

Critical role for adaptive T cell immunity in experimental eosinophilic esophagitis in mice

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Abstract: We have previously developed a murine model of allergen-induced eosinophilic esophagitis (EE), characterized by intraepithelial eosinophils, extracellular granule deposition, and epithelial cell hyperplasia, features that mimic the pathophysiological changes observed in individuals with various forms of EE. We now test the hypothesis that adaptive T cell immunity is critical in initiating experimental EE. We first demonstrate that EE induction is associated with an increase in lymphocyte subpopulations (B^+ , $CD4^+$, and $CD8^+$) in the esophagus. We induced experimental EE in wild-type and various lymphocyte subpopulation-deficient mice by intranasal allergen sensitization. Eosinophil levels and epithelial cell proliferation were determined by performing antimajor basic protein and antiproliferation cell nuclear antigen immunohistochemical analysis. Eosinophil accumulation in the esophagus was ablated completely in RAG1 gene-deficient mice, but no role for B cells or antigen-specific antibodies was required, as B cell-deficient (IgH6) mice developed unabated, experimental EE. In addition, T cell-deficient (forkhead box N1 $^{-/-}$) mice were protected from the induction of experimental EE. It is surprising that CD8 α -deficient mice developed unaltered, experimental EE, and CD4-deficient mice were only protected moderately from disease induction. Taken together, these studies indicate a role of $CD4^+$ and $CD4^-$ cell populations in EE pathogenesis and demonstrate that experimental allergen-induced EE is dependent on adaptive T cell immunity. *J. Leukoc. Biol.* 81: 000–000; 2007.

Key Words: allergen · lymphocytes

INTRODUCTION

The accumulation of eosinophils in the esophagus is a commonly observed medical problem in patients with diverse gastrointestinal diseases such as primary eosinophilic esophagitis (EE) [1–4], an emerging, worldwide disease now found in numerous developed countries [5–7]. Dissection of experimental EE in murine models has revealed that EE can be triggered

by oral and respiratory allergens [8, 9]. It is particularly notable that EE appears to be food antigen-driven, implicating T lymphocyte responses. Early studies have implicated Th2 cell immunity in EE [10, 11]. It is interesting that Th2 cytokines have been demonstrated to be increased in the esophagus of EE patients [11], and murine modeling has established that IL-5 is required for disease pathogenesis [8]. Recent genome-wide analysis has provided substantial evidence that eotaxin-3 is involved in disease pathogenesis [12]. Of note, the Th2 cytokine IL-13 induces eotaxin-3, and indeed, mice genetically deficient in IL-13 are protected from the development of experimental EE, at least in part [10]. Despite these findings, the role of T and/or B cells in experimental or human EE has been largely unexplored.

To provide insight into the molecular and cellular mechanisms that trigger eosinophil trafficking into the esophagus, we now focus our attention on the role of B and T cells in EE induction and pathogenesis. We first demonstrate that intranasal allergen exposure induces B and T cell accumulation in the esophagus. Second, we dissect the mechanism involved by focusing on the role of specific cell populations by inducing experimental EE in B and T cell-deficient mice. As the experimental protocol (induced by respiratory allergen exposure) induces lung airway and esophageal inflammation, we compared pulmonary airway and esophageal responses. We demonstrate that T cell (but not B cell) responses are required for the induction of eosinophilic inflammation in the airway and esophagus. Although CD4 cells contribute to esophageal eosinophils, their role in regulating lung airway eosinophilia is relatively more critical, highlighting that CD4 dependency is less important in the esophagus compared with the lung.

MATERIALS AND METHODS

Mice

Specific, pathogen-free BALB/c, C57Bl6, and lymphocyte (RAG1) $^{-/-}$, B cell (IgH6) $^{-/-}$, T cell [forkhead box N1 (Foxn1)] $^{-/-}$, CD4 $^{-/-}$, and CD8-deficient mice were

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obtained from Jackson Laboratory (Bar Harbor, ME, USA). All the experiments were performed on age- and gender-matched, 6- to 8-week-old mice, which were maintained in a pathogen-free barrier facility, and animals were handled according to institutional guidelines. A minimum of three to four mice/group was used in each experiment, and each experiment was repeated three times.

Experimental EE induction

A mouse model of allergic EE was established using methods described previously [13]. In brief, mice were anesthetized lightly with isoflurane (IsoFlo, Abbott Laboratories, North Chicago, IL, USA), and 100 μ g (50 μ l) *Aspergillus fumigatus* (Bayer Pharmaceuticals, Spokane, WA, USA) or 50 μ l normal saline alone was applied to the nares using a micropipette with the mouse held in the supine position. After instillation, mice were held upright until alert. We also delivered intratracheal allergen to the mice anesthetized by i.p. injection of 500 μ g Ketaject (Ketamine HCL, Phoenix Pharmaceutical, Inc., St., Joseph, MO, USA). These mice were hung upright at a 60° angle on a vertical platform. Using flat forceps, the tongue was extended gently, and a long-loading pipette tip was inserted directly into the trachea of anesthetized mice, followed by the delivery of 25 μ l allergen or saline. After three treatments per week for 3 weeks, mice were killed between 18 and 20 h after the last intranasal or intratracheal allergen and saline challenge.

Bronchoalveolar lavage fluid (BALF) collection and analysis

The mice were killed by CO₂ inhalation. Immediately thereafter, a midline neck incision was made, and the trachea was cannulated. The lungs were lavaged three times with 1.0 ml PBS containing 1% FCS and 0.5 mM EDTA. The recovered BALF was centrifuged at 400 *g* for 5 min at 4°C and resuspended in 200 μ l PBS containing 1% FCS and 0.5 mM EDTA. Lysis of RBCs was carried out using RBC lysis buffer (Sigma Chemical Co., St. Louis, MO, USA), according to the manufacturer's recommendations. Total cell numbers were counted with a hemacytometer. Cytospin preparations of 5×10^4 cells were stained with Giemsa-Diff-Quick (Dade Diagnostics of P.R., Inc., Aguada, PR), and differential cell counts were determined. The BALF eosinophil counts were expressed as an indication of lung airway eosinophilia.

Total leukocyte analysis in the esophagus

The esophagus of adult mice were fixed with 4% paraformaldehyde in phosphate buffer, pH 7.4, embedded in paraffin, cut into 5 μ m sections, fixed to positive-charge slides, and immunostained for CD45, a pan-leukocyte marker for total leukocyte counts, as described previously [14].

Esophageal T and B cell analysis

The esophagus of adult mice were embedded in optimal cutting temperature compound (Sakura, Torrance, CA, USA) on dry ice; 5 μ m frozen tissue sections were cut and fixed to positive charge slides. The sections were immunostained with antimouse CD45R/B220 mAb (BD Biosciences, San Jose, CA, USA) for B cells and antimouse CD4 and CD8 α mAb (BD PharMingen, San Diego, CA, USA) for CD4⁺ and CD8⁺ lymphocytes, respectively, using an immunostaining kit following the manufacturer's protocol (Zymed Laboratories Inc., San Francisco, CA, USA). Negative controls included replacing the primary antibody with normal rat IgG to check the specificity of the antibodies.

Eosinophil analysis in the esophagus

The 5- μ m esophageal paraffin tissue sections were immunostained with anti-serum against mouse eosinophil major basic protein (anti-MBP; kindly provided by Drs. Nancy and James Lee, Mayo Clinic, Scottsdale, AZ, USA), as described [14, 15]. 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 rabbit anti-MBP (1:5000) overnight at 4°C, followed by 1:200 dilution of biotinylated goat antirabbit IgG secondary antibody and avidin-peroxidase complex (Vector Laboratories, Burlingame, CA, USA) for 30 min each. These slides were developed further with nickel diaminobenzidine-cobalt chloride solution to form a black precipitate and counter-stained with nuclear fast red. Negative controls included replacing the primary antibody with normal rabbit serum.

Quantification of eosinophils

Eosinophils were quantified by counting the anti-MBP, positive-stained cells in each tissue section with the assistance of digital morphometry using the Metamorph imaging system (Universal Imaging Corp., West Chester, PA, USA) and expressed as eosinophils/mm² tissue area as described earlier [16, 17].

Endotoxin-free Aspergillus

A. fumigatus antigen (2 mg/ml) was prepared endotoxin-free by using the endotoxin-removing column (AffinityPack™ Detoxi-Gel™ endotoxin-removing gel, Pierce, Rockford, IL, USA) following the manufacturer's protocol. The samples were collected, and protein concentration was measured using a bacillus Calmette-Guerin kit (Pierce). BALB/c mice were challenged nine times in 3 weeks with original *Aspergillus* extract (100 μ g/50 μ l saline), endotoxin-free *Aspergillus* extract (100 μ g/50 μ l saline), or saline (50 μ l) and killed 18–20 h after the last challenge, and esophageal eosinophils were analyzed.

Analysis of epithelial hyperplasia

Epithelial cell hyperplasia was determined by quantification of proliferating esophageal basal cells. The 5- μ m esophageal paraffin tissue sections were immunostained with antiproliferation cell nuclear antigen (anti-PCNA) following an identical protocol as described for eosinophil immunostaining [18] with the addition of an antigen retrieval step. In brief, prior to blocking with serum, the deparaffinized esophageal sections were treated two times in the microwave with boiling citrate buffer at pH 6.0 for 10 min. A PCNA-specific mouse mAb, PC-10 (Dako, Carpinteria, CA, USA) was used as the primary antibody for immunodetection of PCNA. To quantify the immunostaining, at least 200–250 epithelial cells per section were counted. The percent-positive cells is expressed as the mean $\pm$ SD.

Serum IgE and antigen-specific IgG1

Total serum IgE levels were measured using a BD OptEIA ELISA set (BD Biosciences) as per the manufacturer's protocol. Briefly, after blocking non-specific protein binding with 10% FBS, each mouse serum sample or purified mouse IgE was applied to an antimouse IgE mAb-coated, 96-well ELISA plate (Immuron, Dynex Technologies, Chantilly, VA, USA), which was incubated 2 h at room temperature and washed with 0.05% Tween-20 in PBS, and biotinylated antimouse IgE mAb was applied to each well, followed by avidin-HRP conjugate reagent. Finally, tetramethylbenzidine (TMB) substrate solution (BD PharMingen) was added to each well, and the color was developed in the dark at room temperature. The IgE concentration of each sample was calculated by using a standard curve. Further, *A. fumigatus*-specific IgG1 in mouse serum samples was measured as per the protocol. Briefly, sample wells were coated with 100 μ l *Aspergillus* extract (50 μ g/ml), blocked with 10% FBS in PBS, and washed with 0.05% Tween-20 in PBS. Serum samples were diluted 1:3 with 10% FCS in PBS and then serially diluted (1:3). After a 2-h incubation at room temperature, plates were washed with 0.05% Tween-20 in PBS, and 100 μ l HRP-conjugated antimouse IgG1 (1:1500 dilution from 1 mg/ml, Clone X56, BD PharMingen) was added. Using streptavidin HRP detection (100 μ l/well, TMB substrate reagents, BD Biosciences), the OD was read at 450 nm immediately. Data are presented as the serum dilution required to obtain OD = 0.6.

Statistical analysis

Data are expressed as mean $\pm$ SD. Statistical significance comparing different sets of mice was determined by GraphPad InStat *t*-test.

RESULTS

Intranasal and intratracheal aeroallergen exposure induces simultaneous esophageal and pulmonary eosinophilia

We have previously established that intranasal *Aspergillus* extract induces eosinophilic lung and esophageal responses [8], suggesting an interaction between the lung and esophagus.

To explore this further, we compared the effect of intranasal and intratracheal aeroallergen exposure on the induction of eosinophil accumulation in the lung airway and esophagus. A kinetic analysis of eosinophil accumulation in the lung airway and esophagus was performed simultaneously following one to nine exposures to aeroallergen. The eosinophils in the esophagus were evaluated by anti-MBP immunostaining, whereas lung airway eosinophils were evaluated by BALF cell analysis. Allergen challenge induced a significant increase in the level of eosinophils in the airway and esophagus by intranasal and intratracheal inoculations. The level of eosinophils in the lung airway and esophagus continued to increase through the nine exposures (Fig. 1, A and B). After nine intranasal allergen and saline challenges, the BALF eosinophil counts were $197 \pm 26.2 \times 10^4$ and $0.009 \pm 0.006 \times 10^4$, respectively. Eosinophil levels after nine intratracheal allergen and saline challenges were $233 \pm 23.7 \times 10^4$ and $0.01 \pm 0.02 \times 10^4$ (mean $\pm$ SD, $n=6$), respectively. Eosinophil numbers in the esophagus of allergen- and saline-challenged mice following nine intranasal challenges were $48.6 \pm 8.7/\text{mm}^2$ and $0.8 \pm 1.2/\text{mm}^2$; this was comparable with esophageal eosinophils after intratracheal delivery [$66.5 \pm 9.5/\text{mm}^2$ and $1.1 \pm 1.4/\text{mm}^2$, respectively (mean $\pm$ SD, $n=6$, $P<0.01$; Fig. 1B)]. Further, we also aimed to examine if antigen-induced eosinophil accumulation in the esophagus was a result of the presence of endotoxin in the Aspergillus extract. Therefore, we induced experimental EE using endotoxin-free Aspergillus antigen extract. No significant difference in the recruitment of eosinophils to the esophagus of mice after nine challenges with the original Aspergillus extract compared with endotoxin-free Aspergillus extract was observed. The eosinophil level in the esophagus following original Aspergillus extract was $40.3 \pm 8/\text{mm}^2$ and with endotoxin-free Aspergillus, was $41.3 \pm 7.6/\text{mm}^2$ (mean $\pm$ SD, $n=6$), respectively. The saline control-treated mice showed 0.41 ± 0.07 eosinophils/ mm^2 (mean $\pm$ SD, $n=6$).

T and B cells are increased in the esophagus following intranasal allergen exposure

We were next interested in determining the types of inflammatory cells that accumulated in the esophagus, apart from eosinophils, following intranasal allergen exposure. We performed anti-CD45 immunohistochemical analysis on the esophageal tissue sections following allergen and saline exposure. An increase in total leukocytes (CD45^+) in the esophagus of allergen-challenged mice compared with saline-treated mice was observed. The CD45^+ cells in allergen-treated mice were $459 \pm 23/\text{mm}^2$ ($n=8$) compared with $209 \pm 41/\text{mm}^2$ ($n=7$) in saline-treated mice (mean $\pm$ SD, $P<0.001$).

The increase in the number of CD45^+ cells prompted us to analyze the number of lymphocyte subpopulations in the esophagus following allergen exposure. Paraffin-embedded and frozen esophageal tissue sections were immunostained with anti-CD4, anti-CD8, and anti-B220 antibodies to evaluate infiltrating lymphocyte subpopulations. The analysis demonstrated an approximate twofold increase in B cells and a four- to fivefold increase in CD4^+ and CD8^+ cells in the esophagus of allergen-treated mice compared with saline-treated mice (Fig. 2, A–C). The number of B cells in allergen-treated mice

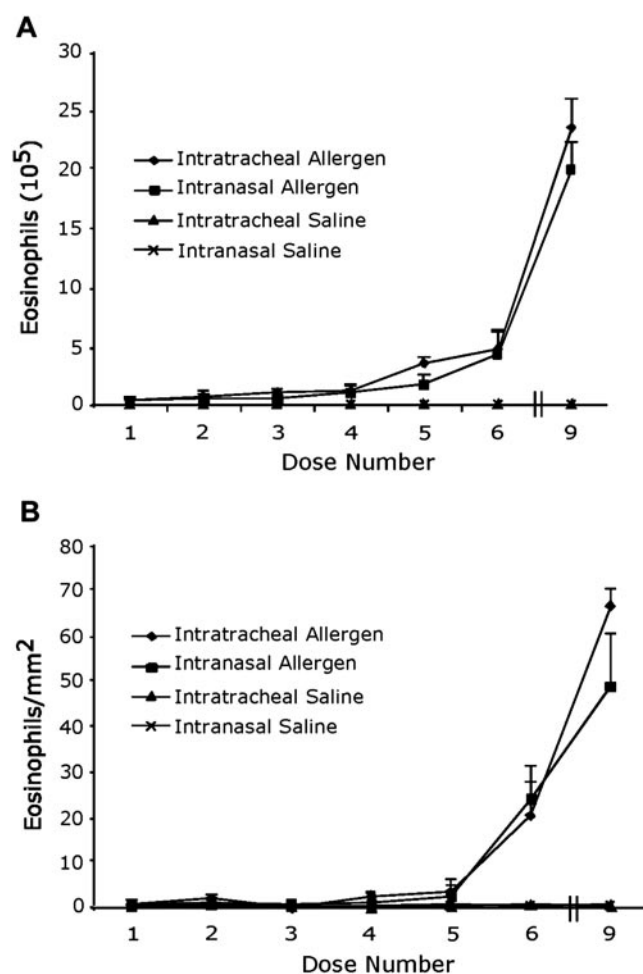


Fig. 1. Eosinophil accumulation in the lung and esophagus following allergen exposure. A kinetic analysis was performed to analyze the number of eosinophils in the lung and esophagus at each dose following intratracheal and intranasal saline and allergen (*A. fumigatus*) exposure. The number of eosinophils in the BALF (A) and esophagus (B) is shown 18–20 h after each treatment. Data are expressed as mean $\pm$ SD ($n=8$ or 10 mice at each dose).

was $22 \pm 7.4/\text{mm}^2$ ($n=9$) compared with $9.4 \pm 5.8/\text{mm}^2$ (mean $\pm$ SD, $n=10$, $P<0.01$) in saline-treated mice. The CD4^+ cells were $30.3 \pm 9.6/\text{mm}^2$ in allergen-treated mice ($n=8$) compared with $7.1 \pm 5/\text{mm}^2$ (mean $\pm$ SD, $n=6$, $P<0.001$) in saline-treated mice, whereas the number of CD8^+ cells was $34 \pm 15/\text{mm}^2$ ($n=8$) compared with $8 \pm 5/\text{mm}^2$ (mean $\pm$ SD, $n=6$, $P<0.001$) in saline-treated mice. As a control, the allergen-treated mice had a large influx of eosinophils in the esophagus compared with saline-treated, control mice (Fig. 2D). The CD4^+ , CD8^+ , and B220^+ cells were detected in the mucosa and submucosal region of the esophagus (data not shown).

Role of adaptive immunity in the development of experimental EE

To test the role of adaptive immunity in the development of EE, we examined eosinophil recruitment into the esophagus of B and T cell-deficient (RAG-1) mice [19]. It is notable that lung airway and esophageal eosinophilic inflammation were absent

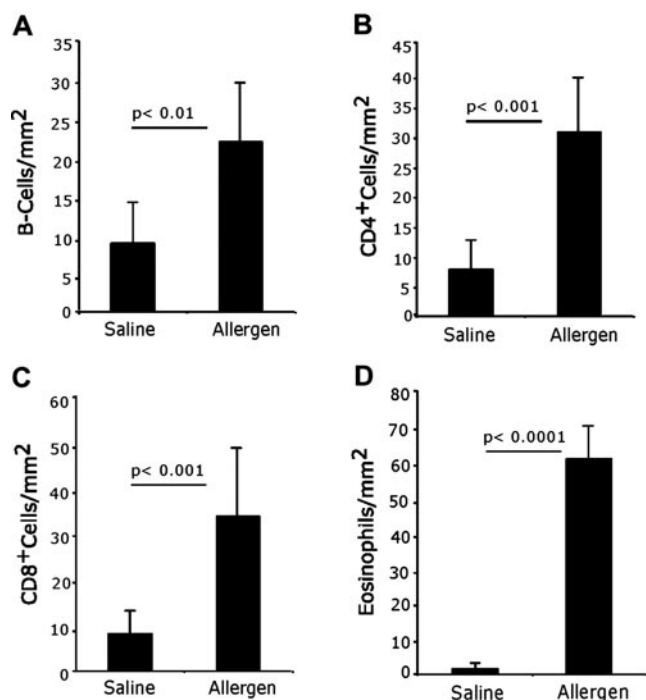


Fig. 2. Lymphocyte and eosinophil levels in the esophagus following allergen exposure. Lymphocyte subpopulations in the esophageal tissue sections were identified by performing immunohistochemical staining and morphometric analysis. The B cell (A), CD4⁺ T cell (B), CD8⁺ T cell (C), and eosinophil (D) levels, 18–20 h following nine saline and allergen (*A. fumigatus*) exposures, are shown. Data are expressed as mean $\pm$ SD ($n=6-10$ mice/group).

in RAG-1 gene-deficient mice (**Fig. 3, A and B**). The number of eosinophils in the lung airway and esophagus of allergen-treated RAG1 gene-deficient mice was $0.11 \pm 0.16 \times 10^4$ and $1.55 \pm 1.8/\text{mm}^2$, respectively, whereas eosinophils in the lung airway and esophagus of wild-type mice were $129 \pm 32 \times 10^4$ and $43 \pm 14.3/\text{mm}^2$, respectively.

Eosinophil accumulation in the esophagus is independent of B cells and allergen-induced antibody responses

Allergen-induced antibodies (IgE or allergen-specific IgG1) may have an important role in experimental EE induction, as total IgE and antigen-specific IgG1 were induced following allergen treatment (**Fig. 4, A and B**). We thus induced experimental EE in wild-type and B cell-deficient (IgH6) mice. Wild-type and B cell-deficient mice developed similar levels of esophageal eosinophilic inflammation following allergen treatment; eosinophilic inflammation in the lung airway was reduced moderately in B cell-deficient mice compared with wild-type, allergen-treated mice (**Fig. 4, C and D**). The number of eosinophils in allergen-treated, wild-type mice was $131 \pm 27 \times 10^4$ and $49 \pm 7.5/\text{mm}^2$ (mean $\pm$ SD, $n=10$) in the lung airway and esophagus, respectively. The number of eosinophils in B cell-deficient mice was $90 \pm 24 \times 10^4$ in the airway and $54 \pm 14/\text{mm}^2$ in the esophagus.

Partial role of CD4⁺ T cells in the induction of EE

We were next interested in determining if EE induction were dependent on CD8⁺ or CD4⁺ T cells. To test this, we

induced experimental EE in CD8 α - and CD4-deficient and control mice. The eosinophil levels in the lung airway of allergen-treated, CD8 α -deficient mice were reduced partially compared with wild-type mice ($P<0.05$); however, eosinophils in the esophagus were at comparable levels [$40.8 \pm 9.4/\text{mm}^2$ and $41.3 \pm 8.5/\text{mm}^2$ (mean $\pm$ SD, $n=9-11$; **Fig. 5, A and B**)]. In CD4-deficient mice, the number of allergen-induced eosinophils in the lung airways was reduced greatly compared with wild-type mice [$56.4 \pm 9 \times 10^4$ vs. $0.03 \pm 0.02 \times 10^4$ (mean $\pm$ SD, $n=9-11$, $P<0.001$) in wild-type and CD4-deficient mice, respectively; **Fig. 5C**]. In contrast, eosinophils accumulated in the esophagus of CD4-deficient mice, albeit at moderately reduced levels compared with wild-type mice (**Fig. 5D**). For example, eosinophil levels in the esophagus of wild-type and CD4-deficient, allergen-treated mice were $39.4 \pm 15.6/\text{mm}^2$ and $24.6 \pm 15.5/\text{mm}^2$, respectively (mean $\pm$ SD, $n=9-11$, $P<0.05$) compared with $1.2 \pm 2.3/\text{mm}^2$ and $1.1 \pm 2.1/\text{mm}^2$ (mean $\pm$ SD,

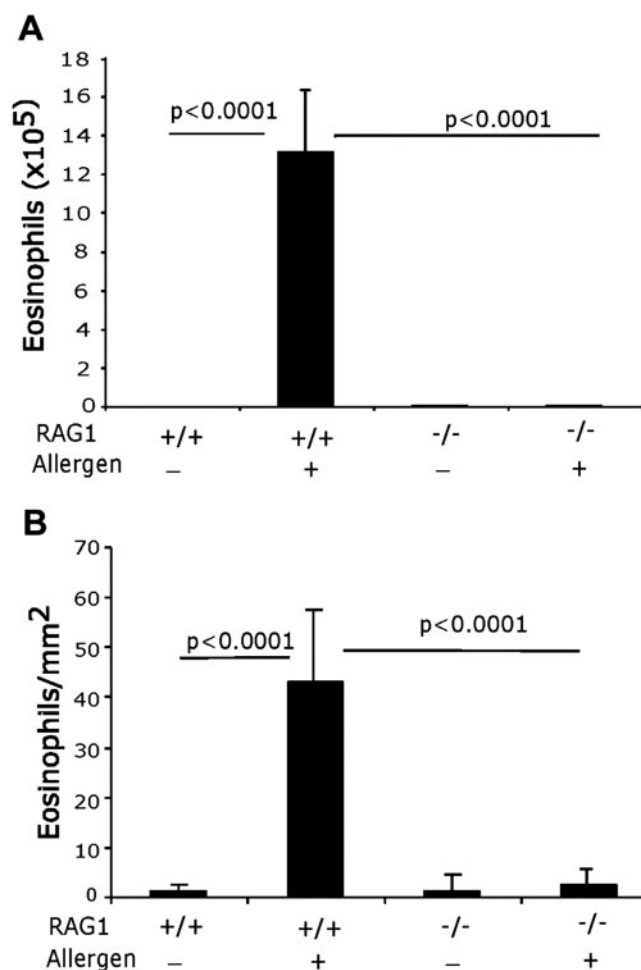


Fig. 3. Allergen-induced eosinophil accumulation in the lung and esophagus of RAG1 gene-deficient mice. RAG1 gene-deficient and wild-type mice were exposed to nine saline and allergen (*A. fumigatus*) exposures. Eosinophil levels in BALF (A) and esophagus (B) after the last treatment are shown. Mice receiving allergen are indicated as +, and mice receiving saline are indicated as -. Data are obtained 18–20 h after the last treatment and are expressed as mean $\pm$ SD ($n=8$ mice/group).

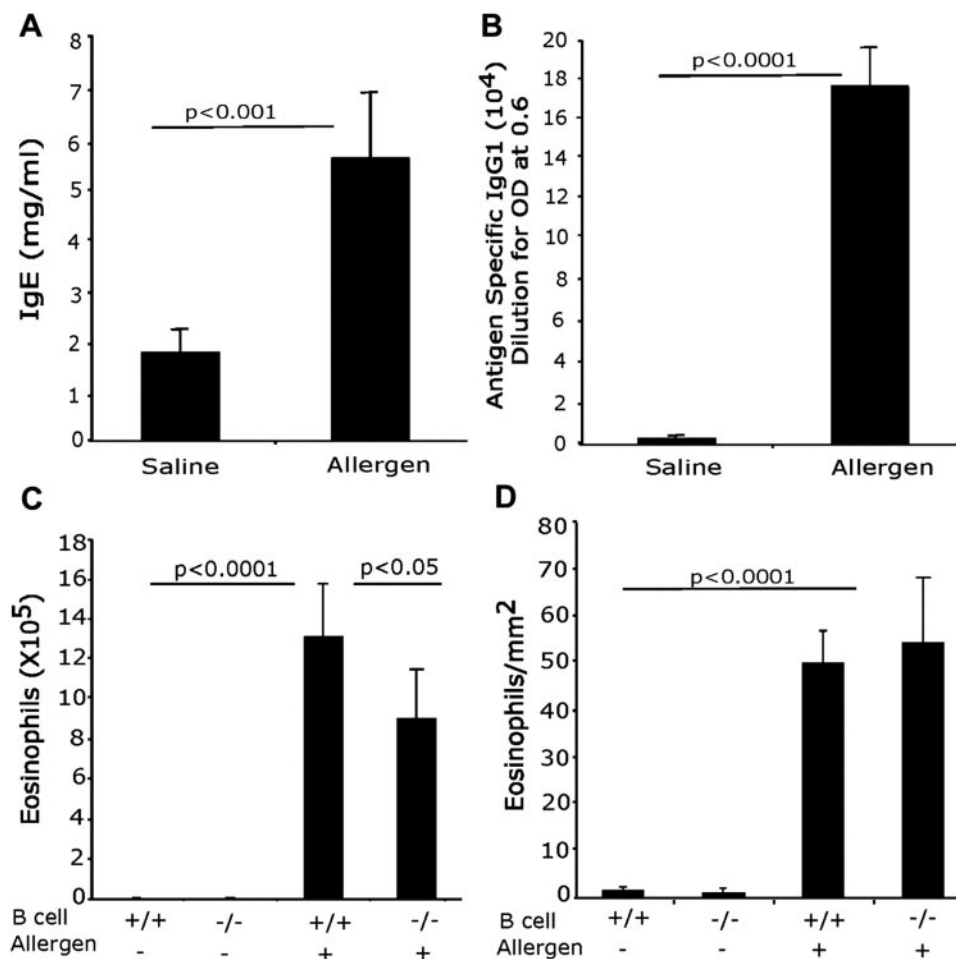


Fig. 4. Induction of experimental disease in B cell-deficient mice. B lymphocyte-deficient (-/-) and wild-type (+/+) mice were exposed to nine saline and allergen (*A. fumigatus*) treatments. The levels of serum IgE (A) and antigen-specific IgG1 (B) in wild-type mice are shown. The eosinophil levels are shown in the BALF (C) and esophagus (D) of wild-type and B cell-deficient mice. Allergen exposure is indicated as +, and saline exposure is indicated as -. Data are obtained 18–20 h after the last treatment and are expressed as mean $\pm$ SD ($n=10$ mice/group).

$n=9-11$) in saline-treated mice. In CD4- and CD8-deficient mice, the location of eosinophils in the esophagus was comparable with that seen in wild-type mice (data not shown). Taken together, these data demonstrate that CD4-positive cells are not primarily necessary for the induction of experimental EE (in contrast to their critical role in the induction of allergen-induced airway eosinophilia).

Allergen-induced esophageal epithelial cell hyperplasia is independent of B, CD4⁺, and CD8⁺ cells

We were next interested in determining if B, CD4⁺, or CD8⁺ lymphocytes had a major role in allergen-induced basal layer hyperplasia, a characteristic finding observed in human EE [20]. To test this, we examined PCNA staining in esophageal tissue sections of saline- and allergen-treated, wild-type, IgH6, and CD4- and CD8 α -deficient mice. A threefold increase of PCNA⁺ cells was observed following allergen treatment. PCNA⁺ cells were detected predominantly in the epithelial layer of the esophagus in wild-type, allergen-treated mice (Fig. 6, A and B). The percent of PCNA⁺ epithelial cells in the esophageal basal layer was comparable in all mice (Fig. 6C); PCNA⁺ cells in wild-type, IgH6, and CD4- and CD8 α -deficient, allergen-treated mice were 36.6 ± 8.1 , 34.5 ± 8.3 , 29.1 ± 9 , and 30.2 ± 7 , respectively (mean $\pm$ SD, $n=9-12$)

compared with 14.8 ± 8 , 12.6 ± 9 , 10.5 ± 8 , and 12.2 ± 9 in saline-treated mice (mean $\pm$ SD, $n=9-12$, $P<0.001$).

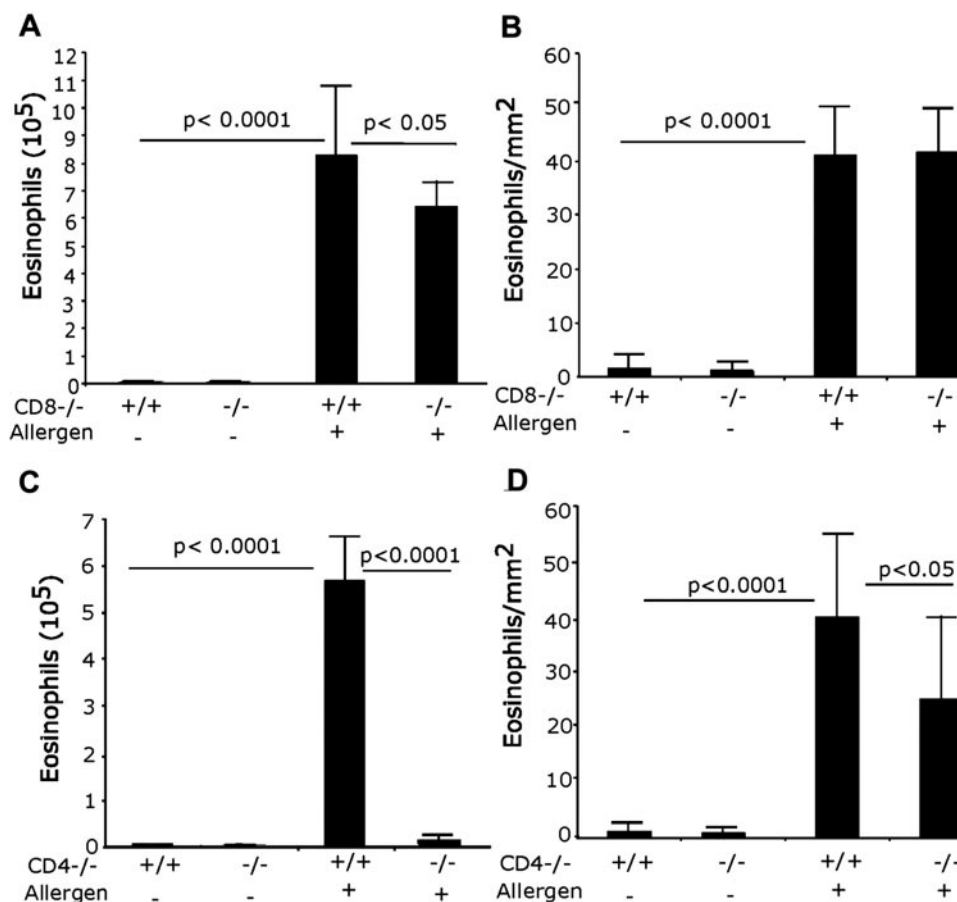
Induction of experimental EE is a T cell-mediated response

To further define the role of T cells, we induced experimental EE in Foxn1 gene-deficient mice. It is notable that Foxn1 gene-deficient mice were protected against disease (Fig. 7, A and B). The number of eosinophils in the esophagus of allergen-treated, wild-type mice was $50.8 \pm 4.8/\text{mm}^2$ compared with $1 \pm 1.5/\text{mm}^2$ (mean $\pm$ SD, $n=9$, $P<0.001$) in saline-treated mice. The eosinophils in the esophagus of allergen-treated Foxn1, gene-deficient mice were $1.7 \pm 0.5/\text{mm}^2$ compared with $1.2 \pm 1/\text{mm}^2$ (mean $\pm$ SD, $n=7-9$) in saline-treated mice (Fig. 7A). It is notable that the percent of PCNA⁺ epithelial cells in the esophageal basal layer of allergen-treated, Foxn1-deficient mice was reduced compared with wild-type mice [38.4 ± 10.9 vs. 19.7 ± 10.7 (mean $\pm$ SD, $n=7-9$, $P<0.001$), respectively; Fig. 7B].

DISCUSSION

EE is now recognized as a medical problem in patients with numerous disorders including gastroesophageal reflux dis-

Fig. 5. Induction of experimental EE in CD4 and CD8 cell-deficient mice. CD4 or CD8 cell-deficient mice as well as wild-type mice were exposed to nine saline and allergen (*A. fumigatus*) treatments, and the induction of eosinophilic lung and esophageal inflammation was monitored. Eosinophil levels in the BALF (A) and esophagus (B) of wild-type (+/+) and CD8 α cell-deficient (-/-) mice are shown. Similarly, eosinophil levels in the lung (C) and esophagus (D) of CD4-deficient mice are shown. Mice receiving allergen are indicated as + and those receiving saline as -. Data are obtained 18–20 h after the last treatment and are expressed as mean $\pm$ SD ($n=9$ –11 mice/group).



ease, drug reactions, eosinophilic gastroenteritis, and primary EE [2–5, 7, 21, 22]. In our previous studies, we developed aeroallergen-induced EE by intranasal administration of *A. fumigatus* antigen [8]. We showed that pulmonary exposure to antigen was critical for disease induction, as oral and intragastric allergen did not induce disease, and aeroallergen-induced esophageal eosinophils have an important role in inducing epithelial hyperplasia [8] consistent with clinical studies correlating the degree of esophageal eosinophil and epithelial hyperplasia in humans [23]. To further examine the connection between the lung airways and esophagus, we compared esophageal responses following intranasal and intratracheal allergen delivery. We hypothesized that intratracheal delivery would be less effective if local delivery of allergen to the esophagus was a critical event in the experimental model. It is surprising that we found that intranasal and intratracheal delivery was equally effective in terms of the kinetics and the magnitude of the esophageal eosinophilia. The induction of esophageal eosinophilia was independent of endotoxin contamination, as endotoxin-free *Aspergillus* antigen induced a similar level of eosinophils in the esophagus as original allergen extract.

Aspergillus antigen is relatively unique compared with a series of other allergens tested for their ability to induce experimental EE. It is notable that *Aspergillus* induces Th1 and Th2 responses, and it is still not understood if EE is completely a Th2 cell-driven disease; therefore, the use of

Aspergillus antigen in experimental EE is relevant in understanding the disease mechanism. In addition, patients with EE have frequent evidence of aeroallergen sensitization, and this has indeed been shown to influence esophageal eosinophil levels [24, 25]. Of note, a clinical study indicated that seasonal pollen exposure induces esophageal eosinophilia [26], consistent with the ability of aeroallergens to induce respiratory tract inflammation and esophagitis in humans [27–29]. Collectively, our present studies add further support to the important role of aeroallergen-induced pulmonary responses in the development of experimental EE.

Furthermore, most prior EE studies have focused on the role of esophageal eosinophils and eosinophil-specific Th2 cytokines in disease pathogenesis [13, 16, 17]; therefore, we now examined lymphocyte subpopulations associated with experimental EE and their role in eosinophil accumulation and tissue pathology. Our observations indicate an increase in B, CD4 $^+$, and CD8 $^+$ cells in the esophagus following allergen exposure. These findings are in accordance with clinical reports that show an increase in CD8 $^+$ lymphocytes in EE patient biopsies [20]. On the basis of our initial observation, we chose to focus on understanding the role of lymphocytes on eosinophil recruitment to the esophagus. We demonstrate that RAG1-deficient mice, which are deficient in functional B cells and CD3 $^+$ TCR-positive cells [19], do not develop experimental EE following allergen

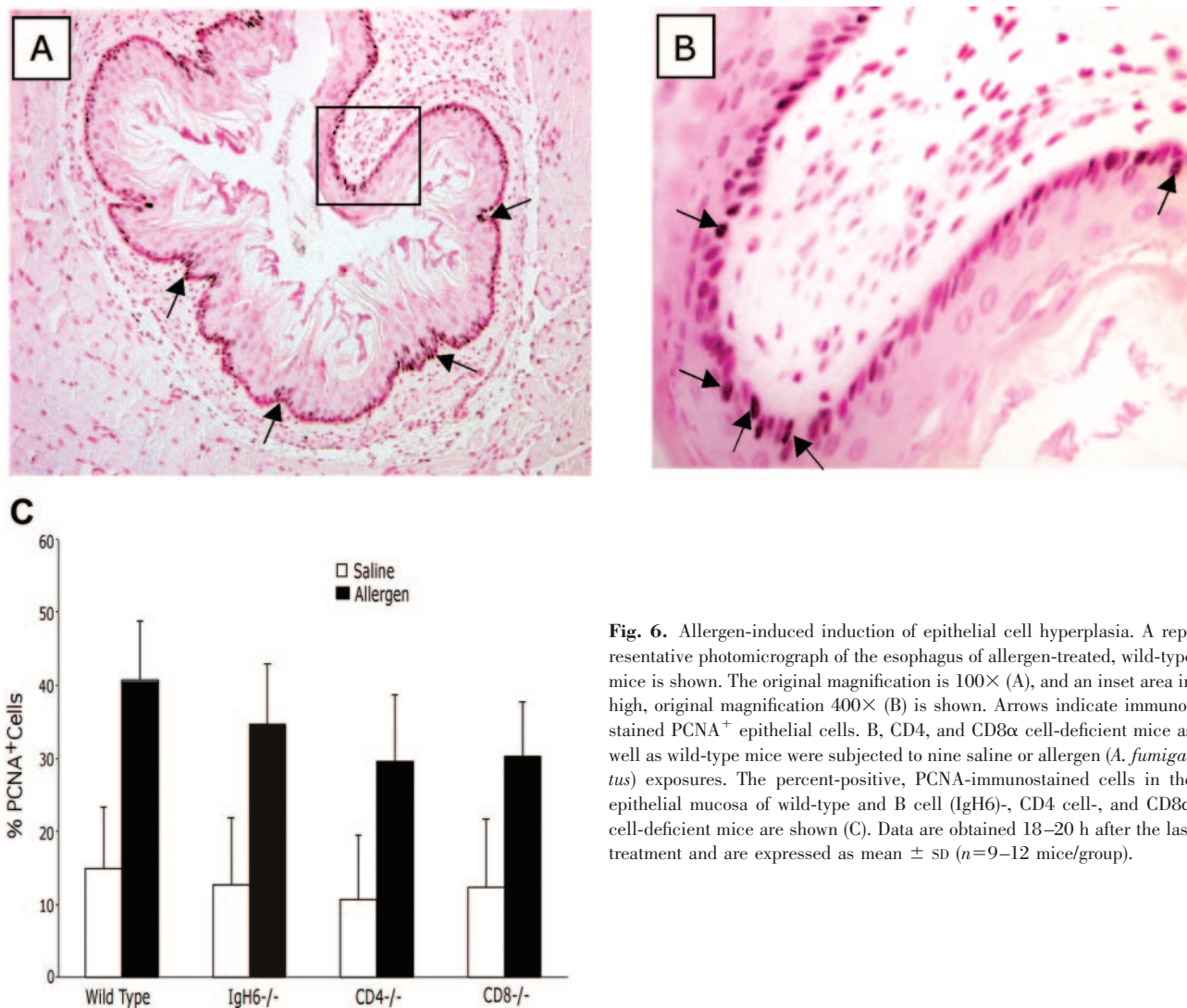


Fig. 6. Allergen-induced induction of epithelial cell hyperplasia. A representative photomicrograph of the esophagus of allergen-treated, wild-type mice is shown. The original magnification is 100 $\times$ (A), and an inset area in high, original magnification 400 $\times$ (B) is shown. Arrows indicate immunostained PCNA⁺ epithelial cells. B, CD4, and CD8 α cell-deficient mice as well as wild-type mice were subjected to nine saline or allergen (*A. fumigatus*) exposures. The percent-positive, PCNA-immunostained cells in the epithelial mucosa of wild-type and B cell (IgH6)-, CD4 cell-, and CD8 α cell-deficient mice are shown (C). Data are obtained 18–20 h after the last treatment and are expressed as mean $\pm$ SD ($n=9$ –12 mice/group).

challenge. This identifies an essential role for lymphocytes in the induction of experimental EE.

Eosinophilic, gastrointestinal diseases are primarily polygenic allergic disorders, which involve mechanisms that fall between pure IgE-mediated and delayed lymphocyte responses [30–32]. To determine whether EE induction is an antibody-mediated response to allergen, we induced experimental EE in homozygous mutant IgH6 mice, which are deficient in B cells and do not express IgM but have normal CD3⁺ cells [33]. We demonstrate that IgH6 mice developed the basic characteristics of EE (esophageal eosinophilia, intraepithelial eosinophils, and epithelial cell hyperplasia). These data demonstrate that the induction of experimental EE is not an antibody-dependent response but rather a T cell-mediated response, at least under these experimental conditions.

To further explore the role of the T cell subsets in experimental EE, we induced EE in cytotoxic T cell (CD8 α)-deficient and Th (CD4)-deficient mice. Our data indicated that CD8⁺ cells have no major role in EE induction, as CD8 α lymphocyte-deficient mice developed esophageal eosinophilia and epithelial hyperplasia at normal levels. However, we observed a partial reduction in esophageal eosinophilic inflammation and complete ablation of eosino-

philic lung airway inflammation in CD4-deficient mice following allergen exposure. Although CD4 cells have a role in allergen-induced EE, their role in the esophagus is less-dominant than in the lung airway. Furthermore, to establish that T lymphocytes have a significant role in EE, we induced disease in Foxn1-deficient mice that lack T cell-mediated immunity but have relatively intact B cell responses [34]. The Foxn1-deficient mice were protected completely from disease etiology, establishing a critical role for T cells in disease induction.

In conclusion, our studies elucidate the role of lymphocytes in the induction of experimental EE in a number of ways. First, increased lymphocyte subpopulations in the esophagus during experimental EE are demonstrated. Second, we identify that antigen-induced antibodies have no role in the development of experimental EE. Third, we demonstrate a partial role for CD4⁺ lymphocytes in EE (but no apparent role for CD8⁺ cells), at least in the setting of the allergen-induced experimental model of EE. Together, these studies indicate a contributory role for adaptive T cell immunity, drawing attention to the need to further elucidate the specific aspects of CD4⁺ and CD4⁻ lymphocytes in experimental EE.

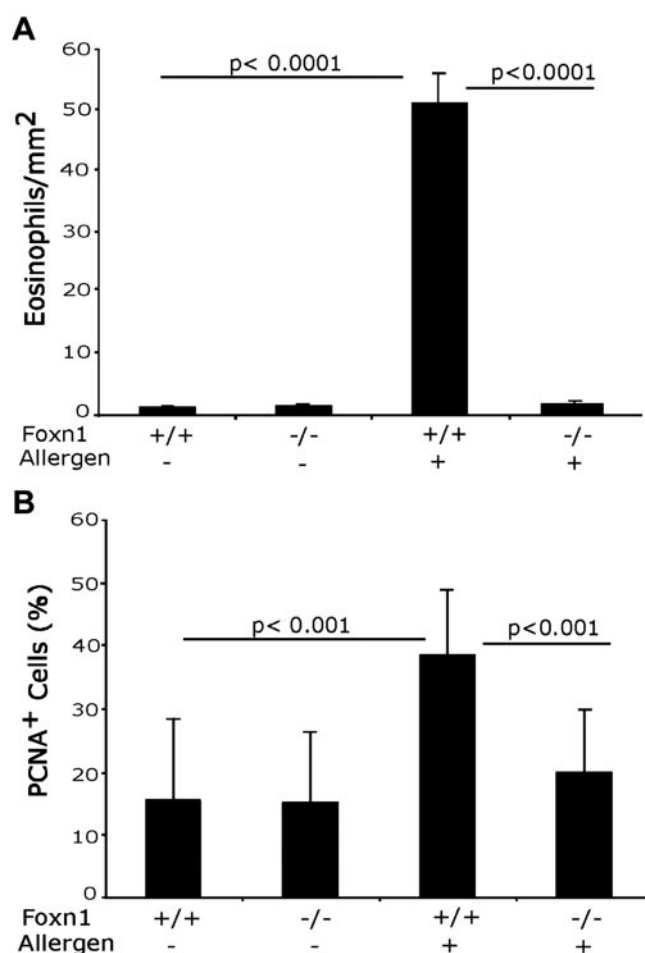


Fig. 7. Induction of experimental disease in Foxn1 gene-deficient mice. T cell-deficient (Foxn1^{-/-}) and wild-type mice were subjected to experimental EE induction following saline and allergen (*A. fumigatus*) exposures. Eosinophil levels in the esophagus (A) of wild-type (+/+) and T cell-deficient (-/-) mice are shown. The PCNA⁺ cells in the epithelial mucosa of the esophagus are shown (B). Mice receiving allergen are indicated as + and those receiving saline as -. Data are obtained 18–20 h after the last of nine treatments and are expressed as mean $\pm$ SD ($n=9$ –10 mice/group).

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