Eosinophils Determine Dermal Thickening and Water Loss in an MC903 Model of Atopic Dermatitis

Karmella Naidoo¹, Ferdinand Jagot¹, Lieke van den Elsen¹,8, Christophe Pellefigues¹, Angela Jones¹, Huijun Luo², Karen Johnston³, Gavin Painter⁴, Ben Roediger⁵, James Lee²,⁴, Wolfgang Weninger⁵,6,⁷, Graham Le Gros¹ and Elizabeth Forbes-Blom¹,⁹

Atopic dermatitis (AD) is a highly debilitating disease with significant health impacts worldwide. It has been a difficult disease to treat because of the wide spectrum of clinical manifestations. Therefore, the current clinical management strategies are nonspecific. Previous studies have documented that AD disease progression is precipitated by a combination of skin barrier dysfunction, itch, and immune dysregulation. However, the precise roles played by effector cells and cytokines have not been fully elucidated. To address this, we established a prolonged model of AD, using MC903. The phenotype of this MC903 model closely resembles the one observed in AD patients, including inflammatory parameters, barrier dysfunction, itch, and histopathological characteristics, thereby providing a platform to evaluate targets for the treatment of AD. This model exposed cells and cytokines that are critically associated with disease severity, including eosinophils, TSLP, and IL-4/IL-13. Indeed, eosinophil depletion significantly ameliorated AD pathology, most notably barrier dysfunction, to a similar extent as blocking of the IL-4/IL-13 axis by genetic deletion of STAT6. Thus, this study has identified eosinophils to be critical for the development and maintenance of AD, thereby proposing these effector cells as therapeutic targets for the treatment of AD.


INTRODUCTION

Atopic dermatitis (AD) is a chronic and relapsing inflammatory disease (Leung, 2016) characterized by pruritic lesions (Hong et al., 2011). This highly debilitating condition has a major emotional and financial impact on patients and their families, thereby significantly affecting their quality of life (Whiteley et al., 2016). The prevalence of AD is estimated between 15% and 30% in children and 2% and 10% in adults and is therefore becoming a substantial health concern worldwide (Bieber, 2010). Several studies into the development of AD have dramatically improved our current understanding of the disease. These investigations have illustrated that genetic predisposition (Saunders et al., 2013), an underlying barrier dysfunction (Irvine et al., 2011; Palmer et al., 2006), environmental factors (Deckers et al., 2012), and immune dysregulation all contribute to the chronic inflammation (Weidinger and Novak, 2014) and contact sensitization (Thyssen et al., 2014). Current management strategies for AD are nonspecific, and experimental immunotherapies have been the only causal treatment of AD (Nowicki et al., 2015; Paller et al., 2017). Dupilumab, a human monoclonal antibody to the alpha subunit of the IL-4 receptor (impairing the signaling of both IL-4 and IL-13) recently approved by the US Food and Drug Administration (Oh et al., 2010), has shown clinical benefit (Beck et al., 2014; Simpson et al., 2016), but only in a subset of patients. Thus, further patient stratification is imperative, along with additional therapeutic options (Bieber et al., 2017).

Our current understanding of AD pathophysiology is limited. Preclinical work has shown that topical application of the vitamin D3 analogue MC903 (known as calcipotriol) was sufficient to induce allergic skin inflammation similar to AD (Kim et al., 2013; Leyva-Castillo et al., 2013; Li et al., 2006; Roediger et al., 2013; Salimi et al., 2013). The resulting phenotype includes epidermal thickening, dermal hyperplasia, and an increased number of inflammatory cells in the skin, most notably mast cells (Roediger et al., 2013), eosinophils (Jenerowicz et al., 2007; Kiehl et al., 2001; Lee et al., 2015; Simon et al., 2004), T helper (Th) type 2 cells (Boguniewicz and Leung, 2011; Li et al., 2006), and innate lymphoid type 2 cells (Kim et al., 2013; Salimi et al., 2013).

References

1Malaghan Institute of Medical Research, Wellington, New Zealand; ²Division of Pulmonary Medicine, Department of Biochemistry and Molecular Biology, Mayo Clinic, Scottsdale, Arizona, USA; ³GlycoSyn, Gracefield, Lower Hutt, New Zealand; ⁴The Ferrier Research Institute, Victoria University of Wellington, Lower Hutt, New Zealand; ⁵The Centenary Institute, Newtown, New South Wales, Australia; ⁶Discipline of Dermatology, University of Sydney, Camperdown, New South Wales, Australia; and ⁷Department of Dermatology, Royal Prince Alfred Hospital, Camperdown, New South Wales, Australia

¹Current address: School of Molecular Sciences, University of Western Australia, Crawley, Australia

²Current address: Nestlé Research Centre, Lausanne, Switzerland

³Deceased.

Correspondence: Elizabeth Forbes-Blom, Malaghan Institute, Wellington 6242, New Zealand. E-mail: elizabeth.forbesblom@rdls.nestle.com

Abbreviations: AD, atopic dermatitis; CRTh2, chemoattractant receptor-homologous molecule expressed on T helper type 2 cells; DT, diphtheria toxin; HPF, high-power field; iPHIL, inducible eosinophil-deficient; PGD2, prostaglandin D2; TEWL, transepidermal water loss; Th, T helper

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However, the effector cells and molecules that drive disease progression are poorly characterized. This is mainly due to the fact that disease manifestation differs according to murine strains and, perhaps more importantly, the amount of MC903 applied and duration of treatment (Kim et al., 2013; Li et al., 2006; Salimi et al., 2013).

The purpose of this study was to establish a unifying MC903 model that would resolve some of the apparent discrepancies between the methodologically diverse MC903 model(s) and human AD pathology. Our focus was to establish a prolonged MC903 model featuring persistent disease parameters but also covering the resolution of the acute inflammatory phase. In particular, TSLP has been described as an early initiator of allergic disease (Liu, 2006; Zhou et al., 2005), and although other AD murine studies have reported high and prolonged systemic levels of TSLP (Kim et al., 2013; Li et al., 2006; Zhang et al., 2009), our AD model phenocopies the reduced levels of TSLP consistent with the TSLP levels seen in cohorts of human AD patients (Alysandratos et al., 2010; Miyagaki et al., 2009; Nakamura et al., 2008). Overall, our work moves the MC903 model closer to the human AD phenotype, including inflammatory parameters, barrier dysfunction, itch, and histopathological characteristics, providing a platform to evaluate targets for the treatment and prevention of AD. Our observations support and confirm the critical role of IL-4 in AD pathogenesis but also show mechanistic insight, in particular with regard to the involvement of eosinophils, that might be useful for the development of AD treatment strategies for patients who do not respond to dupilumab.

RESULTS

Clinical symptoms and inflammatory changes after extended epicutaneous exposure to MC903

We focused on establishing a mouse model that more closely resembled the pathophysiology of human AD. To do this, we explored a time course of epicutaneous MC903 exposure (Figure 1a). Clinical symptoms and inflammatory changes were assessed at various time points and compared with the control treatment (100% ethanol). Ear thickness, a surrogate marker of inflammation and accompanying epidermal thickening, was found to steadily increase over the entire duration of treatment (Figure 1b). Transdermal water loss (TEWL), an established clinical disease parameter (Holm et al., 2006), showed an increase after day 7 and peaked between days 14 and 19 (Figure 1c). In addition, the epidermal water loss and associated dryness correlated with an increased itching frequency, and the expression levels of the tight junction proteins claudin 1 and filaggrin were both reduced after MC903 treatment (see Supplementary Figures S1a and b online). Histopathological assessment of MC903-treated skin showed further hallmarks of human AD, including parakeratosis (presence of nuclei in the cornocytes), presence of spongiosis (intercellular edema), acanthosis (Figure 1d and e), and dermal thickening (Figure 1f). Thus, prolonged MC903 treatment recapitulates multiple clinical features of AD, namely inflammation, itch, and barrier dysfunction.

Potent type 2 inflammatory responses after extended epicutaneous MC903 exposure

Allergic skin inflammation is driven via a coordinated type 2 immune response involving mast cells (Roediger et al., 2013), eosinophils (Lee et al., 2015), basophils, innate lymphoid type 2 cells, and type 2 cytokine responses (Kim et al., 2013; Leyva-Castillo et al., 2013; Li et al., 2006; Salimi et al., 2013). Furthermore, most studies have examined the role of these effector cells and type 2 cytokine responses (Auriemma et al., 2013; Mashiko et al., 2017; Nomura et al., 2003a, 2003b) during the initiation phase of AD-like inflammation.

We sought to establish the kinetics of key effector cells and determine the cytokine profile in the skin throughout the response to MC903, including the chronic inflammatory phase. Mast cells continued to accumulate in the skin (Figure 2a). The degree of eosinophilic infiltration and degranulation in the tissue was determined by an eosinophil peroxidase-monoconal antibody-based immunohistochemistry using a previously described scoring system (Lee et al., 2015; Protheroe et al., 2009) (Figure 2b, and see Supplementary Figure S2 online). Circulating levels of TSLP increased sharply after day 7 of MC903 treatment (Figure 2c), and tissue levels of TSLP increased until day 14 of MC903 application, followed by a significant reduction observed at day 19 (Figure 2d). These data are consistent with the reported role for TSLP in the initiation but not the maintenance of allergic skin inflammation (Ziegler and Artis, 2010). The IL-17 response in the skin after MC903 exposure inversely correlates with TSLP production (Figure 2f). In concordance with the mast cell and eosinophil responses, IL-4 production in the skin tissue after MC903 exposure continued to increase over time (Figure 2e). We could not detect any significant changes to the levels of IL-25, IFN-γ, or IL-13, and IL-5 expression remained undetectable in the tissue (data not shown). However, significantly elevated levels of IL-22 were observed on day 7, and there was a slight increase in IL-33 seen at day 14 (see Supplementary Figure S3a–e online). Furthermore, we applied MC903 on 4C13R dual reporter mice to determine the source of IL-4 in the skin. We observed an increase in the frequency of IL-4 (AmCy)−producing basophils after MC903 treatment (see Supplementary Figure S3b online), unlike eosinophils that did not release any detectable levels of IL-4 (data not shown). Moreover, in line with previous studies, we confirmed that allergic inflammation in this prolonged MC903 model is initiated independently of CD4+ T cells, because no changes in ear thickness were detected after CD4+ T cells were depleted in MC903-treated mice (see Supplementary Figure S3a) (Li et al., 2006).

IL-4/STAT6 signaling is essential to the MC903-induced AD phenotype

Previous studies have shown that increased IL-4 levels in the epidermis (Chan et al., 2001) and serum (Herberth et al., 2010) correlate with the development of skin inflammation. However, how IL-4 influences disease progression remains incompletely understood. To further delineate the role of IL-4 in our MC903 model, we used IL-4−/− mice. The genetic deletion of IL-4 resulted in a significant decrease in ear thickness measurements, and mast cell counts remained unchanged in MC903-treated IL-4−/− mice (see Supplementary Figure S5a and b online). To address the fundamental role the IL-4/IL-13 axis in the pathogenesis of AD and the associated clinical efficacy of dupilumab
we conducted our model in mice that lacked STAT6. In these STAT6−/− mice, the signaling of both IL-4 and IL-13 is impaired via the deletion of the common downstream transcription factor STAT6 (Kaplan et al., 1996). MC903-treated STAT6−/− mice showed a dramatic reduction in ear thickness compared with the MC903-treated C57BL/6 mice (Figure 3a). TEWL, acanthosis, and dermal thickening were significantly reduced as well (Figure 3b–d). However, there was no change in the mast cell counts compared with the MC903 C57BL/6 groups (Figure 3e). Eosinophil peroxidase-monoclonal antibody–based immunohistochemistry showed a drastic reduction in the recruitment and degranulation of eosinophils to the ear tissue of MC903-treated STAT6−/− mice compared with controls (Figure 3f). Furthermore, the levels of eotaxin, a well-characterized eosinophil chemoattractant, were significantly lower in STAT6−/− mice compared with controls after MC903-treatment (Figure 3g). In summary, the absence of IL-4 and IL-13 signaling leads to a significant reduction in AD phenotype, including a substantial reduction in eosinophil recruitment, possibly due to decreased eotaxin levels in the skin.
Eosinophils are necessary for the development and progression of MC903-induced AD phenotype

The substantial reduction of eosinophils and eotaxin levels in the absence of STAT6 suggested a possible mechanism by which IL-4 and IL-13 signaling contribute to AD pathology. Furthermore, it has been reported that eosinophils are associated with disease severity in AD patients (Kiehl et al., 2001; Simon et al., 2004) and itch in a murine model of skin inflammation (Lee et al., 2015). The inducible eosinophil-deficient mice (iPHIL) served as an excellent tool to evaluate the relevance of eosinophils to the development of AD in the MC903 model (Jacobsen et al., 2014). C57BL/6 mice and iPHIL mice were treated with MC903 and diphtheria toxin (DT) (Figure 4a). DT administration was used to deplete eosinophils in the iPHIL mice. Flow cytometry showed a significant increase in the frequency of eosinophils in both the spleen and ear tissue when C57BL/6 mice (no DT) were treated with MC903 (data not shown). However, when treated with DT and MC903, iPHIL mice showed a drastic reduction in eosinophils in both the spleen and ear tissue (see Supplementary Figure S6a and b online). Gated flow plots indicated an efficient depletion system in the iPHIL mice upon treatment with DT. Depletion of eosinophils significantly reduced ear...
thickness in iPHIL mice compared with C57BL/6 DT-treated controls (Figure 4b). Furthermore, TEWL (Figure 4c), acanthosis (Figure 4d), and dermal thickening (Figure 4e) were also markedly reduced after eosinophil depletion. In addition, we assessed the requirement of eosinophils during the maintenance or chronic phases of AD in this model.
To achieve this, iPHIL mice were treated with DT either throughout (for 19 days), during the maintenance phase (from day 6), or only in the chronic phase (from day 12), during extended epicutaneous MC903 exposure (see Supplementary Figure S7a online). Ear thickness was substantially decreased in a group that received DT in only week 3 compared with the wild type. The most profound reduction in ear thickness was seen in the mice that received DT from day 6 (see Supplementary Figure S7b).

After this, TEWL and acanthosis were most effectively reduced in iPHIL mice that received DT throughout weeks 2 and 3 (see Supplementary Figure S7c and d), thereby suggesting that depletion of eosinophils after the initiation of allergic inflammation is sufficient to significantly reduce inflammatory infiltrate and improve barrier function. Functional depletion of eosinophils resulted in an amelioration of disease, making eosinophils critical to the pathogenesis of AD in the MC903 model.

Chemoattractant receptor-homologous molecule expressed on Th2 cells (CRTh2) antagonism therapeutically affects MC903-induced AD

Prostaglandin D2 (PGD2) has been described as a potent chemoattractant for eosinophils, detected in the early phase of allergy (Monneret et al., 2001). PGD2 (Nagata and Hirai, 2003) is produced by activated mast cells and its receptor CRTh2 is ubiquitously present on eosinophils (Lewis et al., 1982; Luna-Gomes et al., 2011; Moon et al., 2014). The PGD2-CRTh2 axis has been implicated in chronic skin inflammation (He et al., 2010; Pettipher et al., 2007; Stebbins et al., 2010), and more recently, Wambre et al. (2017) described a pathogenic Th2 cell subset in allergic individuals who had a high expression of CRTh2, thereby suggesting that CRTh2 antagonism may be a mechanism worth exploring for treatment of allergic diseases. To determine whether PGD2 is involved in the pathogenesis of AD in our MC903 model, we made use of a small molecule CRTh2

Figure 4. Eosinophils are key effector cells in the development of atopic dermatitis in the MC903 model.
Inducible eosinophil-deficient mice (iPHIL) and C57BL/6 mice were topically administered with 1 nmol MC903 or EtOH for 19 days. Mice received 20 ng/g DT intraperitoneally every 3 days from day 0. (a) Schematic of MC903/EtOH and DT treatment regime. (b) Ear thickness was measured at endpoint. (c) TEWL was measured at the endpoint. Hematoxylin and eosin staining was performed, and (d) acanthosis and (e) dermal thickening were evaluated in tissue sections at original magnification of ×20 and quantified by Image J analysis (National Institutes of Health, Bethesda, MD). Plots show mean ± standard error of the mean. Each data point represents an individual mouse. Each graph is representative of three independent experiments. Statistics calculated by one-way analysis of variance with Tukey posttest. ****P < 0.0001. DT, diphtheria toxin; EtOH, ethanol; i.p., intraperitoneally; iPHIL, inducible eosinophil-deficient; TEWL, transepidermal water loss.
antagonist (CRTh2<sup>ant</sup>) (see Supplementary Figure S8 online). This method has been successfully used in the past in preclinical allergy models (Pettipher et al., 2007; Stebbins et al., 2010). A similar approach was carried out by oral administration of CRTh2 antagonist (CRTh2<sup>ant</sup>)/methylcellulose (vehicle control) regime. (b) Ear thickness was measured at endpoint. (c) TEWL was measured at the endpoint. Hematoxylin and eosin staining was performed and (d) acanthosis and (e) dermal thickening assessed in tissue sections at original magnification ×20X and quantified by Image J analysis (National Institutes of Health, Bethesda, MD). (f) EPX-monoclonal antibody-based immunohistochemistry was used to assess eosinophil infiltration. Eosinophils were evaluated, quantified, and scored for degranulation at original magnification ×100 per HPF. The statistics represented are specific to the number of eosinophils per HPF in each treatment group. Plots and bar graphs show mean ± standard error of the mean. Each data point represents an individual mouse. Each graph is representative of at least three independent experiments. Statistics were calculated using one-way analysis of variance with Tukey posttest. **P < 0.01, ***P < 0.005, ****P < 0.0001. CRTh2, chemoattractant receptor-homologous molecule expressed on T helper type 2 cells; EPX, eosinophil peroxidase; HPF, high-power field; NS, not significant; TEWL, transepidermal water loss.

**DISCUSSION**

Skin barrier dysfunction and immune dysregulation are both responsible for AD progression (Dharmage et al., 2014; Nestle et al., 2009; Werfel et al., 2016). These key attributes were recapitulated in the MC903-induced AD model presented here, alongside profound changes in epidermal structure and barrier integrity, increased TEWL, acanthosis, dermal thickening, and the presence of spongiosis and parakeratosis (Holm et al., 2006; Saunders et al., 2013, 2016).
Beyond these clinical AD parameters, our model has established the precise kinetics for the accompanying type 2 cell and cytokine infiltration in the skin. Our experiments highlighted the importance of the IL-4/IL-13 axis in AD, with STAT6−/− mice showing remarkably less inflammation compared with controls. This observation is consistent with the current focus in clinical management of AD and the therapeutic success of dupilumab, leading to its recent approval by the US Food and Drug Administration (Beck et al., 2014; Simpson et al., 2016; Thaci et al., 2016). A recent murine study reported that IL-4Rx plays a significant role in orchestrating the neuro-immune axis mediating chronic itching, suggesting an additional mechanism of action for dupilumab (Oetjen et al., 2017). Furthermore, we report the importance of simultaneously targeting the IL-4 and IL-13 pathways, because MC903-treated IL-4−/− mice still developed significant inflammation. In our study, MC903-treated STAT6−/− mice exhibited a drastic reduction in eosinophilic tissue infiltration, but no associated change in mast cell accumulation, which suggested that the STAT6 pathway is required for the recruitment of eosinophils (Knott et al., 2009).

We subsequently confirmed the critical role of eosinophils in AD disease progression by use of the iPHIL mice (Jacobson et al., 2014). Eosinophils have been previously associated with AD disease pathology (Kiehl et al., 2001; Simon et al., 2004), and our data provide further evidence confirming this. Murine studies have also suggested that eosinophils are associated with itch in the skin (Lee et al., 2015). Here, we have shown that eosinophil depletion resulted in a reduction in inflammation and improved barrier function. Depleting eosinophils in weeks 2–3 of the MC903 model was sufficient to significantly reduce disease phenotype. However, therapeutic blockade of IL-5 via mepolizumab, a humanized antibody targeting IL-5, showed that partial depletion of blood eosinophils offers no clinical benefit. Specifically, Oldhoff et al. (2005) reported that mepolizumab treatment lead to decreased eosinophil survival in the circulation, via mepolizumab, but most likely did not translate into adequate depletion of eosinophils in tissue (i.e., skin) (Oldhoff et al., 2005). Although we were not able to detect measurable amounts of IL-5 in our model of AD, our results support this conclusion. Indeed, the complete blockade of eosinophil function in iPHIL mice, including in tissue, significantly reduced AD pathology, as shown by the reduced TEWL and inflammation. This is further exemplified by our eotaxin results.

A potent eosinophilic chemoattractant, eotaxin-1 has been described for its association with type 2 inflammatory responses. It binds the receptor CCR3, expressed on allergic effector cells (Mackay, 2001) such as eosinophils (Sallusto et al., 1997), basophils (Uguccioni et al., 1997), and mast cells (Ochi et al., 1999). In our model, a significant increase in eotaxin-1 was observed in the tissue after MC903 treatment, and this increase was dependent on STAT6, consistent with STAT6-dependent promoter activity (Hoeck and Woisetschlager, 2001) and the previously reported synergistic effect of IL-4 and IL-13 for the regulation of eotaxin-1 (Yuan et al., 2006). Although the MC903-treated STAT6−/− group showed substantial reduction in eosinophils, no change in the mast cell numbers was observed. At this stage, we hypothesize that although mast cell infiltration increases over time after MC903 application, the mast cell influx is independent of the STAT6 pathway, and further investigations are required to elucidate the role of these cells in this model.

MC903-induced AD models have a predominant type 2 inflammatory response, are independent of classical T and B cells (Li et al., 2006), and are associated with an up-regulation of type 2 innate lymphoid cells (Kim et al., 2013; Roediger et al., 2013; Salimi et al., 2013). In line with the model presented here, these features show that AD skin pathology can occur in the absence of adaptive T cells and thus likely presents as an etiologically heterogeneous condition that could make AD amenable to precision medicine (Bieber et al., 2016). This would entail a tailored approach to treat subgroups of patients with AD and possibly classify them based on a panel of biomarkers.

Recently, a distinct pathogenic subset of human Th2 cells, phenotypically identified as CRTh2+CD49d+CD161+, were documented as being unique to individuals with allergic disease (Wambre et al., 2017). Furthermore, clinical studies have focused on targeting CRTh2 in the treatment of allergic diseases (Horak et al., 2012; Pettipher et al., 2007, 2014; Stebbins et al., 2010). Our findings have suggested the PGD2-CRTh2 axis to be an additional pathway, which contributes to the AD disease pathology, proposing that further studies into this pathway would be worthwhile to determine the precise effect that CRTh2 antagonists could have in AD. Many of the effectors that these findings have highlighted, such as eosinophils and IL-4/IL-13 axis, are highly desirable candidates to serve as biomarkers for AD subtypes.

Collectively, we have established a clinically relevant model of AD that could become instrumental for the assessment of potential targets for the treatment and prevention of AD. Our results point toward eosinophils and the IL-4/IL-13 axis as relevant therapeutic targets for the clinical management of AD.

**METHODS AND MATERIALS**

**Mice**

Specific pathogen-free C57BL/6 mice, STAT6−/− (Kaplan et al., 1996), iPHIL (inducible eosinophil-deficient knock-in) (Jacobson et al., 2014), IL-4−/− (Hu-Li et al., 2001), and dual reporter 4C13R mice with AmCyan under the control of Il4 regulatory elements and DS Red under the control of Il13 regulatory elements (Roediger et al., 2013) were used between 6 and 8 weeks of age and were sex-matched for each experiment. Mice were housed at the Malaghan Institute of Medical Research Biomedical Research Unit, and all experimental procedures were approved by the Victoria University Animal Ethics Committee and performed according to the institutional guidelines.

**MC903 topical application to induce AD**

Mice were anaesthetized using intraperitoneal ketamine/xylazine. MC903 (calcipotriol; Cayman Chemicals, Ann Arbor, MI) was dissolved in 100% ethanol and topically applied on mouse ears (1 nmol in 20 μl, 10 μl per side of ear). As vehicle control, the same volume of ethanol was applied. (See Figure 1a for treatment regimen.)
**Ear thickness measurements**

While mice were sedated, ear thickness was measured with a digital caliper on each treatment day just before topical application of MC903.

**TEWL measurements**

TEWL was measured while mice were sedated, using the DERMA-LAB TEWL probe (Cortex Technology, Hadsund, Denmark) at endpoint. TEWL was measured on both ears of the mice at room temperature, and results were recorded when TEWL regions stabilized. Two readings from each ear were taken and averaged for each mouse.

**Histological evaluation and quantification**

Ear tissue was fixed in 4% paraformaldehyde (Life Technologies, Auckland, New Zealand) for 24 hours, processed, paraffin embedded, and sectioned at 4 μm. Hematoxylin and eosin, chloroacetic esterase, and eosinophil peroxidase-monoclonal antibody (Mayo Clinic, Scottsdale, AZ) staining were conducted on ear sections. Hematoxylin and eosin-stained tissue was evaluated at ×20 magnification, and measurements of acanthosis (epidermal thickening) and dermal thickening were quantified by Image J analysis (National Institutes of Health, Bethesda, MD). Images of hematoxylin and eosin-stained sections were assessed for the presence of spongiosis and parakeratosis at ×40 magnification. Chloroacetic esterase staining was performed to detect mast cells in ear tissue. Images were taken at ×20 magnification, and mast cells were counted consistently on one side of the central cartilage across 10 high-power fields (HPFs). Average counts were determined per HPF for each ear section. Each data point on the graph represented an ear section, that is, the average of 10 HPFs. Eosinophil peroxidase staining was performed, with eosinophil peroxidase-monoclonal antibody used to detect eosinophils and assess their degranulation. Eosinophils were assessed at ×100 objective through 20 HPFs. In each HPF, eosinophils were counted and assigned a degranulation score based on the scoring system defined in Lee et al. (Lee et al., 2015; Protheroe et al., 2009). Images of the tissue sections were taken on an Olympus Brightfield (BX51) microscope (Olympus, Tokyo, Japan).

**Tissue processing**

Single cell suspensions were prepared from ear tissue and spleen by mechanical disruption and passage through 70-μm nylon strainers (BD Falcon, Mountain View, CA). For the skin harvest, ears were split into dorsal and ventral layers and digested with collagenase IV (Sigma-Aldrich, St. Louis, MO) and DNaseI (Roche Diagnostics, Tokyo, Japan). Images were taken at 20 magnification, and mast cells were counted consistently on one side of the central cartilage across 10 high-power fields (HPFs). Average counts were determined per HPF for each ear section. Each data point on the graph represented an ear section, that is, the average of 10 HPFs. Eosinophil peroxidase staining was performed, with eosinophil peroxidase-monoclonal antibody used to detect eosinophils and assess their degranulation. Eosinophils were assessed at ×100 objective through 20 HPFs. In each HPF, eosinophils were counted and assigned a degranulation score based on the scoring system defined in Lee et al. (Lee et al., 2015; Protheroe et al., 2009). Images of the tissue sections were taken on an Olympus Brightfield (BX51) microscope (Olympus, Tokyo, Japan).

**Antibodies and flow cytometry**

Cells were resuspended in phosphate buffered saline (Thermo Fisher Scientific) containing 1% fetal bovine serum, 2 nM/L EDTA (Thermo Fisher Scientific) and 0.01% sodium azide (Sigma-Aldrich) and incubated in anti-mouse CD16/CD32 (generated from 24G2 hybridoma cells) to block nonspecific antibody binding. Cell surface staining was conducted for 30 minutes using the following antibody panel: anti-mouse CD45-BVJ95 (30F11; BD Horizon, San Jose, CA), anti-mouse CD49b FITC (DX5; BioLegend, San Diego, CA), anti-mouse CD3 BV786 (145-2C11; BD Horizon), anti-mouse SiglecF CF594 (E50-2440; BD Pharmingen, Franklin Lakes, NJ), anti-mouse CD11b BV650 (M1/70, BD Horizon), anti-mouse FcεR1 AF647 (MAR-1; eBioScience, Waltham, MA), anti-mouse B220 FITC (RA3-6B2, BD Pharmingen). Dead cells were excluded using DAPI (Molecular Probes; Thermo Fisher Scientific, Waltham, MA). Data were acquired using a custom LSRII SOR flow cytometer with BD Diva Software, version 6.1.1 (Becton Dickinson, Franklin Lakes, NJ), and analyzed using FlowJo, version 9 software (Tree Star, Ashland, OR).

**Measurement of cytokines in tissue**

Ear samples were homogenized in phosphate buffered saline containing protease inhibitors (Halt protease inhibitor; Thermo Fisher Scientific) and 5 mM/L EDTA. Tissue lysates were used to perform ELISA to detect cytokine production (Roediger et al., 2013). TSLP, eotaxin-1 (CCL-11), and IL-17 (Duoset R&D systems, Minneapolis MN); IL-4 (prepared in house); and IL-1β, IL-25, IL-33, and IFN-γ (eBioscience) ELISA kits were used. Tissue concentrations are expressed relative to total protein.

**DT treatment in iPHIL mice to deplete eosinophils**

DT (Sigma-Aldrich) was diluted to a working solution in phosphate buffered saline (Gibco) and 20 ng/g administered by intraperitoneal injection every 3 days from day 0.

**Depletion of CD4+ T cells**

Treatment of 0.5 mg/mL of anti-CD-4 (clone GK1.5; Bio X Cell, West Lebanon, NH) or the isotype control antibody was administered by intraperitoneal injection on days 3, 4, 8, 12, and 16.

**Itching frequency**

Mice were recorded via time-lapse videography, and itch events were determined and quantified. This pathophysiological measurement was obtained from video observation of the relevant treatment groups 24 hours before experimental endpoint, and this was conducted at the Mayo Clinic, Arizona as per specifications in Lee et al. (2015).

**Quantitative real-time PCR**

A quantitative real-time PCR was performed from cDNA of mouse ear tissue using Qiagen DNeasy Mini Kit (Applied Biosystems, Foster City, CA) as per the manufacturer’s protocol. RNA-to-cDNA conversion was carried out using Applied Biosciences High-Capacity RNA to cDNA kit. TaqMan gene expression master mix (Applied Biosystems) and TaqMan primers for claudin1 and filaggrin were used to conduct quantitative real-time PCR analysis from ear tissue. Applied Biosystems software was used to determine the cycle threshold value for each primer. ΔCT values were calculated by normalizing samples to GAPDH control and comparing with the ethanol-treated tissue.

**CRTh2 antagonist treatment**

The CRTh2 antagonist AM156 was chemically synthesized using protocols from Scott et al. (2011) at the Ferrier Institute of Victoria University (Wellington, New Zealand). The chemical methodology used a Suzuki coupling between aryl boronate (4-methoxy-3-(4,4,5,5-tetramethyl-[1,3,2] dioxaborolan-2-yl)-phenyl)-acetic acid ethyl ester) and aryl bromide (2-bromo-5-[trifluoromethyl]benzaldehyde) to form the biphenyl core. Subsequent reductive amination with ethyl amine and installation of the acyl cyclopropene moiety followed by base hydrolysis afforded AM156. Spectroscopic data, including nuclear magnetic resonance and high-resolution mass spectrometry were consistent with the chemical structure (see Supplementary Figure S8). This was resuspended at a concentration of 1 mg/ml in 0.5% methylcellulose (Sigma-Aldrich) and
intragastically administered daily from day 7 to day 18. The control group received 0.5% methylcellulose (vehicle control) by oral administration.

Data visualization and statistics
All graphical representation of data was done with using Prism 7.0 (GraphPad Software, La Jolla, CA). Data presented as mean ± standard error of the mean. Comparison between multiple groups was analyzed by one-way analysis of variance, followed by a Tukey posttest. For comparison between two groups, Student t test followed by a Mann-Whitney posttest was used. P-value of less than 0.05 was considered significant. *P < 0.05, **P < 0.01, ***P < 0.005, ****P < 0.001.

CONFLICT OF INTEREST
The authors state no conflict of interest.

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AUTHOR CONTRIBUTIONS
KN and EBF designed the experiments. KN, FJ, LvDE, CP, and HL conducted experiments. KN analyzed the data. AJ assisted with eosinophil scoring. KJ and GP synthesized the CRTH2 antagonist. BR and WW provided insight into skin pathology and reviewed histology. JL provided the eosinophil peroxidase antibody, iPHIL mice, and expert advice on eosinophils. GLG supervised the research. KN, EBF, and GLG wrote the manuscript with input from all co-authors.

SUPPLEMENTARY MATERIAL
Supplementary material is linked to the online version of the paper at www.jidonline.org, and at https://doi.org/10.1016/j.jid.2018.06.168.

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