

# Induction of Severe Eosinophilic Esophagitis and Multi-Organ Inflammation by Airborne Allergens is Associated with IL-4/IL-13 and CCL11 but Not IgE in Genetic Susceptible Mice

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**Background:** Eosinophilic Esophagitis (EoE) is an increasingly common chronic inflammatory disease. The pathological mechanisms underlying EoE are largely unknown.

**Objective:** We sought to understand the mechanisms underlying aeroallergen-induced EoE in Sharpin gene deficient (Sharpin<sup>-/-</sup>) mice that is prone to inflammatory response.

**Methods:** Sharpin<sup>-/-</sup> mice were exposed with *Aspergillus fumigatus* and ovalbumin intranasally every alternate day for 4 weeks. Wild type (WT) naïve mice, WT exposed, and un-exposed Sharpin<sup>-/-</sup> mice were controls. Histopathological analysis was performed by H&E, trichrome and major basic protein staining. Total and specific IgE, IgG, and IgA levels were measured by ELISA and Th2 cytokine and CCL11 chemokine gene expression were determined.

**Results:** Airborne allergen exposed Sharpin<sup>-/-</sup> mice showed severe eosinophilic inflammation in the esophagus ( $p < 0.001$ ), and markedly increased epithelial thickening ( $p < 0.0001$ ) compared to WT normal controls, whereas airborne allergen exposed WT mice and unexposed Sharpin<sup>-/-</sup> mice only showed mild eosinophilic inflammation in the esophagus. These exposed Sharpin<sup>-/-</sup> mice also showed over 7-fold increase in blood eosinophils ( $p < 0.0001$ ), 60-fold increase in eosinophils in bronchoalveolar lavage fluid ( $p < 0.0001$ ) and 4-fold increase in eosinophils in the skin ( $p < 0.0001$ ) compared to normal controls. Surprisingly, exposed Sharpin<sup>-/-</sup> mice did not show elevation of serum total or antigen-specific IgE levels but reduced total IgA and IgG levels than normal controls. There was a marked increase in IL-4, IL-13 and CCL11 gene expression in esophageal tissue ( $p < 0.001$ ) in exposed Sharpin<sup>-/-</sup> mice compared to WT normal mice.

**Conclusion:** Th2 cytokines and chemokines, but not IgE may play an important pathologic role in aeroallergen-induced EoE. This study may provide insight into new therapeutics for EoE.

**Keywords:** aeroallergen, eosinophilic esophagitis, non-IgE, Th2

## Introduction

EoE is a chronic allergic inflammatory disease of the esophagus characterized by reflux symptoms, feeding difficulty in young children, pain on swallowing, food impaction, esophageal stricture formation and esophageal rupture.<sup>1</sup> Histologically, it is characterized by a defective epithelial barrier and eosinophilic infiltration (>15 eosinophil/HPF) in one or more of at least four esophageal biopsy specimens obtained by endoscopy.<sup>2</sup> Many people still consider the disease relatively rare, but the number of patients diagnosed with EoE is increasing. The incidence of EoE is approximately 7.7/100,000 per year in adults, and EoE affects an estimated 34.4/100,000 people in Europe and North America.<sup>3</sup> There are no FDA-approved treatments for EoE. The current available therapy is either diet, proton pump inhibitors or swallowed

topical steroids. The ideal diet therapy is an elemental diet, which consist of an amino acid based formula and thus excludes the most common food triggers of EoE.<sup>4</sup> The other options for diet therapy are empiric elimination diets which require repeat endoscopies to assess disease response. Diet therapy is difficult and often associated with relapse after discontinuation of the therapy.<sup>5</sup> Swallowed corticosteroids are the mainstay of pharmacologic therapy for EoE; however, some the drawbacks include – lack of an FDA approved preparation and readily available formulations optimized for esophageal delivery.<sup>6</sup> Proton pump inhibitors have also shown efficacy for the treatment of EoE but have potential long-term side effects including kidney disease, osteoporosis and cancer.<sup>7</sup>

EoE is associated with other allergy-induced diseases. Most patients with EoE are atopic.<sup>8</sup> It has been hypothesized that eosinophils buildup in the epithelial lining of the esophagus in response to various ingested and inhaled allergens.<sup>9</sup> Most studies have reported prevalence of different atopic condition in young children<sup>10</sup> and adult<sup>11</sup> with EoE. These studies have indicated that patients with EoE have a higher frequency of asthma, allergic rhinitis, eczema, atopic dermatitis, and food allergies.<sup>9</sup> There also exists a relationship between aeroallergens and EoE.<sup>12</sup> Cases of EoE have reportedly flared during pollen season (spring and summer) and remitted during the winter.<sup>13</sup> Similarly, a twin study has estimated that the risk of EoE due to shared family environment is 2.4% in siblings with the disease. These factors strongly highlight the complexity of the disease and its association with other allergic conditions. Previous evidence that aeroallergen exposure is involved in exacerbating EoE is weak and since it has been suggested that different phenotypes exist in EoE,<sup>14</sup> it is crucial to identify underlying mechanism of EoE where aeroallergen exposure is relevant.

The mechanism by which eosinophils accumulate in the epithelium of the esophagus is the result of complex interplay between genetic, immune, and environmental factors.<sup>15</sup> Previous studies have associated genetic factors such as FLG, DSG1, CAPN14, TSLP and CCL26 with EoE.<sup>16,17</sup> The pathophysiology of eosinophilic infiltration in EoE is initiated by delayed type, a T cell-mediated reaction characterized by Th2 responses involving IL-4 and IL-13.<sup>18,19</sup> It is believed that the esophagus itself is rapidly able to respond to IL-13 by producing CCL11 and CCL24.<sup>20</sup> Eosinophils release many proteins and mediators including MBP, ECP, EPO, EDN and PAF, which play a key role in tissue damage and remodeling.<sup>21</sup> It is still unclear whether IgE contributes to the disease pathogenesis, or it is an associated marker<sup>22</sup> as an anti-IgE treatment (omalizumab) is not effective in human with EoE.<sup>23</sup> Several foods and aeroallergen-induced mouse models of EoE<sup>12,24–29</sup> have led to advances in our understanding of the initiation and progression of the disease. However, how combined exposure of airborne allergen-*Aspergillus*, and food antigen-OVA intranasally in Sharpin deficient mice contributes to severe EoE, and multi-organ inflammation has not been previously investigated.

Sharpin is a widely expressed protein and plays a critical role in the NFκB signaling pathway.<sup>30</sup> These mice develop a chronic proliferative dermatitis that is evident at about four weeks of age<sup>31</sup> followed by multi-organ inflammation in the esophagus, skin, lung, and liver.<sup>31,32</sup> The dermatitis is characterized by epidermal hyperplasia, hyperkeratosis, keratinocyte apoptosis, and accumulation of eosinophils and neutrophils in the dermis and epidermis.<sup>33</sup> Previously, intrinsic morphological changes in the esophagus characterized by epithelial hyperplasia, accumulation of eosinophils and expression of CCL11, IL4, IL13 and TSLP with age in Sharpin<sup>-/-</sup> mice have been investigated.<sup>34</sup> However, the consequence of further aeroallergen exposure in these mice is yet to be explored. Therefore, we hypothesized that Sharpin<sup>-/-</sup> mice that are susceptible to develop inflammation will provide a robust eosinophilic inflammatory response in the esophagus along with multi-organ inflammation following airborne allergen exposure. We investigated the effect of *Aspergillus fumigatus* and OVA exposure in these mice and determined the role of Th2 cytokines, chemokines, and IgE in the development of chronic EoE.

## Materials and Methods

Mice: C57BL/KaLawRij-Sharpin<sup>cpdm</sup> (referred to hereafter as Sharpin<sup>-/-</sup>) and WT mice (initially from the Jackson Laboratory, Bar Harbor, ME) were a gift from Dr. Adrian Ting. Mice were maintained in specific pathogen-free conditions on a 12:12 hour light: dark cycle with constant temperature and humidity. The colony was maintained by mating mice heterozygous for the Sharpin (±) allele. At day 28 after birth, a small section of the ear was sent to Jackson Laboratory for genotyping by PCR to distinguish Sharpin homozygous<sup>-/-</sup>, from Sharpin heterozygous (±) and WT (+/+) mice. 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 (IACUC) approved by the Veterinary Services Department of the New York Medical College (IACUC# 37-2-0720).

## Allergen Exposure Protocol

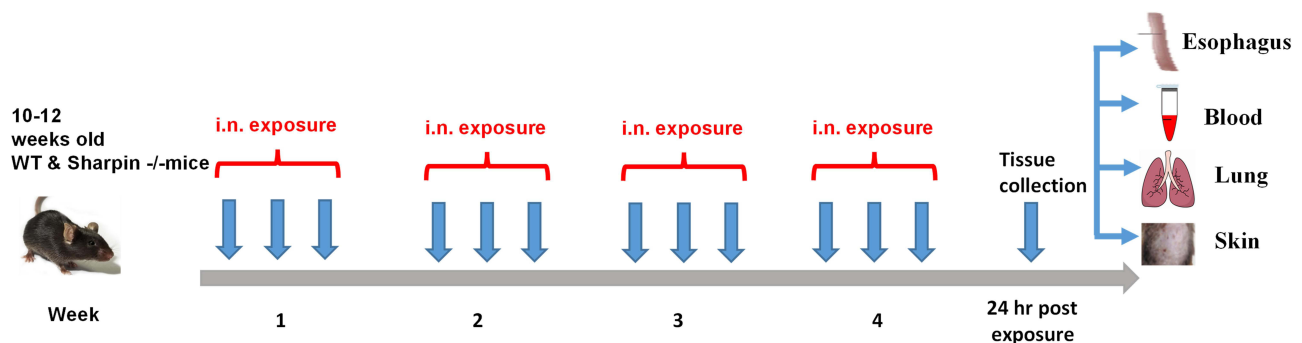
10–12-week-old mice ( $n = 5–7$  per group) were anesthetized with isoflurane, and allergen applied intranasally (i.n.) using a micropipette with the mouse held in supine position. After instillation, mice were held upright until alert. The allergen solution included 100 $\mu$ g of *Aspergillus fumigatus* (GREER, NC) antigen and 100 $\mu$ g of OVA (Grade VI, Sigma, MO) in 50 $\mu$ L of phosphate buffer saline (PBS). The exposure procedure was repeated every alternate day for 4 weeks (Figure 1). The unexposed mice were left alone. Mice were sacrificed after 24 hours from the last i.n. exposure. The experiment was performed with four groups: 1) WT naïve as normal control, 2) WT exposed, 3) Sharpin<sup>-/-</sup> unexposed, and 4) Sharpin<sup>-/-</sup> exposed.

## Esophagus Tissue Processing and Staining

Esophagus tissues ( $n = 5–7$ /per group) were immediately fixed in 10% formalin, paraffin embedded, and cut into 4 $\mu$ M thin section. Tissues were stained with hematoxylin and eosin (H&E), Masson's trichrome and immunohistochemistry using rabbit-antimurine major basic protein tissue (1:10 dilution) at NYMC pathology core facility. The esophageal epithelial thickness was measured at five random sites per section from the basement membrane to the top of the stratum granulosum (10x objective) using a Nikon microscope. Based on H&E staining, eosinophil was morphologically distinguished from neutrophils and counted based on eosinophil morphology (correct size, bilobed nucleus, prominent and distinct magenta granules), as well as positive major basic protein staining in the esophagus as previously described.<sup>35</sup> Eosinophils in each section was counted by examining five random fields using the 40X objective. The esophagus was scored using severity of eosinophilic inflammation (0, absent; 1, scattered eosinophils within the epithelium; 2, moderate and consistent presence of eosinophils in the epithelium; 3, severe, dense eosinophilic infiltration; and 4, very severe, dense infiltration with submucosal expansion). The collagen deposition was accessed using Masson's trichrome stain (Abcam, MA) on esophageal sections according to the manufacturer's instruction. The percent collagen of the esophageal wall was determined by imaging the entire cross-section of trichrome-stained esophageal tissue at 4X. The area of trichrome stain in each image was selected by adjusting the color threshold manually to select for blue trichrome staining using ImageJ. The selected area of collagen, and the total area of the esophagus were measured. The area of collagen deposition was calculated by dividing the total area of trichrome stain by the total area of the esophagus. Eosinophils in each section were counted using immunohistochemistry by examining five random sites using the 40X objective.

## BALF Collection and Lung Histology

The BALF was collected by lavaging once with 1.0 mL phosphate-buffer saline as previously described.<sup>36</sup> 40,000 cells were cytopun and stained with Diff Kwik stain (Thermo Fisher Scientific, MI). The percentage of eosinophils,



**Figure 1** Experimental Design for exposure. 10–12 weeks-old WT and Sharpin<sup>-/-</sup> were intranasally (i.n.) exposed with 100 $\mu$ g of *Aspergillus* and 100 $\mu$ g OVA, every alternate day for 4 weeks. 24-hour post last exposure, mice were sacrificed, and different organs were harvested.

macrophage, lymphocyte, and neutrophils were represented by counting 300 cells/slide. The severity of lung inflammation (perivascular and peribronchial) was graded and scored, by an investigator blinded to the experimental groups, semi quantitatively for the following features, 0, normal; 1, few cells; 2, a ring of inflammatory cells 1 cell layer deep; 3, a ring of inflammation cells 2–4 cells deep; 4, a ring of inflammatory cells of >4 cells deep.

## Skin sampling and histology

Epidermal thickness was measured from basement membrane to the top of epidermis using a 10X objective (Nikon microscope). Eosinophils in each section were counted in a blind manner by examining five random sites using the 40X objective.

## Blood Sampling, cell differential counts

Blood was collected in an EDTA tube (BD Biosciences, NJ) and lysed using ACK Lysing Buffer (Life Technologies, NY). 40,000 cells were cytopun on to a glass slide. Blood differential count were determined by counting 500 cells stained with Diff Kwik (Thermo Fisher Scientific, MI) under microscope (Nikon). Total number of blood lymphocytes, macrophages, eosinophils, and neutrophils were obtained by multiplying the differential count by total blood count per mL.

## Measurement of serum IgE, IgG and IgA

Serum total IgE levels were measured using the BD OptEIA ELISA. The optical density was immediately read at 405nm. OVA<sup>37</sup> and Aspergillus-specific<sup>38</sup> IgEs were measured as described previously by coating sample wells with 100µL of OVA (50µg/mL) and *A. fumigatus* (100µg/mL) respectively. The optical density was read at 450nm. The mouse t-IgG levels were measured by ELISA (Thermo fisher, Austria) and t-IgA levels were measured by coating sample wells with 100µL of rat-anti mouse IgA (1µg/mL, B&D).

## RNA extraction and qRT-PCR

In a separate experiment, mice (n = 5 per group) were exposed with Aspergillus and OVA for 4-weeks. Twenty-four hours after the last exposure esophagus, lung and skin tissue were collected. Tissues were then homogenized in Trizol (Ambian Technologies, CA), and RNA was extracted using Qiagen mini kit. For each qRT-PCR, a 25µL reaction was run with 12.5µL Maxima SYBR Green/ROX qPCR Master Mix 2X, 1.8µL of 0.3µM assays on demand primer, and 300ng of RNA. The qRT-PCR was performed at 40 cycles of 25°C for 5 minutes, 42°C for 60 minutes and 70°C for 15 minutes.

**Table 1** Primer Sequence Used for qRT-PCR

Gene	Sequence
mIL-4_F	CATGGGAAACTCCATGC
mIL-4_R	ATGAATCCAGGCATCGAAAA
mIL-5_F	GCTGGCCTCAAACCTGTAATGTA
mIL-5_R	GGCAATGGTTGCATGTCTGTAACCTC
mIL-13_F	AGACCAGACTCCCCTGTGCA
mIL-13_R	TGGGTCTGTAGATGGCATTG
mCCL11_F	AGACCAGACTCCCCTGTGCA
mCCL11_R	TGGGTCTGTAGATGGCATTG
mGAPDH_F	AACTTTGGCATTGTGGAAGG
mGAPDH_R	ACACATTGGGGGTAGGAACA

The Ct values for each gene were normalized by subtracting the Ct values for the housekeeping gene GAPDH ( $\Delta$ CT). The relative fold change in mRNA expression between groups was calculated and expressed as  $2^{-\Delta\Delta CT}$ . The primer sequences are shown in Table 1.

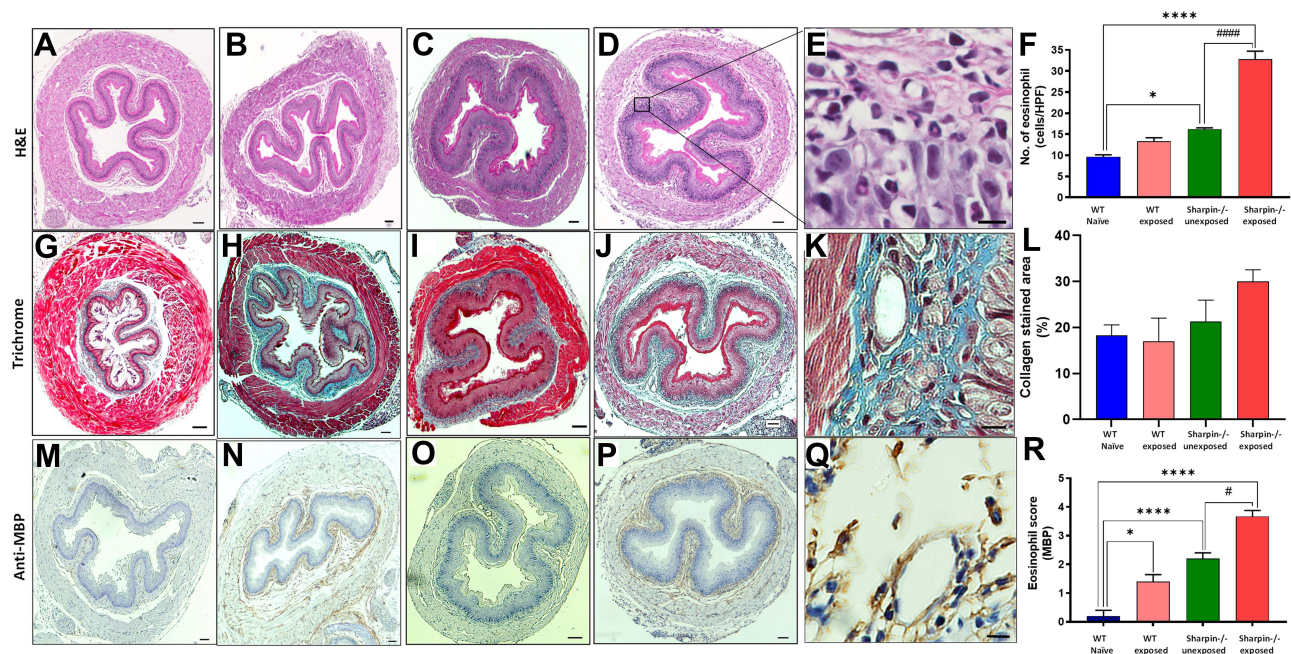
## Statistical analysis

Data were analyzed using Graph Pad (GraphPad Software, San Diego, CA) version 9. Differences between groups were analyzed by one-way ANOVA followed by Bonferroni adjustment for normally distributed data and differences between two groups were analyzed by paired *t* test. *P* values <0.05 were considered significant. Data are expressed as Mean  $\pm$  SD. The computation of sample sizes for power = 0.8 at  $\alpha=0.05$  is based on the formula:  $N = 2 (\text{critical value} + 0.84)^2 (\text{S.D./diff})^2$ , diff. is the difference to be detected, and S.D. the standard deviation. Based on our preliminary data, 5 mice per group will be sufficient.

## Results

### Aeroallergen-Exposure Induced Severe Eosinophilic Infiltration in Esophagus of Sharpin<sup>-/-</sup> Mice

Histopathological analysis of the esophagus by H&E staining revealed that WT naïve mouse had no signs of inflammation (Figure 2A). There were (13 $\pm$ 2 eosinophils/HPF) in WT exposed mice (Figure 2B and F). The Sharpin<sup>-/-</sup> unexposed mice had mild eosinophilia (15 $\pm$ 1 eosinophils/HPF, Figure 2C). Exposing the Sharpin<sup>-/-</sup> mice led to severe inflammation with a rough epithelial lining, intraepithelial micro-abscesses and layers of inflammatory cells surrounding the epithelium (Figure 2D). An enlarged image using the 40x objective highlighted a clear morphology of eosinophil-distinct magenta granules and bilobed nucleus, leading to a conclusion that the majority of the cells were eosinophils with rare mono-nuclear cells (Figure 2E). Eosinophils were counted in a blinded fashion and were found to be as high as 33 $\pm$ 2



**Figure 2** Aspergillus & OVA exposure led to EoE like characteristics in the esophagus. H&E stain of mouse esophagus (4X objective) in a cross-sectional analysis showing different layers in (A) WT naïve, (B) WT exposed, (C) Sharpin<sup>-/-</sup> unexposed and (D) Sharpin<sup>-/-</sup> exposed groups, respectively. Morphology of Eosinophil (E) is clearly highlighted in a partially enlarged image (40X objective). (F) represents average number of Eosinophils per HPF counted in five random sites. Trichrome stain of mouse esophagus (4X objective) showing collagen deposition in (G) WT naïve, (H) WT exposed, (I) Sharpin<sup>-/-</sup> unexposed and (J) Sharpin<sup>-/-</sup> exposed groups, respectively. An enlarged image (K) showing thick deposition of collagen as indicated by blue color. Blue color was quantified using ImageJ and represented as percent collagen-stained area (L). Immunochemical staining of mouse eosinophils with major basic protein in the esophagus of (M) WT naïve, (N) WT exposed, (O) Sharpin<sup>-/-</sup> unexposed and (P) Sharpin<sup>-/-</sup> exposed groups, respectively. A partially enlarged image (Q) showing eosinophils with dark brown color. (R) represents average number of anti-MBP positive cells. Values are means  $\pm$  SD; n=5–7 mice/group. \*\*\*\*p<0.0001, \*p<0.05 vs WT naïve; #####p<0.0001, #p<0.05 vs Sharpin<sup>-/-</sup> unexposed. Scale- 100µm.

eosinophils/HPF, greater than 15/HPF, a diagnostic criterion for human EoE, in the Sharpin<sup>-/-</sup> exposed mice as compared to the WT naïve mice ( $p < 0.0001$ , Figure 2F), which was also greater than exposed WT and unexposed the Sharpin<sup>-/-</sup> mice (Figure 2F).

We performed trichrome staining of formalin fixed esophagus tissue. Area percentage of esophageal trichrome staining (which stains collagen blue) was minimal in WT naïve mice (Figure 2G and L) and was slightly increased but not statistically significant in WT exposed mice (Figure 2H and L). There was less dense trichrome staining in Sharpin<sup>-/-</sup> unexposed mice, which was denser in exposed Sharpin<sup>-/-</sup> mice but not statistically significant (Figure 2I, J and L). An enlarged image (40x) of the trichrome stain showed clear blue coloration (Figure 2K).

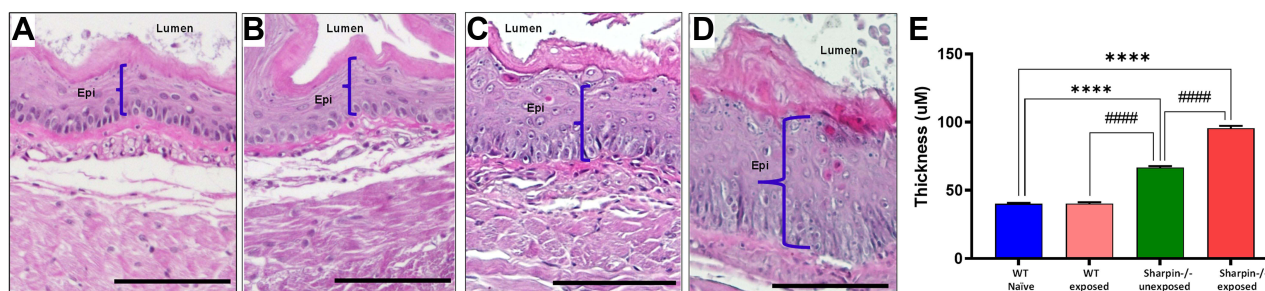
Immunohistochemical staining with anti- MBP, and further scoring eosinophils showed no MBP positive staining in the esophagus of WT naïve mice (Figure 2M and R). Exposed WT showed a few positive stained cells (Figure 2N and R). Sharpin<sup>-/-</sup> unexposed mice had mild eosinophilia as scored by MBP staining (Figure 2O and R). The number of esophageal eosinophils increased remarkably in the Sharpin<sup>-/-</sup> exposed mice as indicated by positive staining for anti-MBP staining (Figure 2P and R,  $p < 0.0001$ ). Figure 2Q is an enlarged image showing positive staining for anti-MBP.

## Aeroallergen-Exposure Induced Severe Mucosal Thickening in Esophagus of SHARPIN<sup>-/-</sup> Mice

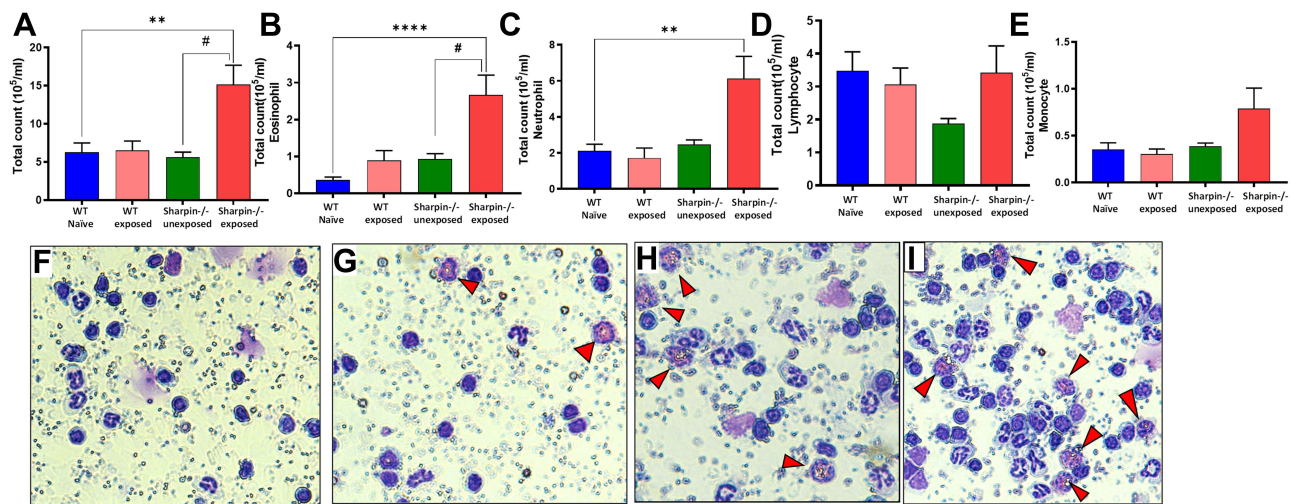
As compared to normal WT naïve mice (Figure 3A), esophageal epithelial lining in the exposed Sharpin<sup>-/-</sup> mice showed remodeling, accompanied by mucosal thickening (Figure 3A–D). There was a 4-fold increase in epithelial thickness in Sharpin<sup>-/-</sup> exposed mice as compared with the WT naïve mice ( $p < 0.0001$ , Figure 3E). Unlike Sharpin<sup>-/-</sup> mice, exposure of WT mice failed to induce epithelial thickening (Figure 3B and E). Although Sharpin<sup>-/-</sup> unexposed mice had intrinsic increased epithelium thickening than WT naïve (1.5-fold increase,  $p < 0.0001$ , Figure 3C and E), exposure in this strain significantly worsened epithelium thickening (Figure 3D and E,  $p < 0.0001$ ).

## Aeroallergen-Exposure Increased Blood Eosinophil Counts in Sharpin<sup>-/-</sup> Mice

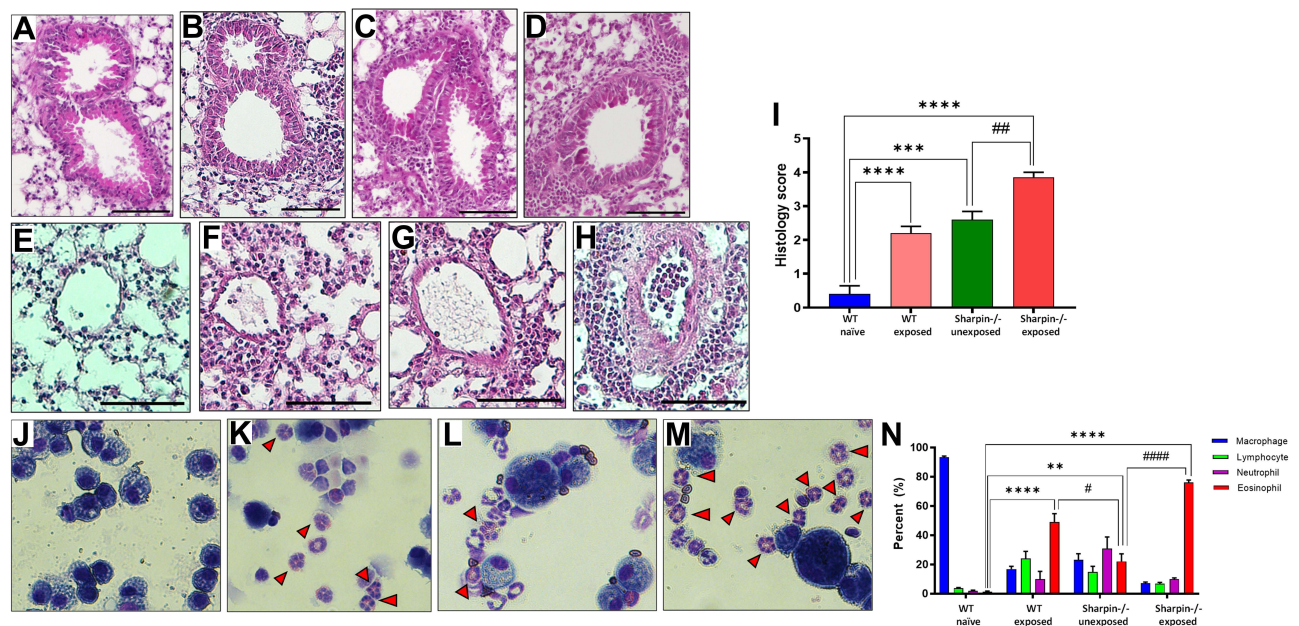
Blood count analysis 24 hrs after the last intra-nasal allergen exposure showed significantly increased total leukocytes in Sharpin<sup>-/-</sup> exposed mice as compared to the normal WT naïve ( $p < 0.01$ ), exposed WT mice, and unexposed Sharpin<sup>-/-</sup> mice ( $p < 0.05$ – $0.01$ , Figure 4A). A blood differential count showed exposure of Sharpin<sup>-/-</sup> significantly increased blood eosinophil count ( $p < 0.0001$ , Figure 4B) and blood neutrophil count ( $p < 0.01$ , Figure 4C) without any difference in blood lymphocyte count and blood monocyte count respectively as compared to WT-naïve mice (Figure 4D and E). Next, blood cytospun slides (Figure 4F–I) showed predominance of eosinophils and neutrophils in the Sharpin<sup>-/-</sup> exposed mice as compared to WT mice. Overall, these findings demonstrate that aero-allergen exposure using *Aspergillus* & OVA increased blood eosinophil and neutrophil numbers. The differences were sharper in the Sharpin<sup>-/-</sup> exposed group as compared to the WT naïve.



**Figure 3** *Aspergillus* and OVA exposure led to increase epithelial thickening. H&E staining of mouse esophagus showing epithelial thickness in (A) WT naïve, (B) WT exposed, (C) Sharpin<sup>-/-</sup> unexposed and (D) Sharpin<sup>-/-</sup> exposed groups, respectively. The blue line indicates the boundary line between the lumen and the submucosa. (E) The thickness of epithelial layer measured using Nikon microscope (10x objective). Values are means  $\pm$  SD;  $n=5$ – $7$  mice/group. \*\*\*\* $p < 0.0001$  vs WT naïve; ##### $p < 0.0001$  vs Sharpin<sup>-/-</sup> unexposed. Scale- 100µm.



**Figure 4** Effect of *Aspergillus* and OVA exposure in blood cell population. Cell differential count was obtained by counting at least 500 cells per cytospin slide by light microscope after differential staining with Diff-Quick: (A) total cell number, (B) eosinophils per milliliter, (C) neutrophils per milliliter, (D) lymphocytes per milliliter, (E) monocytes per milliliter. A representative illustration of Diff Kwik stain of blood cyto-spin smear showing different granulocytes (red arrow pointing to eosinophils) in (F) WT naïve, (G) WT exposed, (H) Sharpin<sup>-/-</sup> unexposed and (I) Sharpin<sup>-/-</sup> exposed groups, respectively. Values are means  $\pm$  SD; n=5–7 mice/group. \*\*\*\*p<0.0001, \*\*p<0.01, vs WT naïve; #p<0.05 vs Sharpin<sup>-/-</sup> unexposed.



**Figure 5** Intranasal allergen exposure led to severe lung inflammation. H&E staining (10X objective) of lung sections of formalin fixed lungs showing peribronchial (PB) inflammation in (A) WT naïve, (B) WT exposed, (C) Sharpin<sup>-/-</sup> unexposed and (D) Sharpin<sup>-/-</sup> exposed groups, respectively. H&E staining showing perivascular inflammation (PV) in (E) WT naïve, (F) WT exposed, (G) Sharpin<sup>-/-</sup> unexposed and (H) Sharpin<sup>-/-</sup> exposed groups, respectively. (I) PV and PB inflammation were graded and scored based on inflammation and number of eosinophils present. Representative illustration of eosinophils (red arrow) of a Diff Kwik stained of BAL cytospin in (J) WT naïve, (K) WT exposed, (L) Sharpin<sup>-/-</sup> unexposed and (M) Sharpin<sup>-/-</sup> exposed groups, respectively. (N) Percent BAL fluid macrophage (blue), eosinophils (red), neutrophils (purple) and lymphocytes green. (Data represented as Means  $\pm$  SD; n=5–7 mice/group. \*\*\*\*p<0.0001, \*\*\* p<0.001, \*\*p<0.01 vs WT naïve; #####p<0.0001, ### p<0.01 and #p<0.05 vs Sharpin<sup>-/-</sup> unexposed. Scale-100 $\mu$ m.

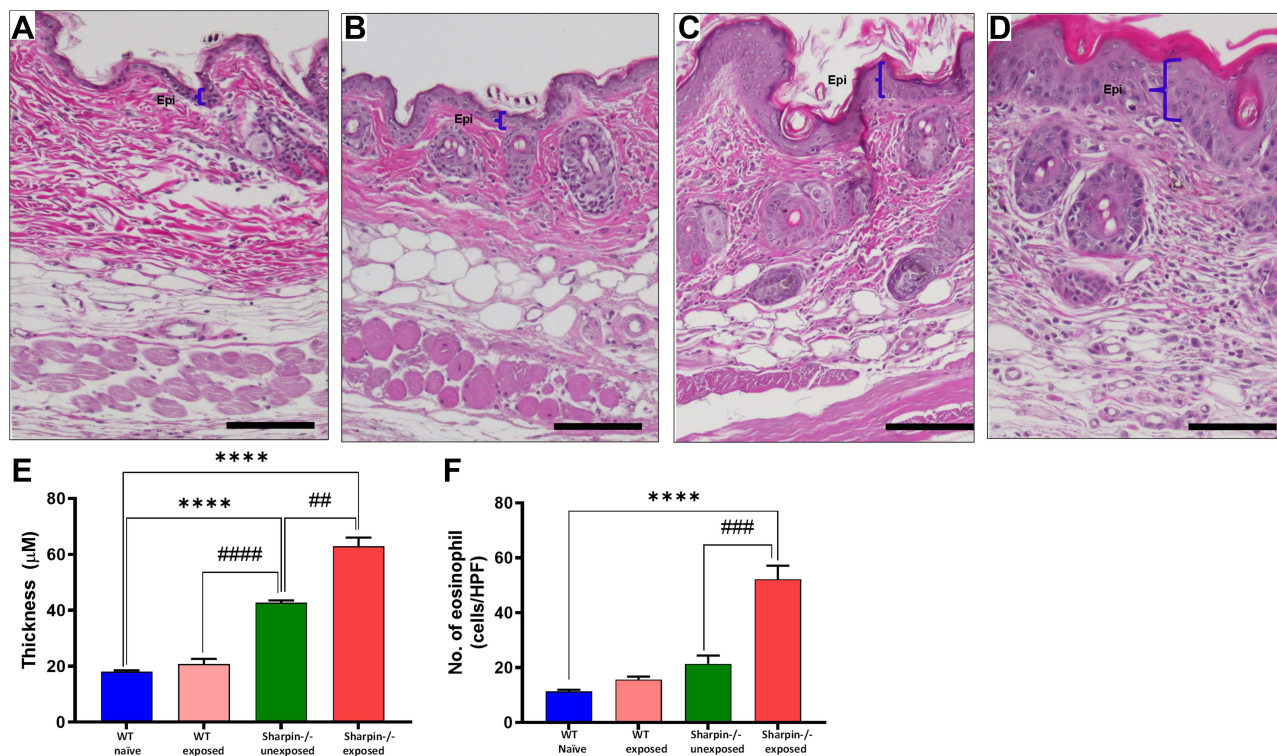
## Aeroallergen-Exposure Increased Leads to Severe Lung Inflammation in Sharpin<sup>-/-</sup> Mice

To analyze the effect of exposure in the lung, we analyzed a lung section stained with H&E. Lung tissue from the Sharpin<sup>-/-</sup> mice demonstrated significant pathological changes, including aggregation of inflammatory cells in airspaces

or vessel walls, and congested alveolar space. Inflammation was not only peribronchial (PB) (Figure 5A–D) but was also clearly observed in perivascular (PV) areas (Figure 5E–H). PV and PB inflammation were graded in a blinded manner and scored where Sharpin<sup>-/-</sup> exposed mice received the highest scores (Figure 5I,  $p < 0.0001$ ). Exposing WT mice led to 10-fold increase in histological scores, which were significantly higher compared to WT naïve mice ( $p < 0.0001$ ). Unexposed Sharpin<sup>-/-</sup> mice displayed elevated histology scores, consistent with spontaneous inflammation associated with these mice,<sup>39</sup> and further became worse with exposure (Figure 5I,  $p < 0.01$ ). Next, BAL fluid cytopun slides (Figure 5J–M) illustrated a predominance of eosinophils in the Sharpin<sup>-/-</sup> exposed mice, which were significantly higher compared to the WT naïve mice ( $p < 0.0001$ , Figure 5N). There was a predominance of macrophages in WT naïve mice with scattered eosinophils. Exposing WT mice led to significant increase in eosinophil infiltration in the lungs ( $p < 0.0001$ , Figure 5K and N). The Sharpin<sup>-/-</sup> unexposed mice intrinsically had  $26 \pm 8\%$  eosinophils in their BAL fluid (Figure 5N), which was boosted to  $76 \pm 2\%$  by exposure ( $p < 0.0001$ , Figure 5M and N). These data suggest that inflammation observed in H&E-stained mice may be related to elevated BALF eosinophil numbers.

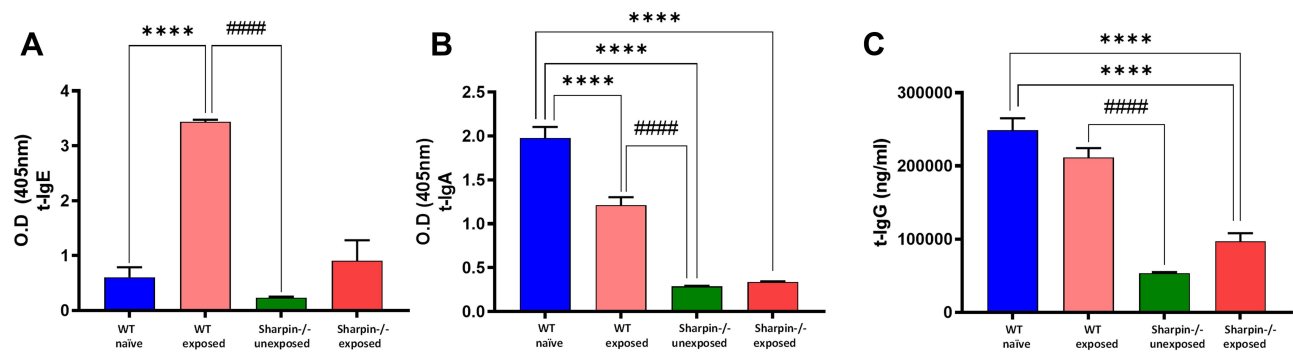
## Aeroallergen-Exposure on Skin Eosinophilic Inflammation and Thickness

Sharpin<sup>-/-</sup> mice develop severe dermatitis, which worsened with age.<sup>40</sup> Therefore, we studied effects of exposure on the extent of inflammation. A schematic showing different layers of skin with the epidermis highlighted (blue) in different experimental groups of mice (Figure 6A–D). H&E staining revealed remarkably increased epidermal thickness in Sharpin<sup>-/-</sup> exposed group compared with the WT naïve ( $p < 0.0001$ , Figure 6E), and was also significantly higher than Sharpin<sup>-/-</sup> unexposed mice ( $p < 0.001$ , Figure 6E). The Sharpin<sup>-/-</sup> unexposed mice, due to its intrinsic skin characteristic had greater epidermis thickening, which was significantly higher compared to the WT exposed mice ( $p < 0.0001$ ). This was further worsened by exposure indicated by significantly increased epidermal thickness ( $p < 0.01$ , Figure 6E). Next, we counted eosinophils in a blinded manner and found that Sharpin<sup>-/-</sup> exposed mice had very high number ( $52 \pm 5$ /HPF) compared to WT naïve ( $11 \pm 1$ /HPF) and Sharpin<sup>-/-</sup> unexposed mice ( $21 \pm 4$ /HPF), which was

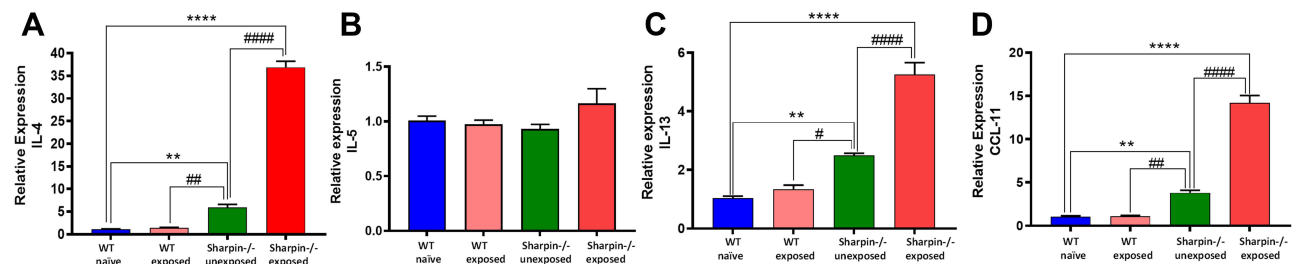


**Figure 6** Intranasal *Aspergillus* and OVA exposure led to severe eosinophilic skin inflammation. Epithelial thickness was presented in H&E-stained section (10x objective) in (A) WT naïve, (B) WT exposed, (C) Sharpin<sup>-/-</sup> unexposed and (D) Sharpin<sup>-/-</sup> exposed groups, respectively. The blue line indicates the boundary line between the epidermis and the dermis. (E) Measurement of epidermal thickness (F) represents average number of Eosinophils per HPF counted in five random sites. Results are expressed as the mean  $\pm$  SD;  $n=5-7$  mice/group. \*\*\*\* $p < 0.0001$  vs WT naïve; #####  $p < 0.0001$ , ####  $p < 0.001$ , ###  $p < 0.01$  vs Sharpin<sup>-/-</sup> unexposed. Scale-100μM.





**Figure 7** Measurement of serum antibody levels in blood. Serum t-IgE (A), t-IgA (B) and t-IgG (C) were measured by ELISA. Data expressed as Mean  $\pm$  SD. n=5–7 mice/group. \*\*\*\*p<0.0001 vs WT naïve; ##### p<0.001 vs Sharpin-/- unexposed.



**Figure 8** Mechanism of EoE was Th2 and CCL11 dependent. Fold change in mRNA expression levels of type 2 cytokine IL-4 (A), IL-5 (B), IL-13 (C) and CCL11 (D) in the esophagus tissue. n=5 mice/group. \*\*\*\*p<0.0001, \*\*p<0.01 vs WT naïve; #####p<0.001, ###p<0.01, #p<0.05 vs Sharpin-/- unexposed.

statistically significant (to Sharpin-/- unexposed and WT naïve mice ( $p < 0.0001$ – $0.001$ , Figure 6F). These data indicate that exposure indeed led to significant eosinophilic inflammation in the skin of Sharpin-/- mice.

## Induction of EoE by Airborne Allergen Exposure Was Independent of Serum IgE

Exposure of WT mice led to significantly increased serum total-IgE (t-IgE) ( $p < 0.0001$ , Figure 7A), which was also significantly higher than Sharpin-/- unexposed mice ( $p < 0.0001$ ). However, exposure of Sharpin-/- mice led to slightly increased, but statistically insignificant, serum t-IgE. ( $p > 0.05$ , Figure 7A) There were no detectable levels of serum *Aspergillus* and OVA specific IgEs in any group (data not shown). Moreover, exposure led to significantly decreased serum t-IgA and IgG in WT mice ( $p < 0.0001$ , Figure 7B and C). Sharpin-/- showed intrinsic lower IgA and IgG than wild type mice, which as not altered by exposure ( $p < 0.0001$ , Figure 7B and C). These results indicate that mechanism of EoE in Sharpin-/- exposed mice is independent of IgE but may be associated with impaired IgA and IgG.

## Induction of EoE is Associated with Upregulation of Th2 Cytokine IL4, IL13 and Th2 Chemokine CCL11 Responses

To further understand the EoE mechanism of this mouse model, we evaluated Th2 cytokine gene expression in the esophagus tissue. The expression of IL-4 ( $p < 0.0001$ ) and IL-13 ( $p < 0.0001$ ) were significantly increased in the esophagus of Sharpin-/- exposed mice as compared to the WT mice (Figure 8A and C). Consistent with the previous results, Sharpin-/- unexposed mice showed intrinsic lower expression levels of IL-4 and IL-13 respectively which was significantly higher than WT naïve and WT exposed mice respectively ( $p < 0.05$ – $0.01$ , Figure 8A and C). Interestingly, there was no IL-5 expression difference in the esophagus tissue between all four groups (Figure 8B).

We found similar results in the expression of chemokine CCL11. There was significant increase in CCL11 expression in esophagus of Sharpin-/- exposed mice compared with WT naïve and Sharpin-/- unexposed mice ( $p < 0.001$ , Figure 8D). Further, we found significant increase in expression of IL-4, and IL13, and CCL11 but not IL-5, in the

lung of Sharpin<sup>-/-</sup> exposed mice respectively ( $p < 0.05$ , [Supplementary Figure 1A–D](#)). There was a similar pattern of cytokine expression in skin tissues as in lung tissues, but CCL11 expression showed no difference in the skin of Sharpin<sup>-/-</sup> exposed mice as compared to the WT mice ([Supplementary Figure 1 E–H](#)).

## Discussion

In this study we were able to show aero-allergen exposure induces EoE like disease associated with multi-organ inflammation. We for the first time demonstrated that Sharpin<sup>-/-</sup> mice exposed with airborne allergens developed severe eosinophilic inflammation. Eosinophil numbers were markedly increased in the esophagus and even more evident in the epithelial layer and submucosa with remarkably increased epithelial thickening, a sign of epithelial hyperplasia much as in human EoE. Further, trichrome staining indicated increasing collagen deposition. We found that eosinophilic inflammation was not limited to the esophagus but also involved organs including the lungs and skin. Eosinophils were evident in BAL fluid and further histopathological analysis showed severe inflammation in PB and PV regions. This further supports that allergic disease precedes EoE and that airway exposure may lead to EoE.<sup>41</sup> Next, our model showed that intranasal aeroallergen exposure contributed to skin pathology as evident by significantly increased epidermal thickness and eosinophil number in the H&E staining. It is well known that there is significant allergic predisposition in the EoE population with many patients having concurrent allergic eczema, asthma and/or a history of atopy.<sup>41,42</sup> Our study is concordant with another study by HogenEsch et al where the Sharpin<sup>-/-</sup> mice develop marked dermatitis as early 5 to 6 weeks.<sup>31</sup> This predisposing atopy may be an important factor leading to severe EoE, which we saw in our model as majority of adult EoE patients are poly exposed to environmental allergens and suffer from atopic illness.<sup>43–45</sup> However, this was not the case in the WT mice as exposure induced less severe inflammation compared to the Sharpin<sup>-/-</sup> exposed mice

Reports claim EoE association with elevated serum IgE in 70% of cases<sup>46</sup> with specific IgE to aeroallergens, food allergens or both in 80%. However, a subset of patients may not have elevated aeroallergen specific IgE or elevated total IgE. Based on the assumption that IgE plays a key role in the pathogenesis of EoE, a pilot study with an anti-IgE antibody, Omalizumab in EoE patient resulted in reduction in tissue IgE levels but with remission rate of only 33%.<sup>47</sup> Furthermore, a recent study using anti-Siglec-F antibody showed reduced numbers of eosinophils without any effect on IgE levels.<sup>48</sup> This indicates IgE might not be playing a key role in all EoE patients. Furthermore, most patients with EoE are atopic.<sup>49</sup> However, this study also found the possibility of EoE without atopy, either alimentary or pulmonary. Currently, no published studies document correlation of specific IgE with EoE severity. Taken together, this data in combination with our data in the Sharpin<sup>-/-</sup> model, further validates that EoE is not simply an IgE-mediated disease but involves a complex interplay between the epithelial barrier and adaptive immune response.

Further, we found significantly decreased t-IgA by exposure of the WT mice. However, regardless of exposure, the Sharpin<sup>-/-</sup> mice had significantly lower serum t-IgA. The mucosal lining in the gastrointestinal tract is exposed to variety of microorganisms and to maintain barrier integrity, these bacteria stimulate the mucosal immune system to produce IgA, which acts as an important first line of defense.<sup>50</sup> It could be possible that exposure alter microbial communities to lower IgA, leading to a compromised mucosal barrier. Further studies are needed to better understand the role of microbiota in EoE pathogenesis. Furthermore, we found significantly decreased total IgG in the Sharpin<sup>-/-</sup> group regardless of exposure. As IgG4 is reported to increase in patients with EoE,<sup>51</sup> however, we did not measure it and needs to be further investigated. The impaired IgA and IgG levels further may exacerbate inflammation in this model.

It is well established that Th2 responses play a vital role in EoE,<sup>19</sup> but it is important to understand unique esophageal immune responses. In this study, we were able to show EoE mechanisms were strictly regulated by Th2 responses- IL-4 and IL-13. These results partially agree with previous reports that allergen-induced EoE is independent of IL-13 without reporting on IL-4.<sup>52</sup> Consistent with a previous study,<sup>34</sup> we did not see differences in the expression of IL-5. However, this contradicts another study that highlighted the role of IL-5 in regulating eosinophilic accumulation,<sup>12</sup> in which there was no direct IL-5 measurement. These differences may be due to the strain of mice used, allergen exposure protocol, and intrinsic esophagus tissue regulatory factors. Given remarkable eosinophilic inflammation in the esophagus of Sharpin<sup>-/-</sup> exposed mice and lack of association with IL-5, we determined expression of CCL11/Eotaxin, an important eosinophil-specific chemokine associated with the recruitment of eosinophils. Previous study by Chien et al has shown

CCL11 expression in different weeks of age in Sharpin<sup>-/-</sup> mice.<sup>34</sup> However, we are first to show increased CCL11 expression in Sharpin<sup>-/-</sup> exposed mice. It has been suggested that IL-13 and CCL11 are involved in chronic liver inflammation in chronic hepatitis B viral infection<sup>53</sup> and IL4/IL13, which with TGF- $\beta$ , has been shown importance in airway fibrosis in asthma.<sup>54</sup> Whether IL-4/IL-13 and CCL11 were synergistically involved in inducing chronic inflammation and tissue remodeling in esophagus in our model of EoE require further investigation. The cytokine profile in the lung and skin tissue showed similar results with increased expression of Th2 responses-IL-4 and IL-13, but with no difference in IL-5 expression. Furthermore, there are two types of EoE disease: inflammatory and fibrous.<sup>55</sup> IL-13 and IL-4 are thought to be central regulators of EoE and are classical Th2 inflammatory cytokine in the pathophysiology of EoE.<sup>18</sup> TGF- $\beta$ 1, CCL-18, FGF-9, VEGF, and VCAM-1 appears to be a profibrotic factor. IL-5 and the TGF- $\beta$ 1 signaling pathway for EoE-associated fibrosis has been reported.<sup>56</sup> Our results showed a trend but not significant increase in collagen stained area in exposed sharping<sup>-/-</sup> deficient mice than controls, and there was no difference in IL-5 production between groups, indicating our model is less likely to be fibrosis type of EoE. However, further research is needed to classify different types of EoE.

There was a difference, however, in the expression of chemokine CCL11, which showed no difference in Sharpin<sup>-/-</sup> exposed mice skin tissue. This further indicates other factors play a role in eosinophilic inflammation in the skin tissue. Furthermore, based on our network pharmacology and computation analysis, other cytokines such as IL1 $\beta$ , TNF- $\alpha$ , IL-6, may also be associated with EoE (Manuscript in preparation, Maskey et al 2022). However, further analysis and validation of these genes are necessary to understand complex pathways underlying EoE.

## Conclusion

We demonstrated for the first time that aeroallergen-exposure to Sharpin<sup>-/-</sup> mice leads to severe eosinophilic inflammation and epithelial thickening in the esophagus, but that associated skin and lung inflammation. Mechanism of EoE in this model was independent of IgE. Elevated expression of Th2 cytokines IL4 and IL13, but not IL-5, and Th2 chemokine CCL11, together with impaired IgA and IgG, may contribute to severe pathogenesis of EoE and multiple organ inflammation in Sharpin<sup>-/-</sup> mice. Future research may uncover the complex interplay of Th2 cytokines, chemokines in the esophagus of our model. This model will facilitate clinical study design, help direct therapeutic strategies and management of patients with EoE, including a potential role for allergen immunotherapy.

## Abbreviation

Sharpin, SHANK associated RH domain interacting protein; EoE, Eosinophilic Esophagitis; qRT-PCR, quantitative real-time PCR; MBP, major basic protein; ECP, eosinophilic cationic protein; EPO, eosinophil peroxidase; EDN, eosinophil-derived neurotoxin PAF, platelet activating factor; H&E, Hematoxylin & Eosin.

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## Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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