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The pathogenesis of chronic eosinophilic esophagitis in SHARPIN-deficient mice

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Abstract

An increased number of eosinophils in the esophagus is common in several esophageal and systemic diseases, and a prominent feature of eosinophilic esophagitis. Mouse models can provide insight into the mechanisms of eosinophil infiltration and their pathogenic role. SHARPIN-deficient *cpdm* mice develop a chronic proliferative dermatitis and an esophagitis characterized by epithelial hyperplasia and the accumulation of eosinophils in the serosa, submucosa, lamina propria and epithelium of the esophagus. We conducted a detailed investigation of the pathogenesis of the esophagitis by light microscopy, immunohistochemistry, and gene expression as the mice aged from 4 to 10 weeks. The thickness of the esophageal epithelium and the number of eosinophils in the esophagus both increased with age. There were scattered apoptotic epithelial cells in mice at 6 – 10 weeks of age that reacted with antibodies to activated caspase 3 and caspase 9. The expression of CCL11 (eotaxin-1), IL4, IL13 and TSLP was increased in *cpdm* mice compared with wild type (WT) mice, and there was no change in the expression of CCL24 (eotaxin-2), IL5 and IL33. The expression of chitinase-like 3 and 4 (YM1 and YM2) proteins, markers of type 2 inflammation, was greatly increased in *cpdm* mice, and this was replicated *in vitro* by incubation of WT esophagus in the presence of IL4 and IL13. Immunohistochemistry showed that these proteins were localized in esophageal epithelial cells. The severity of the esophagitis was not affected by crossing SHARPIN-deficient mice with lymphocyte-deficient *Rag1* null mice indicating that the inflammation is independent of B and T lymphocytes.

Keywords

Mouse; SHARPIN; esophagitis; eosinophils; chemokines; chitinase-like proteins

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Introduction

An increased number of eosinophils in the esophagus is a feature of a wide spectrum of organ-specific and systemic diseases, including eosinophilic esophagitis (EoE), gastroesophageal reflux disease (GERD), Crohn's disease, and hypereosinophilic syndromes (Atkins et al., 2009; Liacouras et al., 2011). EoE is a recently recognized chronic disease in children and adults with a predisposition for males characterized by the presence of many eosinophils throughout the epithelium of the esophagus. It is thought to be caused by an allergic reaction to airborne or food antigens (Blanchard, 2015; Rothenberg, 2015). The light microscopic features include accumulation of eosinophils in the esophageal epithelium, fibrosis of the subepithelial lamina propria, and epithelial cell hyperplasia. Eosinophils accumulate superficially in the epithelium sometimes forming microabscesses. EoE may be differentiated from GERD by the larger number of eosinophils ($> 15/\text{HPF}$), distribution of eosinophils along the length of the esophagus, and lack of response to proton pump inhibitors (PPI) (Liacouras et al., 2011). However, it was recently recognized that a substantial subpopulation of patients with clinical symptoms and histologic features indistinguishable from EoE, responds to PPIs (Dellon et al., 2013; Moawad et al., 2014). This is considered a separate disease entity termed PPI-responsive esophageal eosinophilia. EoE and PPI-responsive esophageal eosinophilia have a very similar gene expression profile except for a small subset of genes that appears to distinguish the two groups of patients (Wen et al., 2015).

The mechanisms by which eosinophils accumulate in the epithelium of the esophagus are not completely understood and likely vary depending on the disease. The epithelium of the esophagus is normally devoid of eosinophils, but eosinophils can accumulate as a non-specific response to various types of injuries (Odze, 2009). Gene expression analysis revealed greatly increased expression of *Ccl26* mRNA in the esophageal epithelium of patients with EoE. Furthermore, a single nucleotide polymorphism in the 3' untranslated region of *Ccl26* correlated with increased susceptibility to the disease supporting a role of this chemokine in the accumulation of eosinophils (Blanchard et al., 2006). Clinical trials with anti-IL5 monoclonal antibodies demonstrated a partial reduction of the number of intraepithelial eosinophils in the esophagus suggesting the involvement of this cytokine in eosinophil accumulation in EoE (Assa'ad et al., 2011; Spergel et al., 2012; Straumann et al., 2010).

Mouse models may provide further insight into the pathogenesis of EoE and related diseases characterized by esophageal eosinophilia. Intranasal administration of fungal or house dust mite antigens, ovalbumin, and peanut allergens to mice resulted in eosinophil infiltration of the esophagus accompanied by increased epithelial cell proliferation and accumulation of mast cells (Mishra et al., 2001; Rajavelu et al., 2012; Rubinstein et al., 2011). The eosinophils were predominantly localized in the submucosa and lamina propria and occasionally in the basal layer of the esophageal epithelium in contrast to the more superficial localization of eosinophils in human patients with EoE. Using these models, it was shown that eosinophil accumulation was dependent on T cells, whereas B cells were dispensable (Mishra et al., 2007). Mice deficient in either CD8^+ T cells or CD4^+ T cells still developed esophageal eosinophilia and recent studies suggest a role for NKT cells (Rajavelu

et al., 2012; Rayapudi et al., 2014). In another mouse model, transgenic mice with overexpression of IL5 in the esophageal epithelium were sensitized cutaneously and challenged via gavage with a hapten (Masterson et al., 2014). Eosinophils accumulated in the esophageal connective tissue and the epithelium and formed superficial microabscesses similar to human EoE (Masterson et al., 2014).

SHANK-associated RH domain-interacting protein (SHARPIN) is a widely expressed protein and a component of the linear ubiquitination assembly complex that plays a critical role in the NF κ B signaling pathway (Walczak et al., 2012; Wang et al., 2012). SHARPIN is also a negative regulator of the β 1 integrin and decreases the activity of the tumor suppressor protein PTEN (He et al., 2010; Jung et al., 2010; Rantala et al., 2011). SHARPIN-deficient mice carry a spontaneous mutation resulting in a premature stop codon in exon 1 of the *Sharpin* gene (Seymour et al., 2007). These mice develop a chronic proliferative dermatitis that becomes clinically manifest at about four weeks of age (HogenEsch et al., 1993). The dermatitis is characterized by epidermal hyperplasia, hyperkeratosis, scattered keratinocyte apoptosis, and accumulation of eosinophils and fewer macrophages, mast cells, and neutrophils in the dermis and epidermis (HogenEsch et al., 1993). The esophagus of mice is lined by stratified squamous cell epithelium similar to the skin. Here, we report on the pathogenesis of the esophagitis in SHARPIN-deficient mice. We investigated whether the morphologic changes and gene expression were similar to those in the skin and we determined the role of B and T lymphocytes in the development of the inflammation.

Materials and Methods

Mice

In this study, C57BL/KaLawRij-*Sharpin*^{cpdm}/*Sharpin*^{cpdm}RijSunJ (JR# 7599; referred to hereafter as *cpdm*) and CByJ. Cg-*Sharpin*^{cpdm-Dem}/*Sharpin*^{cpdm-Dem} (hereafter *cpdm-Dem*) mutant and wild type (WT, +/+) mice (The Jackson Laboratory, Bar Harbor, ME) were maintained in specific pathogen-free conditions (http://jaxmice.jax.org/genetichealth/health_program.html) on a 12:12 hour light:dark cycle with constant temperature and humidity. Each double-pen polycarbonate cages (330 cm² floor area) housed a maximum of four per pen. Commercial autoclaved food (NIH 31, 6% fat; LabDiet 5K52, Purina Mills, St. Louis, MO) and acidified water (pH 2.8–3.2) were fed *ad libitum*. Mice were genotyped by PCR as previously described to distinguish –/– or +/+ mice at day 10 after birth (Potter et al., 2014). *Sharpin*^{cpdm-Dem}, *Rag1*^{–/–} double mutant mice were generated by intercrossing homozygous male BALB/c-*Rag1*^{tm1Mom}/J mice with heterozygous C. Ocb3-*Sharpin*^{cpdm-Dem} females. Progeny that genotyped as heterozygous for both alleles were then intercrossed until the *Rag1*^{tm1Mom} allele was fixed to homozygosity. The colony was maintained by mating mice homozygous for the *Rag1*^{tm1Mom} allele and heterozygous for the *Sharpin*^{cpdm-Dem} allele. All work was approved by The Jackson Laboratory and Purdue University Animal Care and Use Committees.

Esophagus Collection

Age and gender matched mice were euthanized by CO₂ asphyxiation at 4, 6, 8, and 10 weeks of age. Euthanized mice were placed ventral side up. A small incision was made along the ventral midline. The skin and peritoneal wall were reflected back to expose the internal organs. Lifting up the sternum with forceps, the diaphragm cut followed by the ribs at the costo-chondral junction. The liver was removed to expose the esophagus and the stomach. The esophagus was then gently dissected free of the trachea to the hyoid apparatus, removed from the mouse, placed on a piece of aluminum foil, and coiled to form a “Swiss roll” with the distal segment, near the stomach, being in the center of the coil. The esophagus was fixed by immersion in Fekete’s acid-alcohol-formalin (for routine histology and immunohistochemistry). Tissues were processed routinely, embedded in paraffin, sectioned at 6 µm, and stained with hematoxylin and eosin (H&E), toluidine blue, Masson’s trichrome, or processed for immunohistochemistry. Alternatively, esophagus was embedded in Optimal Cutting Temperature (OCT) medium, snap frozen in liquid nitrogen, and stored at –80 °C, or collected in cold HBSS for culture.

Immunohistochemistry

Serial paraffin sections from tissues fixed in Fekete’s acid alcohol formalin were processed using a Ventana autostainer (Tucson, AZ). Cleaved caspase 3 (Cell Signalling Technologies; Danvers, MA) and cleaved caspase 9 (Novus Biologicals; Littleton, CO) were used to evaluate apoptosis in the esophagus, and anti-major basic protein (MBP) antibody (obtained from J. Lee, Mayo Clinic, Scottsdale, AZ) to identify eosinophils. Diaminobenzidine (Sigma, St. Louis, MO) was used as chromogen.

For immunofluorescent labeling, frozen sections of esophagus were fixed in 100% acetone and incubated with fluorochrome-labeled antibodies against CD11B (Biolegend) or rabbit antibodies against CHIL3/4 proteins (Stemcell Technologies, Vancouver, CA) followed by Alexafluor 488-labeled donkey anti-rabbit IgG (Jackson ImmunoResearch, Westgrove, PA). The sections were cover slipped with ProLong® Gold Antifade solution (Thermo Fisher Scientific, Grand Island, NY) with 4′,6-diamidino-2-phenylindole (DAPI).

Histopathological scoring

To quantify the severity of esophageal lesions, three criteria were used to measure the cellular changes: (1) mucosal thickness, (2) number of apoptotic keratinocytes, and (3) number of eosinophils. Each criterion was measured on ten randomly selected areas of the esophagus as described below.

Epithelial thickness—The esophageal epithelium was measured from the basement membrane to the top of the stratum granulosum (malpighian layer) and to the top of the stratum corneum (full thickness) using the 40x objective on an Olympus BH-2 microscope with a DP70 digital camera and DP Controller (3.2.1.276 version software, Olympus Corp., Tokyo, Japan).

Apoptotic keratinocytes—The number of apoptotic keratinocytes were counted within a field a using a 63x high dry objective on an Olympus BH2 microscope. The field had a total area of 139 μm^2 .

Eosinophil scoring—All lesions were subjectively graded using a 63x high dry objective on an Olympus BH-2 microscope (0 = normal, 1 = few widely scattered eosinophils, 2 = small clusters of eosinophils below the basement membrane, 3 = a linear arrangement of eosinophils under basement membrane, and 4 = rows of eosinophils below the basement membrane with a moderate number in the mucosa; 0 = normal, 1 = mild, 2 = moderate, 3 = severe, 4 = extreme).

Quantitative RT-PCR

Quantitative RT-PCR was performed as previously described (Renninger et al., 2010). Briefly, Skin from age and sex-matched mice was collected and stored in RNA Later (Qiagen, Valencia, CA) at -80°C until samples from all replicates were collected. RNA was then extracted using a PureLink RNA MiniKit (Invitrogen, Grand Island, NY). For each qRT-PCR, a 15 μl reaction was run with 7.5 μl Taqman One-Step RT-PCR MasterMix 2X (Life Technologies, Grand Island, NY), 0.4 μl 40x Multiscribe and RNAase Inhibitor Mix, 0.75 μl of 20x Assays on Demand Taqman primer and probe set, and 100 ng RNA. The qRT-PCR was performed at 40 cycles of 42°C for 50 minutes, 90°C for 10 minutes, 95°C for 15 seconds, 60°C for 1 minute, and 72°C for 1 minute. The Ct values for each chemokine were normalized by subtracting the Ct values for the housekeeping gene *Gapdh* (Ct). The relative fold change in mRNA expression between wild-type mice and mutant mice was calculated and expressed as $2^{-\Delta\text{Ct}}$ (Livak and Schmittgen, 2001)

Culture of esophagus tissue

Segments of the esophagus, about 2–3 mm in length, were collected from WT mice in cold HBSS with 50 $\mu\text{g}/\text{ml}$ gentamycin and washed extensively. The segments were cultured in RPMI-1640 medium (Thermo Fisher Scientific) supplemented with 5% fetal calf serum, 50 μM 2-mercaptoethanol and antibiotics. Cultures were incubated with or without 20 ng/ml recombinant mouse IL4 and 20 ng/ml IL13 (Peprotech, Rocky Hill, NJ). After 24 hours, the tissues were collected for RNA extraction or immunofluorescence as described above.

Statistical analysis

The statistical significance of differences between means of experimental groups for epithelial thickness and number of mitotic figures was determined by two-tailed Student's t-test. The number of apoptotic cells and eosinophils were compared using the Mann-Whitney U-test. Linear regression was used to determine the relation between epithelial thickness and number of eosinophils. The fold increases of mRNA expression are presented as geometric means with 95% confidence intervals. The statistical difference of differences in normalized Ct values between WT and mutant mice were calculated by two-tailed Student's t-test. All statistical analyses were performed using GraphPad Prism 6.0.

Results

The mouse esophagus is lined by stratified squamous epithelia with a prominent stratum corneum (Fig. 1). There were no changes in the control mice between four and ten weeks of age nor obvious differences between male and female mice. However, progressive changes were present in both sexes in homozygous mutant mice. The esophageal epithelium was slightly thicker at 4 weeks of age with a few scattered eosinophils in the adventitia and scattered apoptotic keratinocytes. These changes progressed as the mice aged (Fig. 2). There was marked acanthosis and hyperkeratosis of the epithelium. The thickness of the epithelium increased with age (Fig. 2). Inflammatory cells consisted primarily of eosinophils with rare mononuclear cells. Eosinophils were initially present in the adventitia surrounding the esophagus. This progressed to eosinophils accumulating in a linear manner under the basement membrane and infiltrating the epithelium. The eosinophils reached the superficial epithelium and occasionally formed intraepithelial microabscesses. These histological observations were confirmed by immunohistochemistry for eosinophil major basic protein (Fig. 1). The number of eosinophils was positively correlated with the thickness of the esophageal epithelium (Fig. 2C; $P < 0.001$). The presence of apoptotic keratinocytes was confirmed by positive expression of cleaved caspase 3 and 9 (Fig. 1). Their number increased from 4 to 6 weeks of age and did not further increase as the mice got older (Fig. 2). The alterations in the esophageal epithelium are similar to those in the epidermis of the skin of *cpdm* mice (Gijbels et al., 1996; HogenEsch et al., 1993). The dermatitis in *cpdm* mice is further characterized by an increased number of mast cells and fibrosis. In contrast, toluidine blue staining showed a few scattered mast cells in the submucosa and serosa of control and *cpdm* mice (not shown). Masson's trichrome staining revealed less dense collagen in the esophageal submucosa of *cpdm* mice compared with WT mice, but no evidence of fibrosis (Fig. 1). There were few CD11B-positive cells consistent with the paucity of mononuclear cells observed by light microscopy.

The expression of *Ccr3* mRNA was increased in the esophagus of *cpdm* mice (Fig. 3). This chemokine receptor is expressed on eosinophils and mast cells in mice, and, in the absence of a significant number of mast cells, the increase is consistent with the accumulation of eosinophils in the esophagus. The chemokines CCL11 (eotaxin) and CCL24 (eotaxin-2) are ligands of CCR3 and selective chemo-attractants for eosinophils. The expression of *Ccl11* mRNA was increased significantly at all 4, 6, 8, and 10 weeks of age, whereas the expression of *Ccl24* mRNA was modestly increased only at 4 and 6 weeks of age. The Th2 cytokines IL4, IL5, and IL13 can play an important role in eosinophilic inflammation by inducing the expression of eotaxins and enhancing the production of eosinophils. The expression of *Il4* and especially *Il13* mRNA was increased, but there was no expression of *Il5* mRNA in *cpdm* esophagus. The cytokines TSLP and IL33 are thought to play a role in the pathogenesis of type 2 inflammatory diseases and the expression of both cytokines was increased in the skin of *cpdm* mice (Potter et al., 2014). The expression of *Tslp* mRNA in *cpdm* mice was about 4-fold the expression in WT mice at 4 weeks of age (Fig. 3). The expression increased to more than 16-fold at 6, 8 and 10 weeks of age. In contrast, the expression of *Il33* mRNA was only slightly increased in the esophagus of 10 week old *cpdm* mice.

The chitinase-like proteins CHIL3 and CHIL4 (also known as YM1 and YM2) are markers of type 2 inflammation induced by IL4 and IL13. There was significantly increased expression of *Chil3* and an even greater expression of *Chil4* mRNA in the *cpdm* esophagus (Fig. 4). The CHIL3 and CHIL4 proteins share more than 95% of their amino acid sequences, and antibodies do not distinguish between the two proteins. Immunofluorescence with antibodies against CHIL3/4 identified protein expression in the epithelium of the esophagus of *cpdm* mice and very little reactivity with epithelial cells in WT mice (Fig. 4A). The proteins were localized in the cytoplasm of epithelial cells and the expression appeared to increase towards the lumen of the esophagus (Fig. 4B). To determine if IL4 and IL13 could induce expression of *Chil3* and *Chil4*, esophageal tissue from WT mice was cultured in the presence of IL4, IL13 or both. IL4 and IL13 induced greatly increased expression of *Chil4* mRNA and a modest increase of *Chil3* mRNA (Fig. 4C). The combination of IL4 and IL13 did not further increase the expression. Immunofluorescence with anti-CHIL3/4 antibodies showed localization of these proteins in epithelial cells similar to the in vivo presence in the *cpdm* esophagus (not shown).

Two different mutations in exon 1 of the *Sharpin* gene underlie the absence of SHARPIN protein in *cpdm* and *cpdm-Dem* mice (Seymour et al., 2007). The skin disease is more severe in mice with the *cpdm-Dem* allele compared with mice with the *cpdm* allele likely due to differences in genetic background. The esophagus of *cpdm-Dem* mice revealed similar changes as in *cpdm* mice but the changes were more severe at 4 and 6 weeks of age (not shown). To determine if B and T lymphocytes play a role in the EoE, *cpdm-Dem* mice were crossed with *Rag1*^{-/-} mice which lack T and B cells to produce compound homozygous mice and appropriate controls. There were no significant differences between the mice that were homozygous for *cpdm-Dem* regardless of the *Rag1* genotype indicating that B and T lymphocytes play no role in this disease process as was the case with the skin lesions in these mice (Potter et al., 2014).

Discussion

The epithelium of the esophagus and the epidermis of the skin of mice are both cornified stratified squamous cell epithelia. In SHARPIN-deficient *cpdm* mice, both epithelial tissues are markedly altered and characterized by hyperplasia, hyperkeratosis, scattered keratinocyte apoptosis, and infiltration by eosinophils. However, the detailed studies presented here revealed several differences which suggest that the pathogenesis of the inflammation between the two types of tissues may be different. The number of mast cells was not increased in the esophagus whereas mast cells are abundant in the skin of *cpdm* mice (HogenEsch et al., 1993). Furthermore, the expression of *Il5* mRNA was not increased in the *cpdm* esophagus in contrast to the significant overexpression of this cytokine in the skin (HogenEsch et al., 2001). The absence of *Il5* mRNA expression was surprising because IL5 is intimately associated with recruitment and activation of eosinophils which are prominent in the *cpdm* esophagus. However, the lack of *Il5* mRNA expression in the esophagus does not exclude a role of IL5 in the eosinophilic inflammation in the esophagus, because systemically increased IL5 enhances the production of eosinophils in the bone marrow (Yamaguchi et al., 1988). We previously reported that treatment with IL5-neutralizing antibodies and genetic deletion of IL5 greatly reduced the number of circulating

eosinophils and the number of eosinophils in the skin (Renninger et al., 2010). Inhibition of systemic IL5 would be expected to reduce the accumulation of eosinophils in the esophagus as well.

The infiltration of tissues by eosinophils is regulated in part via the chemokine receptor CCR3. Ligands for CCR3 include CCL11, CCL24, and, in humans, CCL26 (Rothenberg and Hogan, 2006). *Ccl26* is not expressed in mice and is probably a pseudogene (Pope et al., 2005). CCL26 is thought to be the major chemokine driving the infiltration of eosinophils in human EoE patients based on the high expression of CCL26 in EoE and the association of EoE with polymorphisms in CCL26 (Blanchard et al., 2006). Similar to the *cpdm* skin, the expression of *Ccl11* mRNA in the *cpdm* esophagus was significantly increased, while there was only a modest increase in the expression of *Ccl24* mRNA suggesting an important role for CCL11. The importance of CCL11 is supported by the effect of genetic deletion of *Ccl11* on eosinophil accumulation in other mouse models of eosinophilic esophagitis, whereas deletion of *Ccl24* had no effect (Mishra et al., 2001; Zuo et al., 2010).

The expression of *Il13* mRNA was increased in 4 week old mice and remained elevated as the mice aged. IL13 can contribute to eosinophil accumulation by inducing the expression of CCL11 (Zuo et al., 2010). Transgenic overexpression of IL13 in the airways was sufficient to induce eosinophil accumulation in the esophagus and epithelial hyperplasia (Zuo et al., 2010). The epithelial hyperplasia induced by overexpression of IL13 was not dependent on eosinophils. A role for IL13 in EoE in human patients is supported by the increased expression of several genes that are targets of IL13 (Blanchard, 2015). Furthermore, treatment of EoE patients with an anti-IL13 monoclonal antibody in a small clinical study resulted in a decrease of intra-epithelial eosinophils in the majority of subjects, and a trend to clinical improvement (Rothenberg et al., 2015). In contrast, there was no difference in eosinophil accumulation and epithelial hyperplasia between wild-type and IL13-deficient mice in a mouse model of esophagitis induced by intranasal *Aspergillus* antigen (Niranjan et al., 2013). This suggests that the role of IL13 in eosinophil accumulation of the esophagus may vary between mouse models and between human patients with conditions characterized by eosinophil accumulation in the esophagus.

The eosinophilic inflammation in the *cpdm* esophagus developed in the absence of B and T lymphocytes similar to the dermatitis in the *cpdm* skin (Potter et al., 2014). Studies in experimentally induced esophagitis in mice indicate that the inflammation was absent in RAG1-deficient mice, but developed independently of B cells, CD4⁺ T cells and CD8⁺ T cells (Mishra et al., 2007). Recent studies suggest that iNKT cells play an important role in the pathogenesis of EoE in human patients and in experimentally induced mouse models (Rayapudi et al., 2014). Indeed, activation of iNKT cells by intranasal or intravenous administration of iNKT cell agonists in mice induced eosinophil infiltration into the esophagus (Rayapudi et al., 2014). The iNKT cells are absent in RAG1-deficient mice, and the data obtained from *cpdm* mice crossed with RAG1-deficient mice suggest yet another mechanism of eosinophil accumulation that does not involve B and T lymphocytes. Application of calcipotriol to the skin of mice induces type 2 inflammation with eosinophils independent of B and T lymphocytes (Li et al., 2006). Instead, the dermatitis is dependent partially on TSLP, IL25, and IL33 (Kim et al., 2013; Li et al., 2009; Salimi et al., 2013).

These cytokines are released from keratinocytes upon exposure to environmental stimuli, and induce and orchestrate type 2 inflammatory responses. Increased expression of TSLP was also observed in the esophageal epithelium of EoE patients and in mice with experimentally induced eosinophilic esophagitis (Noti et al., 2013; Rothenberg et al., 2010). The eosinophilic inflammation in the experimental mouse model was dependent on TSLP (Noti et al., 2013). The increased expression of *Tslp* mRNA in the *cpdm* esophagus suggests that TSLP may be involved in the B and T lymphocyte-independent inflammation in these mice.

The chitinase-like proteins CHIL3 and CHIL4 are two nearly identical proteins with different constitutive tissue expression patterns. CHIL3 is primarily expressed in the lung, spleen, and bone marrow, whereas CHIL4 is expressed in the squamous epithelial cells of the fore stomach at the limiting ridge (Nio et al., 2004; Ward et al., 2001). Both proteins are highly expressed by alternatively activated macrophages and IL4-stimulated mast cells (HogenEsch et al., 2006; Lee et al., 2005; Loke et al., 2002; Raes et al., 2002). Their increased expression is a feature of type 2 inflammatory reactions. There was high expression of *Chil3* and *Chil4* mRNA in the *cpdm* esophagus at as early as 4 weeks of age consistent with a type 2 inflammatory reaction. However, in contrast to the skin of *cpdm* mice in which the proteins were present in macrophages and mast cells (HogenEsch et al., 2006), the CHIL3 and CHIL4 proteins were localized in esophageal epithelial cells. *Chil3* and *Chil4* mRNA can be induced in macrophages and mast cells *in vitro* by incubation with IL4 (HogenEsch et al., 2006; Lee et al., 2005; Nair et al., 2003; Raes et al., 2002; Welch et al., 2002). The receptors for IL4 and IL13 share the IL4RA chain, and IL4 and IL13 have overlapping, but distinct roles in type 2 inflammatory reactions (Van Dyken and Locksley, 2013). Both IL4 and IL13 induced expression of CHIL3 in macrophages (Raes et al., 2002; Welch et al., 2002), but IL13 failed to induce CHIL3/4 proteins in bone marrow-derived mast cells which the authors related to the absence of the IL13 RA1 chain (Lee et al., 2005). We report here that IL4 and IL13 are equivalent in the ability to induce the expression of *Chil3* and *Chil4* mRNA in esophageal tissues. Consistent with the *in vivo* expression in *cpdm* mice, the expression of *Chil4* mRNA was much higher than the expression of *Chil3* mRNA. The function of CHIL3 and CHIL4 proteins is not well understood. A recent report demonstrated that they induce IL1B secretion by macrophages resulting in an increase of IL17-secreting TCRGD T cells and recruitment of neutrophils (Sutherland et al., 2014). The authors speculated that chitinase-like proteins may serve as danger molecules and activate the inflammasome. However, in the *cpdm* esophagus, these proteins were present in the epithelial cells and there was no accumulation of neutrophils or macrophages in the tissue. The *cpdm* esophagus may present an interesting alternative model to study the role of CHIL3 and CHIL4 in inflammation.

In summary, the eosinophilic inflammation in the esophagus of *cpdm* mice shares several features with the dermatitis in these mice, including the presence of apoptotic keratinocytes, infiltration of the epithelium by eosinophils, type 2 inflammation, and the independence of T and B lymphocytes. However, notable differences include the absence of mast cells and macrophages and the lack of locally increased *Il5* expression. The inflammation also appears to differ from EoE in humans both in terms of histological features and the involvement of

iNKT cells. Further studies of the esophagitis in *cpdm* mice may shed light on alternative mechanisms of eosinophilic inflammation in the esophagus other than those induced by allergen exposure.

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Highlights

- SHARPIN-deficient mice develop an eosinophilic esophagitis.
- Esophagitis is characterized by intraepithelial accumulation of eosinophils.
- Positive correlation between number of eosinophils and epithelial thickness.
- Pathogenesis of esophagitis is independent of B and T lymphocytes.
- Increased expression of CHIL3 and CHIL4 proteins in esophageal epithelial cells.

Fig. 1. Esophagus of wild type (left panels) and SHARPIN-deficient *cpdm* mice (right panels). (A,B) Increased thickness of the esophageal epithelium with infiltration of eosinophils and an intraepithelial microabscess (arrow). Hematoxylin and eosin (H&E). Bar = 50 μ m (C,D) Lack of an increase of collagen in the lamina propria. Masson's trichrome (TRI). (E,F) Increase of eosinophils in the *cpdm* esophagus identified by immunohistochemistry with anti-Major Basic Protein antibody (MBP). (G–J) Increased number of apoptotic cells in the *cpdm* esophagus identified by expression of activated caspase 3 (C3) and caspase 9 (C9).

Fig. 2.

Morphometric analysis of the esophagus in WT and *cpdm* mice. (A) The thickness of the esophageal epithelium in *cpdm* mice increased with age. (B) Increase of the number of eosinophils with age in the esophagus of *cpdm* mice. (C) Positive correlation ($P < 0.001$) between the thickness of the epithelium and number of eosinophils in the esophagus of *cpdm* mice. Each dot represents a mouse. (D) The number of apoptotic epithelial cells is increased in the esophagus of *cpdm* mice. The box and whisker plots represent the median, 5 – 95% confidence interval, and range of six mice per group. * $P < 0.05$

Fig. 3.

Expression of cytokine mRNA in the esophagus of *cpdm* mice from four to 10 weeks of age. The bars represent the geometric mean of the fold-increase of mRNA expression of six mice compared with age-matched wild-type mice. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Fig. 4.

A. Increased expression of *Chil3* and *Chil4* mRNA in the esophagus of *cpdm* mice from four to 10 weeks of age. The bars represent the geometric mean of the fold-increase of mRNA expression of six mice compared with age-matched wild-type mice. *** $P < 0.001$. B. Increase of CHIL3/4 protein in the esophageal epithelium of *cpdm* mice identified by immunofluorescence with specific antibodies (green). Nuclei are identified by DAPI (blue). The white dotted line indicates the location of the esophageal basement membrane. L = lumen of the esophagus. C. Increased expression of *Chil3* and *Chil4* mRNA in the esophagus of WT mice following 24 h culture in the presence of IL4, IL13 or IL4 and IL13. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.