 34). The finding of a role of iNKT cells in EoE is in accordance with our recently reported studies showing that iNKT cell survival and growth factor IL-15 (12, 30) is induced in human EoE and that the induction of experimental EoE is dependent on IL-15 (46). Interestingly, IL-15 transcripts are also significantly induced in the esophagus in peanut-sensitized and intranasally challenged mice. Furthermore, these findings also provide a rationale for our previously reported studies indicating that T cell-deficient mice were completely protected from disease induction but that conventional T cell subset CD8 and CD4 gene-deficient mice induce EoE despite having an impaired Th2 cell response (29). It is interesting to note that iNKT cells have both CD4⁺ and CD4⁻ T cell subsets (15) and are capable of producing both Th1 and Th2 responses (36). These findings corroborate the significance of iNKT cells to the pathogenesis of EoE. Most importantly, it has also been shown that airway hypersensitivity is dependent on iNKT cells (17, 22), and our findings that iNKT cells are required for the induction of EoE and associated with airway eosinophilic inflammation are in accordance with these reports. Notably, iNKT cells may be activated by glycolipids released by allergen-induced activating macrophages, allergen-derived antigen-presenting cells (APCs), or the allergen-induced iNKT cell-activating genes *MICA* or *MICB*, which have previously been reported as induced in patients with EoE (4). APC activation of iNKT cells leads to rapid production of Th2 cytokines and consequent stimulation of multiple immune components and regulation of dendritic cell function (6, 43). Recently, milk-induced sphingomyelin and house dust mite-induced sphingomyelin-related lipids were identified that acti-

vate iNKT cells in food-allergic patients (16). Therefore, these iNKT-activating antigens may have a critical role in EoE pathogenesis. Taken together, we provide evidence that peanut and corn allergens are effective contributors in promoting experimental EoE and that more than 40–50% of patients with EoE are hypersensitive to peanut and corn allergens (CCED unpublished data included as supplemental document). Importantly, we mechanistically show that para-esophageal lymph nodes are a critical link in food or aeroallergen-induced iNKT cell-mediated airway and esophageal eosinophilia.

ACKNOWLEDGMENTS

We acknowledge and thank Dr. Marc Rothenberg for consistent support and review of the manuscript. The authors also thank Shawna Hottinger for editorial assistance and Drs. James and Nancy Lee (Mayo Clinic, Scottsdale, AZ) for anti-MBP.

GRANTS

This work was supported in part by the following grants and organizations: NIH R01 DK067255 (A. Mishra), R01 AI080581 (A. Mishra), NIH ARRA grant DK067255-05S1 (A. Mishra), and the Digestive Health Center (DHC) grant DK078392.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

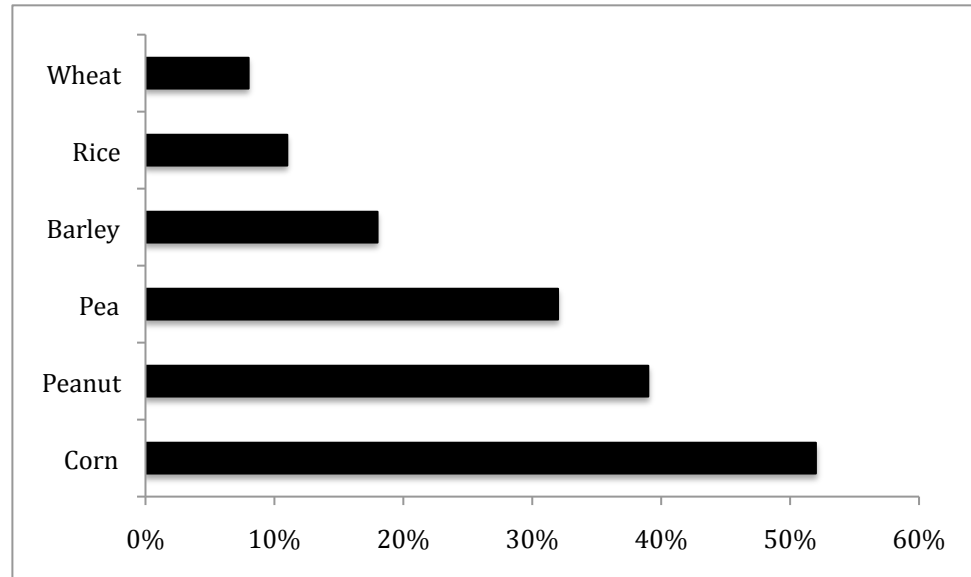
Author contributions: P.R., M.R., M.M., and Akanksha Mishra performed experiments; P.R. and M.R. analyzed data; Anil Mishra conception and design of research; Anil Mishra interpreted results of experiments; Anil Mishra drafted manuscript; Anil Mishra edited and revised manuscript; Anil Mishra approved final version of manuscript.

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Supportive Figure 1



Combined SPT and Patch test (N= 51 Patients)
(Patch test positive for grade ≥ 2)

Most common food allergen hypersensitivity in human EE. The entire enrolled active human EE dataset of allergic patients at Cincinnati Children's Hospital Medical Center allergy clinic (n = 150) was analyzed for food allergen hypersensitivity, and results are shown as percent positive to common food allergens.