

Epicutaneous Antigen Exposure Primes for Experimental Eosinophilic Esophagitis in Mice

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Background & Aims: Eosinophilic esophagitis (EE) is frequently associated with atopic disease, including dermatitis and asthma. Data are emerging that atopic skin may provide an early entry point for antigen sensitization. We aimed to test the hypothesis that epicutaneous exposure to antigen primes for subsequent respiratory antigen-induced EE. **Methods:** Wild-type and genetically engineered mice were subjected to epicutaneous antigen sensitization and the development of experimental EE, and immune responses were examined. **Results:** We show that exposure to antigen via the epicutaneous route primes for marked eosinophilic inflammation in the esophagus triggered by a single airway antigen challenge. The development of experimental EE is associated with significant skin eosinophilia, accelerated bone marrow eosinophilopoiesis, blood eosinophilia, and large increases in serum antigen-specific immunoglobulin G1/immunoglobulin E using ovalbumin or *Aspergillus fumigatus* as the epicutaneous antigen. Mechanistic analysis with gene-targeted mice showed that interleukin-5 was required for esophageal eosinophilia and that interleukin-4, interleukin-13, and STAT6 contributed to a lesser extent. **Conclusions:** These findings provide the first evidence that epicutaneous exposure to allergens potently primes for EE via a Th2-dependent mechanism.

Eosinophil-associated gastrointestinal disorders selectively affect the gastrointestinal tract with eosinophil-rich inflammation in the absence of known causes for eosinophilia.^{1,2} These disorders include eosinophilic esophagitis (EE), eosinophilic gastritis, eosinophilic gastroenteritis, eosinophilic enteritis, and eosinophilic colitis and are occurring with increasing frequency.²⁻⁹ Recent studies have suggested that eosinophils are integral members of the gastrointestinal mucosal immune system and that eosinophilic gastrointestinal disorders are primarily polygenic allergic disorders that involve mechanisms that fall between pure immunoglobulin (Ig) E-mediated and delayed Th2 cell responses.¹⁰ Preclinical studies have identified a contributory role for the cytokine interleukin (IL)-5 and the eotaxin chemokines, providing a rationale for specific disease therapy.¹¹ An es-

sentia question is to determine the cellular and molecular basis for eosinophil-associated gastrointestinal disorders and the means by which antigen sensitization occurs and elicits disease manifestations.

Epidemiologic studies have found that the development of atopic dermatitis (AD) in childhood is a strong predictive factor for persistence of allergic disease into adulthood.¹²⁻¹⁴ Whether this association represents a genetic predisposition for both diseases or an etiologic connection has not been established. Studies by Spergel and Paller and other groups have shown that epicutaneous exposure to the antigen ovalbumin (OVA) under conditions that promote AD-like skin inflammation can prime for subsequent allergic responses in the airway.¹⁵⁻¹⁸ These results strongly support an etiologic link between the development of AD and subsequent atopic responses; however, whether this response is applicable to other end organs (in addition to the respiratory tract) such as the esophagus has not been established. Understanding if this is applicable to EE is important because the majority of patients with EE have preexisting atopic disease, often involving the skin or lungs.^{4,19-21} Experimentation in the allergy field has largely focused on analysis of the cellular and molecular events induced by allergen exposure in sensitized animals (primarily mice) and humans. Although no animal model adequately mimics human disease, experimentation in animals has provided an experimental framework to dissect the key cells and molecules involved in disease pathogenesis. In these studies, mice are typically sensitized with antigen (eg, OVA in the presence of adjuvant [alum]) by intraperitoneal injection. Subsequently, mice are challenged by exposure to local allergen (eg, via intratracheal, intranasal, or nebulized routes for asthma models) and pathologic responses are monitored.²²⁻²⁵ In other murine mod-

Abbreviations used in this paper: AD, atopic dermatitis; BALF, bronchoalveolar lavage fluid; EE, eosinophilic esophagitis; IL, interleukin; OVA, ovalbumin.

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els, unsensitized mice are repeatedly exposed to mucosal allergens (eg, extracts of *Aspergillus fumigatus* antigens) and the development of experimental asthma or EE is monitored.²⁶ Following 9 doses of intranasal *A fumigatus* antigen, but not OVA, concomitant eosinophilic inflammation of the lung and esophagus develops with features that mimic the human diseases asthma and EE, respectively.²⁶ It is important to note that the standard OVA/alum experimental asthma model does not induce EE, highlighting that *A fumigatus*, which does induce EE, may have a special etiologic role. Additionally, while aeroallergen sensitization is commonly seen in patients with EE, food sensitization is comparably present.^{4,7,19–21}

The experimental systems in the lung have established a strong correlation between the presence of CD4⁺ Th2 cells, eosinophils, and disease severity, suggesting an integral role for these cells in the pathophysiology of allergic disease. IL-4 and IL-13 signaling involves utilization of the IL-4 receptor α chain^{27,28} and the induction of Janus kinase 1 and STAT6.^{29–32} Indeed, mice deficient in IL-4, IL-5, IL-13, or STAT6 have reductions in select features of OVA-induced experimental asthma.^{33–37} Recently, IL-13 delivery to the lung has been shown to also induce experimental EE by a STAT6-dependent pathway.³⁸ However, the role of IL-13 and STAT6 in antigen-induced EE has not been established. Notably, studies with the *A fumigatus*-induced model have shown the importance of STAT6-independent pathways for the development of certain features of allergic lung disease.^{37,39} It remains to be determined if Th2 cytokines and STAT6 are required for the development of EE triggered by *A fumigatus* exposure.

In this study, we aimed to determine if epicutaneous exposure to antigen would prime for subsequent allergen-induced responses in the esophagus of mice. Additionally, we aimed to dissect the mechanism involved, focusing on the role of the Th2 cytokines IL-4, IL-5, and IL-13 and the transcription factor STAT6. Our results show that epicutaneous exposure to *A fumigatus* and OVA potentially primes for systemic allergic disease manifested by large increases in antigen-specific IgG1 and IgE, eosinophilia in the blood and bone marrow, and, following a single antigen challenge, significant eosinophilia in the esophagus. Furthermore, atopic disease elicited by this protocol induces priming of eosinophilic inflammation in the esophagus and lung that is largely dependent on IL-5 and, to a lesser extent, IL-4, IL-13, and STAT6. As such, these studies provide a new paradigm concerning the potential etiology of EE, involving cutaneous antigen sensitization as an initiating event.

Materials and Methods

Mice

Four- to 8-week-old BALB/c mice (National Cancer Institute, Frederick, MD), STAT6-deficient BALB/c mice (Jackson Laboratory, Bar Harbor, ME), and IL-5-deficient (originally obtained from Drs K. Matthaei and P. S. Foster, John Curtin School of Medical Research, Australian National University, Canberra, Australia), IL-13-deficient, IL-4/IL-13 double-deficient, and their littermate F6 BALB/c controls (originally obtained from A. MacKenzie, Medical Research Council Laboratory of Molecular Biology, Cambridge, England) were kept in a specific pathogen-free environment. All procedures were performed in accordance with the ethical guidelines in the Guide for the Care and Use of Laboratory Animals of the Institutional Animal Care and Use Committee approved by the Veterinary Services Department of the Cincinnati Children's Hospital Medical Center Research Foundation.

Allergen Sensitization Protocol

Mice were sensitized as previously described with some modification.^{16,40} Briefly, mice were anesthetized with isoflurane (IsoFlo; Abbott Laboratories, North Chicago, IL), their backs shaved with an electric razor, and allergen applied via a 1 × 1-cm patch of sterile gauze that was previously immersed in a diluted allergen solution. The allergen solutions were made as follows: 100 μ g of OVA (grade V; Sigma Chemical Co, St Louis, MO) was diluted in 100 μ L of normal saline, and 100 μ g of *A fumigatus* antigen (Allergenic Extract; Hollister-Stier, Spokane, WA) was diluted to 100 μ L with normal saline. The allergen diluents consisted of 100 μ L of normal saline as a control for OVA or 100 μ L of 5% glycerin diluted with normal saline as a control for *A fumigatus*. The patch was secured by a transparent Bioclusive dressing (Johnson & Johnson Medical Inc, Arlington, TX) and a Band-Aid (Johnson & Johnson) for 1 week. Following a rest period of 2 weeks (no patch), this procedure was repeated 3 times over the course of 7 weeks for OVA sensitization or repeated 2 times over the course of 4 weeks for *A fumigatus* sensitization. The experiments were performed with 4 groups: (1) OVA group, (2) saline group (as the control for the OVA group), (3) *A fumigatus* group, and (4) control group (as the control for the *A fumigatus* group).

Blood Eosinophil Counts

Eosinophil counts were measured by Discombe's analysis using 5 μ L of blood obtained from the lateral tail vein as previously described.⁴¹

Serum IgE and Antigen-Specific IgG1

Serum total IgE levels were measured using the BD OptEIA ELISA Set (BD Biosciences, San Diego, CA). The optical density was immediately read at 450 nm. The IgE concentration of each sample was calculated by using a standard curve. OVA- and *A fumigatus*-specific IgG1 were measured as described previously by coating sample wells with 100

μL of OVA (100 $\mu\text{g}/\text{mL}$) and *A. fumigatus* (50 $\mu\text{g}/\text{mL}$), respectively.⁴² The optical density was read at 450 nm. Data are presented as the serum dilution required to obtain an optical density of 0.6.

Airway Challenge

Two days after the end of epicutaneous sensitization, mice were deeply anesthetized with isoflurane inhalation and given a single intranasal challenge with 25 μL of allergen (1 mg/mL OVA or 1 mg/mL *A. fumigatus*) or 25 μL of each vehicle alone.

Bronchoalveolar Lavage Fluid Analysis

Twenty-four hours after the airway challenge, mice were killed and the recovered bronchoalveolar lavage fluid (BALF) was collected immediately by lavaging once with 1.0 mL phosphate-buffered saline, cytopinned (Cytospin 3; Thermo Shandon, Sewickley, PA), and stained with Diff-Quick (Dade Behring Inc, Deerfield, IL), and differential cell counts were determined.

Bone Marrow Analysis

Twenty-four hours after the last epicutaneous sensitization, mice were killed and bone marrow cells were obtained from the femoral bone; cytopsin slides were prepared as previously described⁴³ and then stained with Diff-Quick, and eosinophil cell counts (expressed as the percentage of total bone marrow cells) were determined by counting 1000 cells/slide. The eosinophil maturation assay was performed in 1 mL methylcellulose culture, which was made up of methylcellulose medium (Methocult M3234; Stemcell Technologies, Seattle, WA), 20% fetal calf serum, and 5 ng/mL of recombinant mouse IL-5 (R&D Systems, Minneapolis, MN) with 1×10^5 nonadherent mononuclear cells to identify the differentiated cells from bone marrow precursor cells. Sample cells in 10-day culture were evaluated as previously described⁴³ according to the criteria established by Lee et al.⁴⁴

Tissue Processing and Staining

Esophagus, lung, and skin tissue were immediately fixed in 10% formalin, paraffin embedded, and cut into 5- μm -thin sections. To identify tissue eosinophils, immunostaining for eosinophil major basic protein was performed as previously described¹¹ using rabbit anti-murine major basic protein (kindly provided by Dr. James Lee, Mayo Clinic, Scottsdale, AZ). Diff-Quick staining was also used to assess eosinophils and mast cells in a modified fashion after deparaffinization. Briefly, slides were fixed in acetone/methanol at room temperature for 5 minutes and then stained with Diff-Quick solutions, followed by a single wash in tap water and a 1-minute exposure to methanol and xylene. The count was performed under light microscopy. Quantification of immunoreactive cells in the lamina propria was performed by morphometric analysis using the Metamorph Imaging System (Universal Imaging Corp, West Chester, PA) and Image Pro Plus System (Olympus America, Melville, NY).

Statistical Analysis

For all cell counts, stained slides were read randomly and in a blinded fashion. The nonparametric Mann-Whitney *U* test was used for comparison of data between groups, and analysis of variance was used to evaluate the differences in clinical symptoms and time studies.

Results

Epicutaneous OVA Induces Cutaneous and Systemic Th2 Responses

Previous studies have shown that epicutaneous exposure to the food antigen OVA induces AD-like eosinophilic inflammation locally in the skin accompanied by production of Th2-associated Ig and lymphocyte responses.^{15,16,40,45} Furthermore, following a single respiratory OVA challenge, epicutaneously sensitized mice developed marked lung inflammation.^{16,18} Related to this observation, we tested several hypotheses concerning EE. First, we were interested in determining if epicutaneous administration of antigen alone would induce EE. To examine this, we exposed mice to repeated doses of epicutaneous OVA or *A. fumigatus* antigen, the latter being the antigen that has previously been associated with the development of experimental EE following repeated intranasal administration. Notably, exposure to either epicutaneous antigen alone failed to induce eosinophil accumulation in the esophagus even though they promoted marked AD-like skin inflammation. For example, following 51 days of epicutaneous OVA or control, histologic analysis of the local skin site showed 40.0 ± 7.31 versus 6.67 ± 2.56 eosinophils/ mm^2 ($P < .01$) and 158 ± 29.4 versus 51.5 ± 7.98 mast cells/ mm^2 ($P < .05$), respectively, even though esophageal eosinophil levels were barely detectable (data not shown). Cutaneous eosinophils were mostly located in the dermis and were primarily not degranulated (data not shown). We next hypothesized that epicutaneous administration of antigen followed by intranasal antigen would induce EE. Notably, mice exposed to epicutaneous OVA developed marked EE following a single administration of intranasal OVA (Figure 1A). Additionally, mice exposed to epicutaneous *A. fumigatus* developed marked EE following a single administration of intranasal *A. fumigatus*. As a control, nonsensitized control mice failed to develop EE following intranasal allergen challenge. Furthermore, allergen-sensitized mice failed to develop EE when exposed to intranasal control saline (Figure 1A). Histologic analysis showed readily detectable eosinophils in the lamina propria of the esophagus of mice (Figure 1B). Scattered eosinophils were seen in the epithelial and muscle layers.

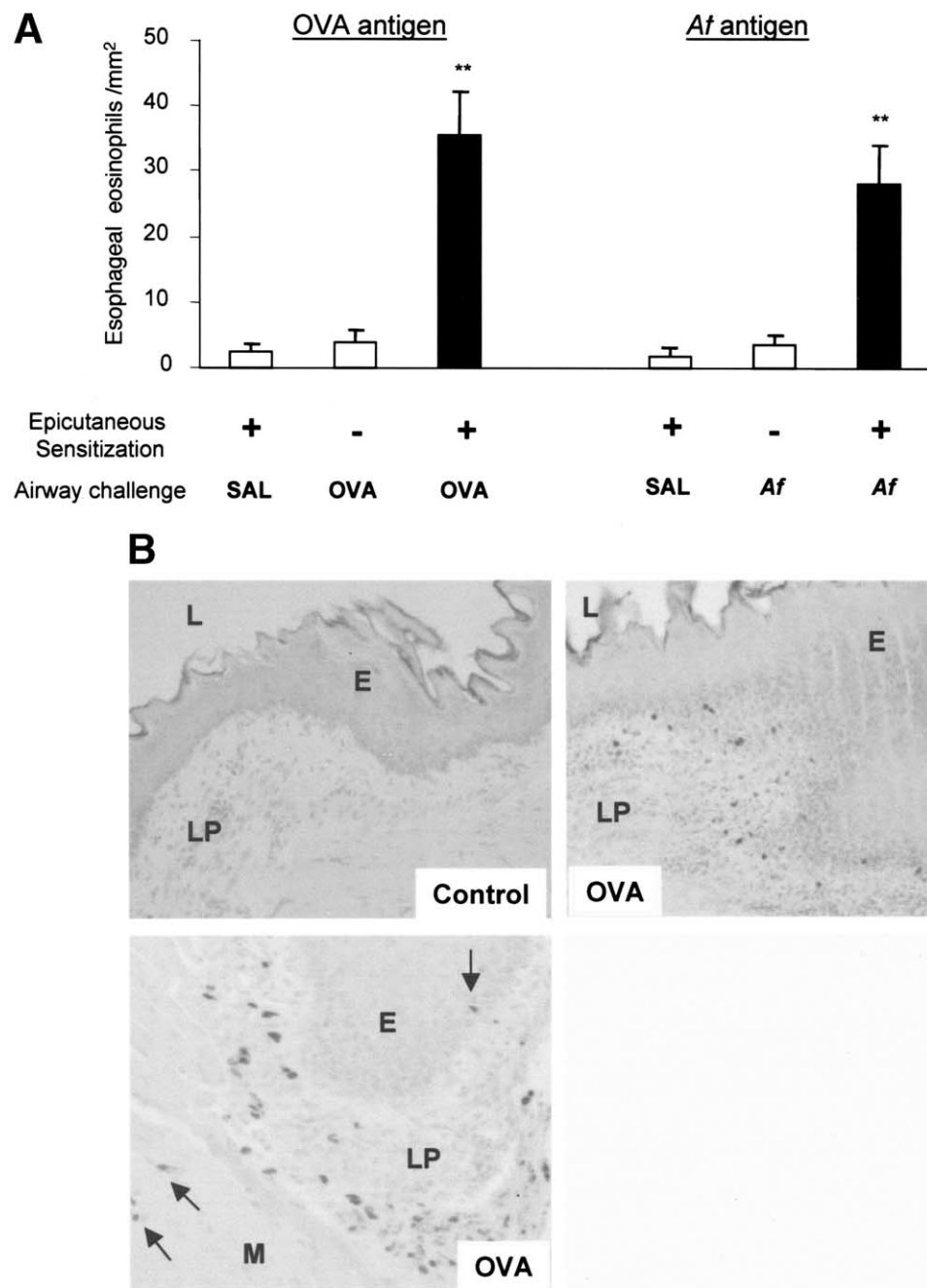


Figure 1. Esophageal eosinophilia in epicutaneously sensitized mice. (A) The eosinophil level in the esophagus of mice sensitized with OVA or *A fumigatus* (+) or control vehicle (-). Mice were exposed to epicutaneous antigen for 1 week; following a rest period of 2 weeks, this procedure was repeated over the course of 7 weeks (OVA) and 4 weeks (*A fumigatus*). Two days following the last day of epicutaneous antigen exposure, mice were exposed to intranasal allergen or control saline (SAL). Data are expressed as mean $\pm$ SEM with 8 mice/group. ** $P < .01$ compared with control or saline group. (B) Eosinophils were identified by immunostaining with anti-major basic protein. Representative eosinophils are indicated by arrows. Significant eosinophilia was observed in the OVA group but not in the control group. Eosinophils were observed mainly in the lamina propria (LP), but occasional eosinophils were observed in epithelial (E) or muscle layers (M). L, lumen. (Original magnification: upper figures, 100 $\times$; lower figure, 400 $\times$.)

Kinetic Study on Eosinophil Accumulation in the Lung and Esophagus

The development of experimental EE was associated with the development of marked lung inflammation. A kinetic analysis of eosinophil accumulation in the lung and esophagus after a single antigen challenge with OVA or *A fumigatus* is shown in Figure 2A and B, respectively. Eosinophil accumulation in both tissues was notable between 1 and 4 hours following respiratory antigen challenge. Interestingly, eosinophilia peaked by 4 hours for both OVA and *A fumigatus* in the esophagus.

In the lung, eosinophilia peaked between 18 and 24 hours. Notably, barely detectable eosinophils were present in the lung and esophageal tissue before antigen challenge.

Systemic Effect of Epicutaneous Antigen Administration

To examine the events associated with epicutaneous priming for EE, we analyzed the consequences of epicutaneous antigen exposure on eosinophil levels in the hematopoietic organs. As shown in Figure 3A, the eo-

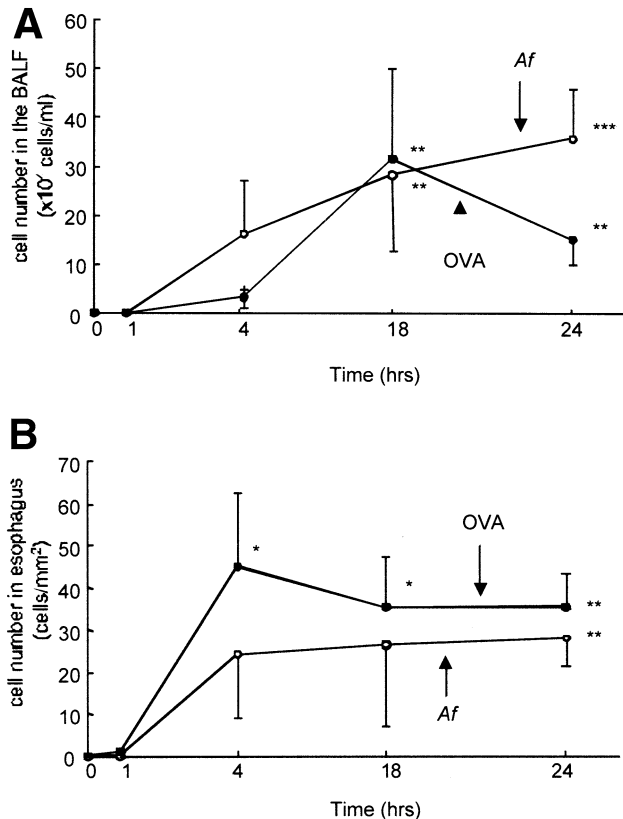


Figure 2. Kinetic analysis of eosinophil accumulation in the (A) lung and (B) esophagus following epicutaneous antigen sensitization. Two days following the last day of epicutaneous antigen exposure, mice were exposed to intranasal allergen or control saline, and the level of eosinophils was kinetically determined for both the OVA (closed circles) and *A fumigatus* (open circles) groups. * $P < .05$, ** $P < .01$, *** $P < .001$ compared with control group. Data are expressed as mean $\pm$ SEM; $n = 5-8$ mice per condition.

sinophil count in the bone marrow was significantly increased following epicutaneous OVA. In addition, the number of mature eosinophils in ex vivo cultures of bone marrow mononuclear cells (an index of eosinophilopoiesis) of OVA-sensitized mice was significantly increased (Figure 3B). Furthermore, following epicutaneous OVA exposure, the blood eosinophil counts were elevated compared with the saline group on days 7, 28, and 50 (Figure 3C). The reason for the decrease in eosinophils at day 21 is not known. However, it is interesting to speculate that this may represent dwindling Th2 immunity after the first antigen patch because day 21 is the last of 14 rest days after the first antigen exposure. These data suggest that epicutaneous antigen exposure induces a systemic Th2 response, as measured by the expansion of eosinophils. Consistent with this, mice exposed to epicutaneous OVA had significant increases in antigen-specific IgG1 and IgE notable by days 19 and 29, respectively (data not shown). For example, following 50 days of epicutaneous OVA, the titer of OVA-specific IgG1 and IgE increased

from 0.0 ± 0.0 to $30,970 \pm 8506$ and 0.0 ± 0.0 to 41.8 ± 31.9 , respectively (mean $\pm$ SEM; $n = 8$; $P < .001$).

The Role of IL-5 in Epicutaneous Antigen-Associated EE

We were next interested in examining the role of IL-5 in the induction of epicutaneous antigen-induced eosinophil responses. To examine this, we monitored

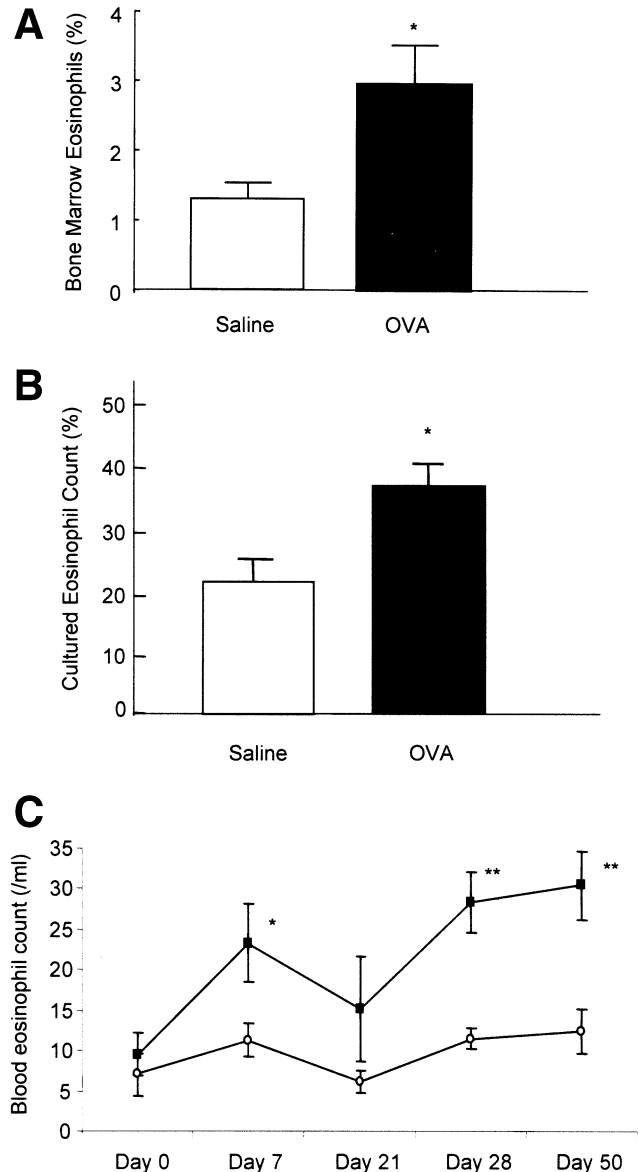


Figure 3. Epicutaneous antigen-induced eosinophilopoiesis. (A) The eosinophil count in the bone marrow was assessed by differential staining following OVA or control sensitization. (B) Eosinophil development in methylcellulose in ex vivo cultures after 50 days from the initial antigen sensitization. (C) The level of eosinophils in the peripheral blood during the first 50 days of epicutaneous OVA (closed squares) or control saline (open squares) exposure is shown. * $P < .05$ compared with the saline group. Data are expressed as mean $\pm$ SEM; $n = 8$ mice per condition.

eosinophil responses in mice deficient in IL-5. Notably, following epicutaneous OVA or *A fumigatus*, IL-5-deficient mice developed markedly attenuated eosinophilia in the esophagus following respiratory allergen challenge compared with wild-type mice (Figure 4A and B). To further examine eosinophil responses in these mice, eosinophil levels in the skin, blood, and lungs following epicutaneous sensitization were determined. In the skin, antigen-induced eosinophilia was attenuated, although inflammation with the other cell types was still observed. The data for OVA-induced skin inflammation are shown in Table 1. Additionally, allergen-induced blood eosinophilia was absent in these mice (Figure 5A). In the lung, allergen-induced eosinophilia was markedly decreased in these mice (Figure 5B). Taken together, these results show an essential role for IL-5 in promoting eosinophil responses following epicutaneous sensitization.

The Role of Th2 Cytokines in Epicutaneous Antigen-Induced EE

We were next interested in testing the role of the classic Th2 cytokines (IL-4 and IL-13) and their signaling molecule STAT6 in the development of epicutaneous antigen-associated EE. To examine this, we induced experimental disease in mice with genetic deficiencies of either IL-13, IL-4 and IL-13 together, or STAT6, as well as control BALB/c mice. Notably, mice deficient in IL-13 alone or IL-4 and IL-13 together had reduced antigen-induced EE following OVA (Figure 4A) or *A fumigatus* antigen (Figure 4B) exposure and challenge compared with wild-type mice. Notably, the effect was only modest compared with IL-5-deficient mice (Figure 4A and B). To further examine eosinophil responses in these mice, eosinophil levels in the skin, blood, and lungs following epicutaneous sensitization were determined. In the skin, antigen-induced eosinophilia was attenuated but similarly not as significantly as the decrease seen in IL-5-deficient mice (Table 1). Additionally, allergen-induced blood eosinophilia was diminished in these mice (Figure 5A). Similar trends were seen in the *A fumigatus* model. For example, the eosinophil blood level increased from 8.8 ± 2.1 to $17.9 \pm 2.7 \times 10^4$ cells/mL between days 0 and 30 of *A fumigatus* exposure in wild-type mice ($P < .05$); in contrast, the values changed from 0.3 ± 0.2 to 0.7 ± 0.4 (not significant), 2.6 ± 1.1 to 8.1 ± 1.8 ($P < .05$), 6.7 ± 2.8 to 6.4 ± 3.0 (not significant), and 4.0 ± 1.2 to $9.3 \pm 1.6 \times 10^4$ cells/mL ($P < .05$) in mice deficient in IL-5, IL-13, IL-4/IL-13, and STAT6, respectively. In the lung BALF, allergen-induced eosinophilia was also decreased in these mice (Figure 5B).

Finally, we were interested in determining the role of Th2-associated molecules in antigen-induced Ig re-

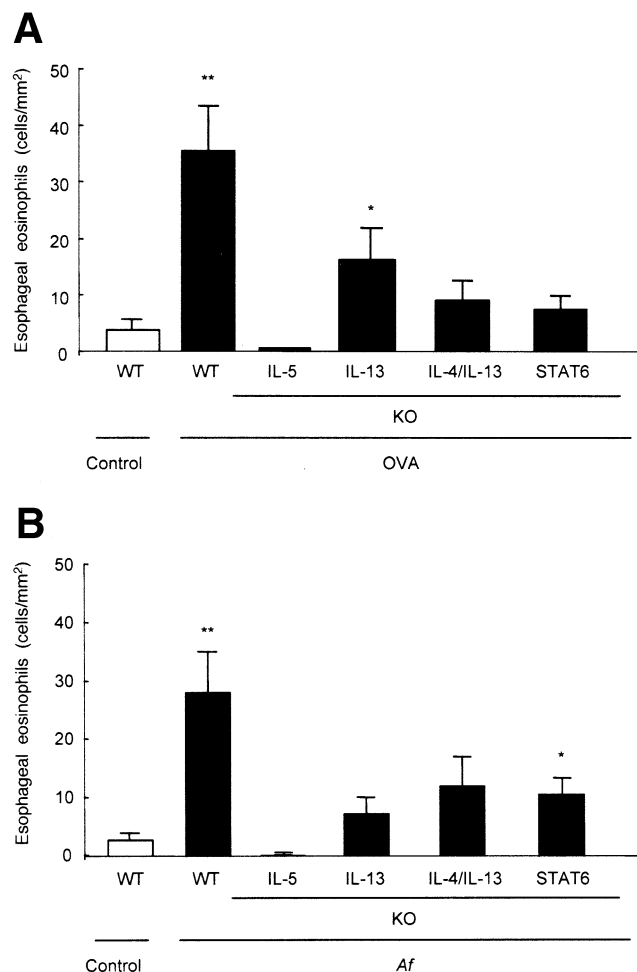


Figure 4. Esophageal eosinophil levels in Th2 cytokine-deficient mice. Epicutaneously sensitized mice were challenged with (A) control saline or OVA or (B) diluent control or *A fumigatus*. Mice were wild-type (WT) control or deficient in IL-5, IL-13, IL-4/IL-13, or STAT6 and analyzed after 29 and 50 days from the initial antigen sensitization for *A fumigatus* and OVA, respectively. * $P < .05$, ** $P < .01$ compared with wild-type saline group. Data are expressed as mean $\pm$ SEM; $n = 8$ mice per condition.

sponses. Notably, OVA-specific IgG1 and IgE were strongly dependent on IL-13, but not completely (Figure 6A and B). Furthermore, IL-4/IL-13 double-deficient mice and STAT6-deficient mice failed to develop detectable levels of OVA-specific IgG1 and IgE. In contrast, IL-5-deficient mice developed normal levels of OVA-specific IgG1 and IgE (Figure 6A and B).

Discussion

Eosinophil infiltration into the esophagus is a commonly observed medical problem in patients with diverse conditions, including gastroesophageal reflux disease, drug reactions, eosinophilic gastroenteritis, and primary EE.¹⁻¹⁰ Importantly, recent clinical studies have suggested that these disorders (especially primary EE) are

Table 1. Cutaneous Allergic Inflammation in Th2 Cytokine-Deficient Mice

Mice	Eosinophils	Mast cells	Neutrophils	Mononuclear cells
Wild-type	40.0 ± 7.31	158 ± 29.4	95.8 ± 18.2	296 ± 55.5
IL-5 KO	0.56 ± 0.56 ^a	144 ± 19.7	71.9 ± 17.1	323 ± 20.1
IL-13 KO	12.1 ± 5.31 ^b	95.8 ± 12.0	75.9 ± 11.8	347 ± 37.0
IL-4/IL-13 DKO	15.2 ± 7.42 ^c	115 ± 9.79	94.3 ± 19.7	346 ± 37.0
STAT6 KO	17.0 ± 9.12 ^c	120 ± 33.4	117 ± 15.6	347 ± 34.6

NOTE. Values represent mean ± SEM of the cell count in the subepithelial region/mm² (n = 8 mice per condition) following 51 days of epicutaneous OVA exposure.

KO, gene-deficient knockout mice; DKO, double gene-deficient knockout mice.

^aP < .001, ^bP < .01, ^cP < .05 compared with wild type.

occurring with increasing frequency.^{6,9} Additionally, the level of eosinophils in the esophagus negatively correlates with response to conventional therapy for gastroesophageal reflux.⁴⁶ Furthermore, a causal role for esophageal

eosinophils in the development of epithelial hyperplasia, a cardinal feature of primary EE, has been shown in an experimental model of intranasal aeroallergen-induced EE,²⁶ consistent with the presence of activated eosinophils in human EE.⁴⁷ Taken together, these studies highlight the importance of further dissecting the pathogenesis of EE. Accordingly, the present study was designed to examine the means by which antigen sensitization might lead to EE and the molecular and cellular processes involved in the development of experimental EE. Based on the high reported concordance between atopy and EE in clinical studies,^{10,19,20} we hypothesized that AD may precede the development of EE in an experimental model system in mice. Notably, EE and AD share common features, including eosinophil infiltration, eosinophil degranulation, and squamous epithelial cell hyperplasia,^{48–50} suggesting that common pathogenetic mechanisms may be operational. To address this, we tested the ability of epicutaneous antigen exposure to induce EE. First, we showed that epicutaneous exposure to the allergens OVA or *A fumigatus* alone induces AD-like skin inflammation and a strong systemic Th2 response. Despite chronic cutaneous antigen exposure and the development of accelerated bone marrow eosinophilopoiesis and circulating eosinophilia, eosinophils do not migrate into the esophagus. However, when we subsequently expose epicutaneously sensitized mice to a single dose of intranasal antigen, this induces a marked degree of EE (and lung inflammation). Notably, the development of allergen-induced experimental EE has been previously observed only following multiple doses of intranasal *A fumigatus* (not OVA exposure).²⁶ Thus, these data establish that epicutaneous sensitization is a highly efficient process for the development of experimental EE (compared with intranasal antigen sensitization alone). Furthermore, these data establish that the development of experimental EE can be triggered by the classic experimental allergen OVA, not just the respiratory aeroallergen *A fumigatus*. As such, this experimental system opens up new approaches for dissecting the pathogenesis

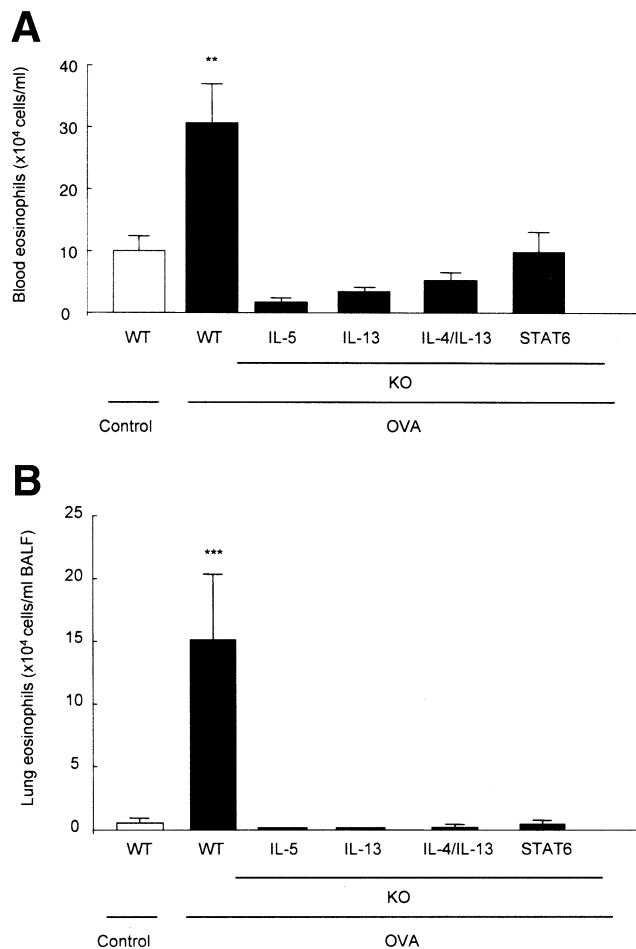


Figure 5. Blood and BALF eosinophil levels in Th2 cytokine-deficient mice. (A) Blood eosinophil levels were measured 24 hours after the end of epicutaneous sensitization. (B) Two days after the end of epicutaneous sensitization, mice were challenged with control saline or OVA and eosinophil levels were measured in the BALF 24 hours after one intranasal OVA challenge. Mice were wild-type (WT) control or deficient in IL-5, IL-13, IL-4/IL-13, or STAT6. **P < .01, ***P < .001 compared with wild-type saline group. Data are expressed as mean ± SEM; n = 8 mice per condition.

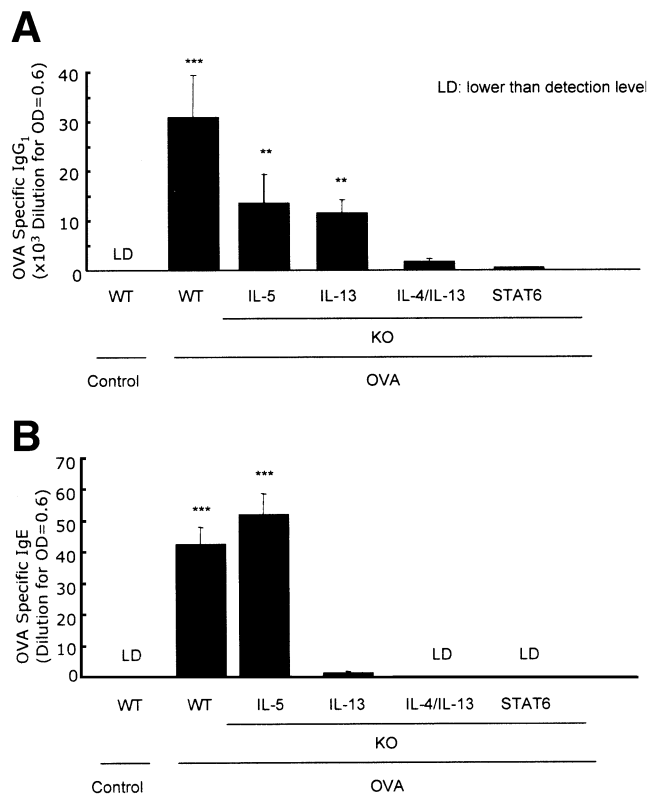


Figure 6. Systemic Th2 responses in epicutaneously sensitized Th2 cytokine-deficient mice. (A) OVA-specific IgG1 and (B) IgE levels were measured 24 hours after the end of epicutaneous sensitization. Mice were wild-type (WT) control or deficient in IL-5, IL-13, IL-4/IL-13, or STAT6. ** $P < .01$, *** $P < .001$ compared with wild-type saline group. Data are expressed as mean $\pm$ SEM; $n = 8$ mice per condition. LD, lower than detection limit.

of experimental EE, because numerous tools are available for analysis of OVA-specific immunity. Our finding that the systemic Th2 response is associated but not sufficient for EE development is consistent with our prior finding that intratracheal IL-13 can elicit experimental EE without inducing a systemic Th2 response and/or peripheral blood eosinophilia.³⁸ Our results are in agreement with those of Spergel et al, who have shown that epicutaneous OVA can promote a Th2-associated skin response and lung inflammation following only a single dose of respiratory antigen.¹⁶ Notably, we extend these results by reporting that *A fumigatus* can promote this response as well and that epicutaneous antigen sensitization primes for a Th2-associated response in the esophagus.

We report an essential role for IL-5 in mediating the effects of antigen-induced EE following epicutaneous sensitization. Prior studies have shown that IL-5 is required for the induction of experimental EE by aeroallergens and that IL-5 overexpression (by transgenesis or pharmacologic delivery) is sufficient to promote eosinophil trafficking to the esophagus.^{11,26} In addition, esoph-

ageal eosinophilia induced by exposure of OVA-sensitized mice to enteric-coated OVA beads is IL-5 dependent.¹¹ This latter finding is particularly significant because these oral antigen-challenged IL-5-deficient mice still mount intestinal eosinophilia,⁴² highlighting that there is a differential requirement of IL-5 in regulating esophageal and intestinal eosinophilia. IL-5 is known to prime eosinophils to respond to chemoattractants and to induce eosinophil adhesion molecule expression and activation.⁵¹ Thus, IL-5 may induce eosinophil trafficking to the esophagus by enhancing eosinophil responsiveness to endogenous chemokines expressed by the esophagus, such as the eotaxins, or by up-regulating homing receptors specifically involved in eosinophil trafficking to the esophagus. It also remains possible that the decreased level of esophageal eosinophils seen in IL-5-deficient mice may be related to the decreased circulating pool of eosinophils, at least in part. Of note, eotaxin-1 is a constitutively expressed chemokine in the esophagus⁵² that is induced by the Th2 cytokines IL-4 and IL-13. To evaluate the potential role of IL-4, IL-13, and STAT6 in mediating allergen-induced eosinophil trafficking to the esophagus, we examined mice that were genetically deficient in either IL-4 and IL-13 together or STAT6. It is noteworthy that although antigen-induced EE was largely dependent on these signaling molecules, the requirement was only partial because allergen-treated IL-4/IL-13 double-deficient and STAT6-deficient mice still had higher esophageal eosinophil counts compared with saline-treated wild-type mice. These results provide the first evidence that allergen-induced EE is dependent on classic Th2 cytokine signaling. Prior studies have only shown that IL-13-induced experimental EE is STAT6 dependent. Furthermore, the finding that IL-5 is absolutely required for the development of experimental EE (compared with the partial requirement for IL-4, IL-13, and STAT6) draws increasing attention to the importance of IL-5 in disease pathogenesis. Indeed, recent clinical studies have reported overexpression of IL-5 in the esophageal lesions of patients with primary EE⁵³ and the beneficial role of anti-IL-5 in a patient with EE.⁵⁴

Our study highlights the intimate connection between the development of allergic respiratory responses and EE, because EE only developed following respiratory allergen exposure. Furthermore, our study is the first to show that mechanistic differences may exist between the lung and the esophagus. For example, the kinetic analysis following allergen challenge shows that eosinophil infiltration into the esophagus peaks within 4 hours following the single allergen challenge, whereas eosinophil infiltration into the lung continues to peak for at least 24 hours. Furthermore, our results show that the development of

EE is less dependent on IL-4, IL-13, and STAT6 compared with the development of allergic airway inflammation. However, it is important to note that a direct comparison may not be valid because our studies in the lung are primarily based on BALF analysis.

In summary, these investigations dissect the cellular and molecular mechanisms involved in eosinophil homing to the murine esophagus. These data show that allergen delivery to the skin potently primes for respiratory antigen-induced EE. In addition, we show a critical role for IL-5 and (to a lesser extent) IL-4, IL-13, and STAT6, strongly implicating Th2-associated immune responses in the pathogenesis of EE. Prior studies have implicated Th2 cytokines based on detection of the Th2 cytokines IL-4 and IL-13 in EE tissue biopsy specimens^{53,55} and the ability of IL-13 delivery to the lung to induce experimental EE.³⁸ Our study is the first to show that the classic Th2 signaling molecules IL-4, IL-13, and STAT6 are indeed required for antigen-induced disease pathogenesis, at least in part. Taken together with our prior findings that EE is often associated with a history of atopy,^{4,9,21} these results draw attention to the potential role of epicutaneous antigen sensitization in the etiology of experimental EE in mice.

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