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## Signaling through FcR $\gamma$ -associated receptors on dendritic cells drives IL-33 dependent Th2-type responses

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### Abstract

**Background**—While allergic sensitization can be generated against various allergens, it is unknown how such a diversity of antigens is able to promote type 2 helper cell (Th2)-mediated inflammation leading to atopy. Our previous studies demonstrated that allergen-specific IgG immune complexes (ICs) and house dust mite (HDM) extract both induced dendritic cells (DCs) to drive Th2-mediated inflammation, but the mechanism by which these diverse stimuli produce similar responses are unknown.

**Objective**—To identify the DC signaling pathways utilized by Th2 stimuli to promote Th2-mediated inflammation.

**Methods**—C57BL/6, Fc $\gamma$ RIII<sup>-/-</sup>, FcR $\gamma$ <sup>-/-</sup>, and ST2<sup>-/-</sup> mice were sensitized and challenged with HDM, and inflammation was assessed by flow cytometry, histology, and cytokine production. Bone-marrow derived DCs (BMDCs) from these strains were utilized in signaling and adoptive transfer experiments.

**Results**—Our findings indicate that two distinct Th2 stimuli, ICs and HDM, both utilize FcR $\gamma$ -associated receptors, Fc $\gamma$ RIII and Dectin-2 respectively, to promote Th2-mediated lung

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inflammation. In this study we demonstrate that both ICs and HDM induce expression of IL-33, a critical mediator in asthma pathogenesis and the differentiation of Th2 cells, in DCs. Upregulation of IL-33 in DCs is dependent on FcR $\gamma$ , toll-like receptor 4 (TLR4), and phosphoinositide 3 (PI3)-kinase. Exogenous IL-33 is sufficient to restore development of Th2 responses in FcR $\gamma$ -deficient mice. Finally, adoptive transfer of allergen-pulsed FcR $\gamma^{+/-}$  BMDCs restores development of Th2-type inflammation in FcR $\gamma$ -deficient mice, demonstrating the necessity of this signaling pathway in DCs for allergen-induced inflammation.

**Conclusion**—These data identify a mechanism whereby Th2 stimuli signal through FcR $\gamma$ -associated receptors on DCs to upregulate IL-33 production and induce Th2-mediated allergic airway inflammation.

### Keywords

Dendritic cells; immune complexes; house dust mite; FcR $\gamma$ ; Th2; allergic airway inflammation; lungs

## INTRODUCTION

Atopic asthma is a chronic inflammatory disease of the lungs that causes recurrent narrowing of the airways resulting in wheezing, shortness of breath, and coughing (1). This disease presents with a variety of phenotypes but is most commonly associated with a Th2-type response (2). Development of a Th2-type response is dependent on respiratory DCs that produce specific cytokines, including IL-10 and IL-33, to promote skewing towards Th2 differentiation (3–5). Allergic responses can be triggered by a variety of structurally diverse allergens with varying biological functions including pollen, animal dander, fungal spores, and dust mites (6). These allergens can be characterized based on whether or not they exhibit enzymatic activity. It has been suggested that allergens be divided into Class I allergens that have enzymatic activity (i.e. dust mite and cockroach) and Class II allergens that do not have enzymatic activity (i.e. pet dander) (7). While allergic sensitization can be generated against various allergens, it is unknown how these diverse allergens can promote Th2-skewing and differentiation leading to atopy.

Protein allergens that lack enzymatic activity are often modeled in mice using the inert protein ovalbumin (OVA). Using this model allergen, we previously demonstrated that during secondary responses, OVA-specific IgG formed ICs with OVA instilled in the airways. These ICs signaled through Fc $\gamma$ RIII on DCs, and in the presence of a TLR4 stimuli, upregulated IL-33 expression and promoted Th2-mediated lung inflammation (8). While ICs represented one mechanism by which homogenous protein allergens could promote Th2-mediated allergic responses, it was unclear whether this model was relevant for other complex allergens such as HDM that are comprised of many immunogenic proteins in association with glycans and endotoxin. This allergen contains a wide variety of components that may contribute to its allergenicity including cysteine proteases, chitinases, serine proteases, and others (9). We recently found that HDM induced IL-33 expression in BMDCs in the absence of specific IgG suggesting alternative pathways for the induction of Th2 responses (5). This study was designed to further clarify how diverse allergens are able to induce similar Th2-type responses in the lungs.

In this study, we determine that Th2 stimuli (i.e. ICs and HDM) signal through FcR $\gamma$ -associated receptors on DCs to promote Th2 responses in the lungs by increasing expression of IL-33. FcR $\gamma$  is a common signaling component of multiple receptors including Fc $\gamma$ RIII and Dectin-2 that have been shown to bind ICs and HDM, respectively (10, 11). Using these two different Th2 stimuli, we demonstrate that ICs and HDM both induce expression of IL-33 in BMDCs in an FcR $\gamma$ , TLR4, and PI3-kinase dependent manner. HDM-induced allergic airway inflammation is FcR $\gamma$  dependent, and this inflammatory response was restored in FcR $\gamma^{-/-}$  mice upon administration of recombinant IL-33 during sensitization with HDM. Moreover, adoptive transfer of allergen-pulsed FcR $\gamma^{+/+}$  BMDCs is sufficient to restore development of Th2-type inflammation in FcR $\gamma^{-/-}$  mice. These data identify a mechanism that can be engaged by different Th2 stimuli to promote atopic asthma by activating DCs through FcR $\gamma$ -associated receptors to induce IL-33 production and drive Th2 responses.

## METHODS

### Mice

C57BL/6 mice (WT) were purchased from Harlan Laboratories. B6.Fc $\gamma$ RIII $^{-/-}$  and B6.FcR $\gamma^{-/-}$  mice were purchased from Jackson Laboratory. B6.ST2 $^{-/-}$  mice were provided by Dr. A. McKenzie (Medical Research Laboratory, University of Cambridge, UK) (12). B6.Dectin-2 $^{-/-}$  mice were generated by Dr. Y. Iwakura (The Institute of Medical Science, Tokyo, Japan) (13). B6.TLR4 $^{-/-}$  were provided by Dr. C. Nagler (University of Chicago, Chicago, IL). All animal procedures and housing were approved by the University of Chicago Animal Resources Center. The studies conformed to the principles set forth by the Animal Welfare Act and the National Institutes of Health guidelines for the care and use of animals in biomedical research.

### Reagents

House dust mite (XPB82D3A25, Greer Laboratories), Grade V chicken egg OVA (Sigma Aldrich), rabbit anti-chicken egg OVA sera (080M4812, Sigma Aldrich), Grade V BSA (Sigma Aldrich), rabbit anti-BSA sera (Sigma Aldrich), and recombinant mouse IL-33 (Biolegend) were used in murine experiments. OVA-ICs were made by mixing a 10:1 excess of anti-OVA:OVA at 37°C for 30 minutes. PI3-kinase inhibitor (Ly294002) or Syk inhibitor (piceatannol) (Sigma Aldrich) were used in cell culture experiments.

### Sensitization and challenge

Mice were sensitized and challenged as described on day 0 (PBS, 100  $\mu$ g of HDM, 500 ng of rIL-33, or both HDM and rIL-33) and day 7 (PBS or 100  $\mu$ g of HDM) and then sacrificed on day 11. For Fig. 5, mice were sensitized by instilling  $1 \times 10^6$  treated BMDCs i.t. into naïve mice. On day 7 all the mice were challenged i.t. with 100  $\mu$ g of HDM and sacrificed on day 11. Using the passive transfer model developed in our previous study (8), 100  $\mu$ L of  $\alpha$ -OVA or  $\alpha$ -BSA sera was injected intravenously into mice followed by a 100  $\mu$ g OVA i.t. sensitization the next day; one week later, animals were challenged with three OVA i.t. administrations. Airway inflammation was assessed as described in the supplementary materials.

## Statistical Analysis

All statistical analyses were performed with GraphPad Prism software, and a P-value less than 0.05 was considered significant (\*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ ; ns = not significant). Experiments with two groups were analyzed using an unpaired Student's two-tailed *t* test. Experiments with greater than two groups were analyzed with a one-way ANOVA and post-hoc Tukey test. Error bars represent the SEM.

## Cytokine Analysis

For Fig. 4, BMDCs were stimulated overnight as described above. For Fig. 5D, single cell suspensions from the mediastinal lymph node were cultured with 25  $\mu\text{g}/\text{mL}$  of HDM at  $2 \times 10^5$  cells/well for 48 hours. The plates were put through a freeze-thaw cycle to release intracellular cytokines before supernatants were collected and analyzed by Multiplex bead array according to the manufacturer's protocol (Millipore).

## RESULTS

### HDM-mediated Th2-type responses are dependent on Fc $\gamma$ RIII but not Fc $\gamma$ RIII

Although we had previously demonstrated that HDM treatment of BMDCs was able to upregulate IL-33 even in the absence of HDM-specific IgG (5), we hypothesized that IC formation during secondary responses could be playing a role in HDM-induced Th2 responses. We first demonstrated that an eosinophilic and CD4<sup>+</sup> T cell response was only seen in WT mice that had received both an intratracheal (i.t.) sensitization and challenge with HDM a week apart; the mice were sacrificed four days after the last challenge (Fig. S1). No significant response was seen in mice that had received a HDM sensitization/PBS challenge, PBS sensitization/HDM challenge, or PBS sensitization/PBS challenge (Fig. S1). We then sensitized and challenged WT and Fc $\gamma$ RIII<sup>-/-</sup> mice with HDM using the protocol described above. Interestingly, we found no difference in the number of eosinophils or CD4<sup>+</sup> T cells in the bronchoalveolar lavage (BAL) between the two strains (Fig. 1A). Histological examination of the lungs showed that both strains had thickening of the airway epithelium and inflammatory cell infiltrates around the airways (Fig. 1B). These results suggested that HDM utilized an Fc $\gamma$ RIII-independent pathway to promote Th2 inflammation in the lungs.

We had previously shown that glycans in HDM were recognized by the Dectin-2/Fc $\gamma$  receptor complex, which led to increased Th2-type responses through the production of cysteinyl leukotrienes (cysLTs), TNF- $\alpha$ , IL-6, IL-10, and IL-23 (14, 15). Given that Fc $\gamma$ RIII and Dectin-2 both signal through Fc $\gamma$ , we hypothesized that Fc $\gamma$ -containing receptors were utilized by Th2 stimuli to induce Th2 responses in vivo. It was previously shown that allergen-induced airway hyperresponsiveness and inflammation was Fc $\gamma$ -dependent using an OVA sensitization and challenge model (16). Thus, it led us to question whether HDM-mediated responses were also Fc $\gamma$  dependent. Heterozygous (Fc $\gamma$ <sup>+/-</sup>) and knockout (Fc $\gamma$ <sup>-/-</sup>) littermates were compared following sensitization and challenge with HDM. The numbers of both eosinophils and CD4<sup>+</sup> T cells were significantly decreased in the BAL of Fc $\gamma$ <sup>-/-</sup> mice demonstrating that this receptor component was important for the induction of Th2 responses to HDM (Fig. 1C). Further, there was decreased inflammation in the airways of the Fc $\gamma$ <sup>-/-</sup> mice as evidenced by histology (Fig. 1D). These findings supported our

hypothesis that FcR $\gamma$  was part of a common signaling pathway that induced allergic airway inflammation.

### IL-33 restores allergic airway inflammation in FcR $\gamma$ <sup>-/-</sup> mice

Our previous work had shown that HDM treatment of BMDCs induced expression of IL-33 suggesting that HDM allergic airway inflammation may be dependent on the ST2/IL-33 pathway (5). To determine if HDM sensitization required ST2, ST2<sup>+/-</sup> and ST2<sup>-/-</sup> littermates were sensitized and challenged with HDM. Compared to the ST2<sup>+/-</sup> mice, the ST2<sup>-/-</sup> mice had a significant reduction in the number of eosinophils and CD4<sup>+</sup> T cells in the BAL as well as decreased lung inflammation and mucus production as shown by histology (Fig. 2A and 2B). Since HDM-induced responses were ST2 dependent, we examined whether administration of recombinant IL-33 (rIL-33) during sensitization with HDM was enough to restore allergic airway inflammation in FcR $\gamma$ <sup>-/-</sup> mice. On day 0, FcR $\gamma$ <sup>-/-</sup> mice received an i.t. of PBS alone, HDM alone, rIL-33 alone, or HDM plus rIL33 combined. On day 7, all mice were challenged with HDM. The FcR $\gamma$ <sup>-/-</sup> mice sensitized with PBS, HDM, or IL-33 alone failed to develop airway or lung inflammation after HDM challenge (Fig. 3A and 3B). Strikingly, the FcR $\gamma$ <sup>-/-</sup> mice that were sensitized with HDM in combination with rIL-33 had increased eosinophilia and CD4<sup>+</sup> T cells in the BAL, and significant thickening of the airway epithelium and lymphocytic infiltration after challenge with HDM (Fig. 3A and 3B). Thus, the addition of IL-33 was sufficient to restore airway and lung inflammation in FcR $\gamma$ <sup>-/-</sup> mice. These data suggested that signaling through FcR $\gamma$ -containing receptors could lead to IL-33 production which was necessary for primary sensitization of mice to HDM.

### HDM-induced IL-33 upregulation in DCs is FcR $\gamma$ and Dectin-2 dependent

To investigate whether signaling through FcR $\gamma$  was required for increased IL-33 expression and production in DCs, WT, Fc $\gamma$ RIII<sup>-/-</sup>, and FcR $\gamma$ <sup>-/-</sup> BMDCs were generated and treated overnight with OVA, ICs, or HDM. IC- or HDM-treatment of WT BMDCs upregulated MHC II and CD86 expression more than OVA suggesting that these stimuli induced greater activation of BMDCs (Fig. S2A). As we had shown in our earlier study, ICs induced IL-33 in an Fc $\gamma$ RIII dependent manner (Fig. 4A)(8). HDM-induced upregulation of IL-33 was Fc $\gamma$ RIII independent supporting our earlier findings that HDM-mediated Th2 inflammation did not require Fc $\gamma$ RIII. Interestingly, similar to the induction by ICs, increased expression of IL-33 following treatment with HDM was dependent on FcR $\gamma$  (Fig. 4A). We determined that after treatment with HDM, there was no significant difference in the expression of MHC II, CD86, or CD40 on WT and FcR $\gamma$ <sup>-/-</sup> BMDCs (Fig. S2B). Furthermore, our previous work had suggested there may be crosstalk between FcR $\gamma$ -associated receptors and TLR4, and indeed we found that IL-33 upregulation following HDM treatment was TLR4 dependent (Fig. S3). To determine if IL-33 upregulation was downstream of the Dectin-2/FcR $\gamma$  receptor complex, Dectin-2<sup>-/-</sup> BMDCs were treated with OVA, ICs, or HDM. In the absence of Dectin-2, there was no significant increase in IL-33 mRNA expression following treatment with HDM (Fig. 4B). From these findings, we determined that IC signaling through Fc $\gamma$ RIII or HDM signaling through Dectin-2 utilized the common signaling component FcR $\gamma$  to promote IL-33 expression.

Since both ICs and HDM upregulated IL-33 in an FcR $\gamma$  dependent manner, but used different co-receptors, we investigated whether they engaged the same downstream signaling molecules for IL-33 induction. Previously, we found that downstream of FcR $\gamma$  signaling on BMDCs, cysLT upregulation was Syk dependent whereas IL-10 upregulation was PI3-kinase dependent (14, 16). In addition, work on mast cells had demonstrated that IL-33 upregulation upon Fc $\epsilon$ RI ligation was PI3-kinase dependent (17). To investigate which pathway led to IL-33 upregulation, WT BMDCs were treated with either ICs or HDM in the presence of a PI3-kinase inhibitor (Ly294002) or Syk inhibitor (piceatannol). Interestingly, compared to the control group that received no inhibitors, IC- and HDM-induced IL-33 production was PI3-kinase dependent. However, the Syk inhibitor failed to reduce the IC- and HDM-induced IL-33 production found in the control groups (Fig. 4C and 4D). Taken together, these data identified a common pathway through FcR $\gamma$  and PI3-kinase which was utilized by different Th2 stimuli to induce production of IL-33 in DCs, which is distinct from the pathway utilized to produce cysLTs.

### **FcR $\gamma$ -sufficient BMDCs reconstitute HDM-mediated inflammation in FcR $\gamma$ -deficient mice**

In our studies on IC-mediated Th2 inflammation, we demonstrated that T cells were necessary and sufficient for development of allergic airway inflammation (8). To determine if T cells were playing a role in our HDM allergen model, WT or Rag $^{-/-}$  were sensitized and challenged with HDM as described above. Notably, there was a significant decrease in the number of total cells and eosinophils in the BAL of Rag $^{-/-}$  mice (Fig. S4A). This inflammatory response was restored in Rag $^{-/-}$  mice that had received an adoptive transfer of nylon-wool nonadherent cells (Fig. S4B). These data demonstrate that T cells are necessary for HDM-mediated allergic airway inflammation.

Development of Th2 inflammation relies on stimulation from antigen presenting cells, primarily DCs, which are able to direct differentiation into specific T-cell lineages (3). Given that HDM-mediated responses were T cell dependent, we investigated whether the lack of inflammation in the FcR $\gamma^{-/-}$  mice was due to a deficient response from the DCs in the lungs. To address this question allergen-pulsed FcR $\gamma^{-/-}$  or FcR $\gamma^{+/-}$  BMDCs were adoptively transferred into naïve FcR $\gamma^{-/-}$  mice (Fig. 5A). FcR $\gamma^{-/-}$  and FcR $\gamma^{+/-}$  BMDCs were stimulated overnight with HDM before  $1 \times 10^6$  BMDCs were transferred i.t. into naïve FcR $\gamma^{-/-}$  mice. On day 7, all the mice received an i.t. challenge of HDM before being sacrificed on day 11. The mice that received the FcR $\gamma^{+/-}$  BMDCs had significantly increased numbers of eosinophils and CD4 $^{+}$  T cells in the BAL as well as increased inflammation as seen on histology (Fig. 5B). Further analysis of the T cells demonstrated that there was a greater percentage of effector memory CD4 $^{+}$  T cells (CD44 $^{+}$ CD62L $^{-}$ ) and a lower percentage of naïve CD4 $^{+}$  T cells (CD44 $^{-}$ CD62L $^{+}$ ) in the mediastinal lymph nodes of mice that had received FcR $\gamma^{+/-}$  BMDCs (Fig. 5C). Moreover, the draining lymph node cells from mice that had received FcR $\gamma^{+/-}$  BMDCs produced significantly more IL-4, IL-5, and IL-13 upon restimulation with HDM (Fig. 5D). Together, these data demonstrated that signaling through FcR $\gamma$ -associated receptors on DCs during sensitization was sufficient to restore effective priming of T cells in the draining lymph node, which in turn, led to the development of Th2-type inflammation.

## DISCUSSION

The focus of this study was to understand how two structurally and biologically diverse allergens were able to induce similar Th2-type responses in the lungs. Utilizing ICs and HDM, we determined that they both signaled through FcR $\gamma$ -associated receptors, TLR4, and PI3-kinase to upregulate IL-33 production in DCs. We further demonstrated that signaling through FcR $\gamma$ -associated receptors on DCs was sufficient to restore Th2 responses in FcR $\gamma$ -deficient mice. These data identify a mechanism that can be engaged by different Th2 stimuli to promote atopic asthma by activating DCs through FcR $\gamma$ -associated receptors to induce IL-33 production and drive Th2 responses.

Our results demonstrate that hematopoietic cells expressing FcR $\gamma$ -associated receptors are able to upregulate IL-33 suggesting that this pathway may contribute to the pathogenesis of Th2-mediated diseases. In humans, significant clinical correlations have been discovered between IL-33 and asthma. Several genome-wide association studies have identified genetic variations in the *IL33* and *IL1RL1* genes (encoding IL-33 and ST2, respectively) as being asthma susceptibility loci (18). These findings support clinical studies demonstrating increased levels of IL-33 in the serum and tissues of patients with asthma or allergies (19–22). However, it has not been as well characterized which human hematopoietic cells can act as a source of IL-33 during allergic airway inflammation. Our studies point to cells that express FcR $\gamma$ -associated receptors, which in humans includes DCs, monocytes, macrophages, mast cells, NK cells, and basophils (23). An association between FcR $\gamma$  and asthma was recently identified by Hinds *et al* in a genome-wide association study of self-reported asthma for rs2070902 in the FcR $\gamma$  locus (*FCER1G*) (24). While further investigations need to be carried out to clarify if IL-33 upregulation in human cells is also downstream of FcR $\gamma$ -associated receptors, taken together with our findings, these studies suggest that binding of allergens to FcR $\gamma$ -associated receptors induce IL-33 production which may represent one mechanism for the promotion of allergic airway inflammation.

Interestingly, our study finds that HDM-mediated allergic airway inflammation does not require the presence of ICs, while our previous findings demonstrate that OVA does require ICs. We propose a model that classifies allergens based on their ability to bind directly or indirectly to FcR $\gamma$ -associated receptors. Allergens like HDM that contain glycans may enhance the Th2 response through direct binding of its components to FcR $\gamma$ -associated receptors such as Dectin-2. On the other hand, homogenous protein allergens like OVA cannot be directly recognized by an FcR $\gamma$ -associated receptor, but in the presence of antigen-specific IgG generated during a primary response, it can form ICs that can ligate and activate Fc $\gamma$ RIII. We have further demonstrated that the IC-mediated Th2 inflammation model works with other homogeneous proteins like bovine serum albumin (BSA). An eosinophilic and CD4<sup>+</sup> T cell response in the lungs was only seen in mice that received antigen that was complementary to the serum administered highlighting the specificity of IC-mediated Th2 inflammation (Fig. S5). Our study demonstrated that the presence of allergen-specific IgG could lead to antigen-specific IC formation that would contribute to the development of allergic responses. In humans, circulating allergen-specific ICs have been detected in allergic individuals, but whether they contribute to development of Th2

responses in humans is still unclear (25–27). Translation of our findings into human studies may help to elucidate whether this dichotomy occurs in humans as well.

Interestingly, our study suggests that FcR $\gamma$ -associated receptors and TLR4 act in concert together to augment allergic airway inflammation. In this study and in our previous work, we have demonstrated that HDM or IC treatment of BMDCs upregulated IL-33 in a manner that was dependent on both an FcR $\gamma$ -associated receptor and TLR4 (8). This finding is consistent with other studies showing that activation of TLR4 led to the rapid upregulation of PI3-kinase, and that IgG-IC-elicited cytokines were dependent on TLR4 and Fc $\gamma$ RIII association and signaling in peritoneal macrophages (28, 29). It is not clear whether TLR4 associates with other FcR $\gamma$ -associated receptors, but our findings suggest a requirement for both signals to upregulate IL-33 upon HDM treatment.

In addition, we demonstrated that Dectin-2 mediated HDM-induced IL-33 production from BMDCs. Canonical signaling downstream of Dectin-2/FcR $\gamma$  is thought to primarily involve activation of Syk, which has been shown to play a critical role in host defense against *Candida Albicans* (13), *Schistosoma Mansoni* (30), *Microsporium Audouinii*, and *Trichophyton Rubrum* (31). Although Dectin-2/FcR $\gamma$  signaling is known to activate Syk and generate NF- $\kappa$ B dependent cytokines through the adaptor protein CARD9, here we demonstrate a novel Syk independent function (14). Overall, our findings identify a pathway downstream of Dectin-2, FcR $\gamma$ , and TLR4 activation which promotes the production of IL-33 that can contribute to the development of Th2 responses.

DCs express a wide variety of receptors that associate and signal through FcR $\gamma$  including Fc $\gamma$ RI, Fc $\gamma$ RIII, Fc $\epsilon$ RI, Dectin-2, activating immunoglobulin-like receptors (LILR, ILT, or LIR), DC activating receptor (DCAR), and paired immunoglobulin-like receptor A (PIR-A) (23, 32). More importantly, several studies have demonstrated or suggested that expression of these receptors on DCs can promote and exacerbate Th2-type responses and allergic airway inflammation including Fc $\gamma$ RI (11, 33), Fc $\gamma$ RIII (8), Fc $\epsilon$ RI (34), and Dectin-2 (35). It has been shown that these receptors can bind to other ligands associated with allergic lung disease including *Candida albicans* (36), *Aspergillus fumigatus* (14), and pollen starch granules (37). Thus, our findings may have broader implications for other allergens that utilize FcR $\gamma$ -associated receptors to promote and exacerbate allergic responses.

Asthma is a heterogenous disease, and a more comprehensive view of how allergens are activating immune cells to promote Th2-type responses must be taken into account in order to develop more effective therapeutics. Several clinical trials have investigated the potential use of anti-cytokine therapy against IL-1, IL-4, IL-5, IL-9, IL-10, IL-12, IL-13, IL-18, and TNF- $\alpha$ ; however, there has been limited success with these approaches (38, 39). Taken together with our previous studies, FcR $\gamma$  signaling on DCs upregulates cysLTs, IL-6, IL-10, IL-23, TNF- $\alpha$ , and IL-33 (14, 16, 35). These results highlight the extensive cascade of inflammatory mediators that can be induced by ligation of receptors that signal through FcR $\gamma$ , and it suggests that a more comprehensive strategy for inhibiting the entire cascade may be more effective than many of the anti-cytokine therapeutic strategies that were previously employed.



## Material and Methods

### Assessment of airway inflammation

For histology, the right middle lobe was removed from the mice after BAL and fixed by immersion into 10% formalin. Fixed sections were stained with hematoxylin and eosin (H&E) or Periodic acid-Schiff (PAS) by the University of Chicago Tissue Resource Center. BAL was performed by delivering sterile PBS into the airway and repeatedly aspirating the fluid for a total recovery of ~3 mL. For FACS analysis,  $5 \times 10^5$  cells were resuspended in 100  $\mu$ L of FACS buffer (PBS containing 0.1% sodium azide and 1% BSA) and blocked with 20  $\mu$ L of 2.4G2 (anti-CD16/32) supernatant for 10 minutes at 25°C. The cells were then stained for 30 minutes at 4°C with fluorescently conjugated antibodies. Eosinophils were identified as SSC<sup>hi</sup>CCR3<sup>+</sup>Grl<sup>-</sup> while CD4<sup>+</sup> T cells were gated as SSC<sup>o</sup>CD3<sup>+</sup>CD4CD8<sup>-</sup>. Flow cytometric analysis was performed on an LSRFortessa (BD Biosciences), and the data were analyzed with FlowJo software (Tree Star Inc.).

Specificity	Clone	Company	Dilution
CCR3	83101	R&D Systems	1 $\mu$ g/mL
CD3	145-2C11	eBiosciences	1 $\mu$ g/mL
CD4	RM4-5	eBiosciences	125 ng/mL
CD8	53-6.7	eBiosciences	250 ng/mL
CD44	IM7	eBiosciences	500 ng/mL
CD62L	MEL-14	eBiosciences	250 ng/mL
Ly6C/G(Grl)	1A8	BD Pharmigen	1 $\mu$ g/mL

### Quantitative PCR (qPCR) primer sequences

	Primer Sequence
IL-33 Forward	5'-GCTGCGTCTGTTGACACATT-3'
IL-33 Reverse	5'-CACCTGGTCTTGCTCTTGGT-3'
$\beta$ 2M Forward	5'-CATACGCCTGCAGAGTTAAGCA-3'
$\beta$ 2M Reverse	5'-GATCACATGTCCGATCCCAGTAG-3'

### Nylon wool non-adherent T cell enrichment and transfer

Brachial, inguinal, cervical, and mesenteric lymph nodes were isolated from mice and made into single cell suspensions. Autoclaved nylon wool columns (Polysciences, Inc.) were equilibrated after which cell suspensions were loaded onto the column. Non-adherent cells were eluted, and  $1.0 \times 10^7$  cells were administered i.v. into naive mice 2 days before being sensitized with HDM. They were then challenged with HDM a week later and sacrificed four days later.

### Isolation of draining (mediastinal) lymph node cells

Draining (mediastinal) lymph nodes were made into single cell suspensions. The tissue was mechanically dissociated and passed through a filter to remove debris. Red blood cells were lysed with ammonium chloride-potassium lysing buffer after which the single cell suspension was stained for flow cytometry.

### Isolation of lung cells

The lungs were perfused with 25 U/mL of heparin (Sigma Aldrich) in PBS and minced with scissors. Tissue dissociation was achieved by incubating the tissue for 30 minutes at 37°C in 5% complete media with 150 U/mL collagenase I (Invitrogen). The digest was passed through a filter to remove debris, and red blood cells were lysed with ammonium chloride-potassium lysing buffer after which the single cell suspension was stained for flow cytometry.

### Production of bone marrow-dendritic cells (BMDCs)

BMDCs were generated as previously described (40). Bone marrow was flushed from the femurs and tibias of mice, and the cells were cultured in 10% complete DMEM supplemented with 20 ng/mL GM-CSF (Shenandoah Biotechnology). On day 3 and 6, the media was replenished with 20 ng/mL GM-CSF. On day 8, the suspension cells were harvested, and  $5 \times 10^5$  BMDCs were cultured in 24-well plates with OVA (percent viability:  $91.9 \pm 3.0$ ), OVA-IC (percent viability:  $93.1 \pm 3.1$ ), or HDM (percent viability:  $91.7 \pm 2.1$ ) at a concentration of 25  $\mu$ g/mL.

### Quantitative PCR (qPCR)

RNA was isolated from cells using an RNeasy Micro Kit according to the manufacturer's protocol (Qiagen) and quantified by nanodrop (Thermo Scientific). cDNA synthesis was done using the Superscript III Reverse Transcriptase kit according to the manufacturer's protocol (Invitrogen). cDNA samples were amplified with the Power Sybr Green PCR master mix (Applied Biosystems) and run on a ABI 7300 cycler (Bio-Rad). Primer sequences used are available in the supplementary materials.

### Statistical analysis

All statistical analyses were performed with GraphPad Prism software, and a P-value less than 0.05 was considered significant (\*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ ; ns = not significant). Experiments with two groups were analyzed using an unpaired Student's two-tailed *t* test. Experiments with greater than two groups were analyzed with a one-way ANOVA and post-hoc Tukey test. Error bars represent the SEM.

### Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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## Abbreviations

<b>DCs</b>	dendritic cells
<b>BMDCs</b>	bone-marrow derived dendritic cells
<b>BAL</b>	bronchoalveolar lavage
<b>OVA</b>	ovalbumin
<b>ICs</b>	immune complexes
<b>HDM</b>	house dust mite
<b>WT</b>	wildtype
<b>cysLTs</b>	cysteinyl leukotrienes
<b>i.t</b>	intratracheal
<b>TLR</b>	toll-like receptor
<b>Th</b>	T helper cell

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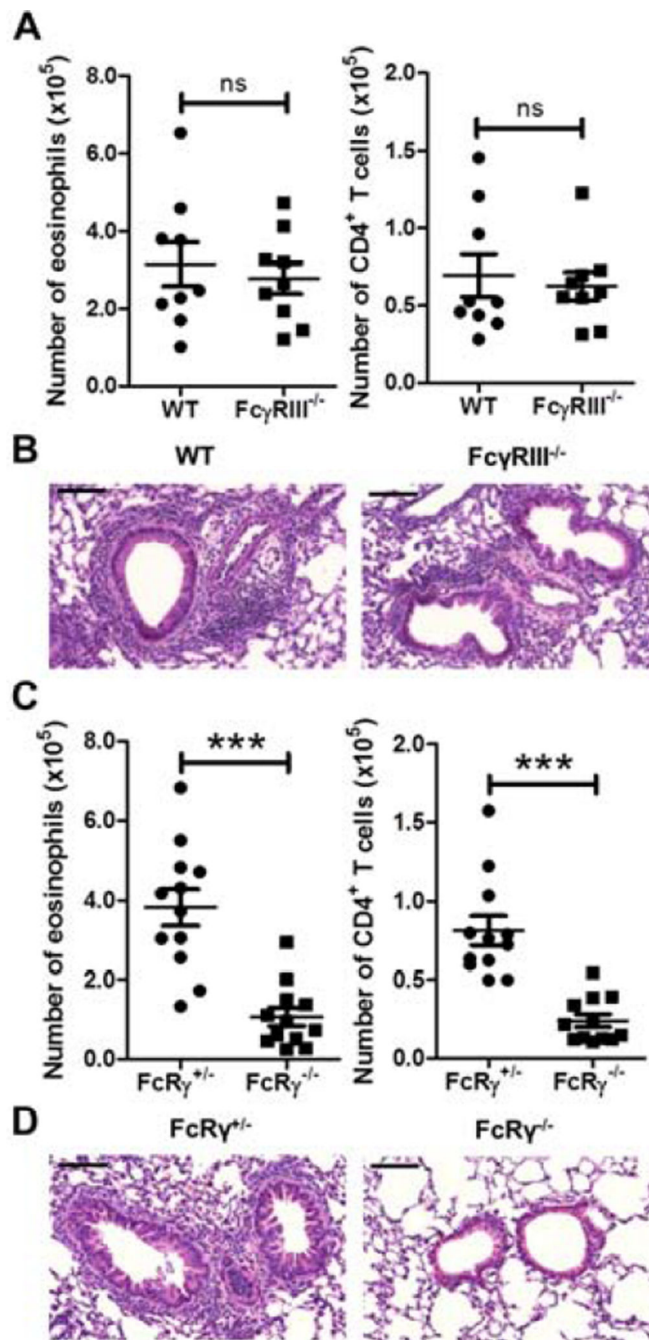
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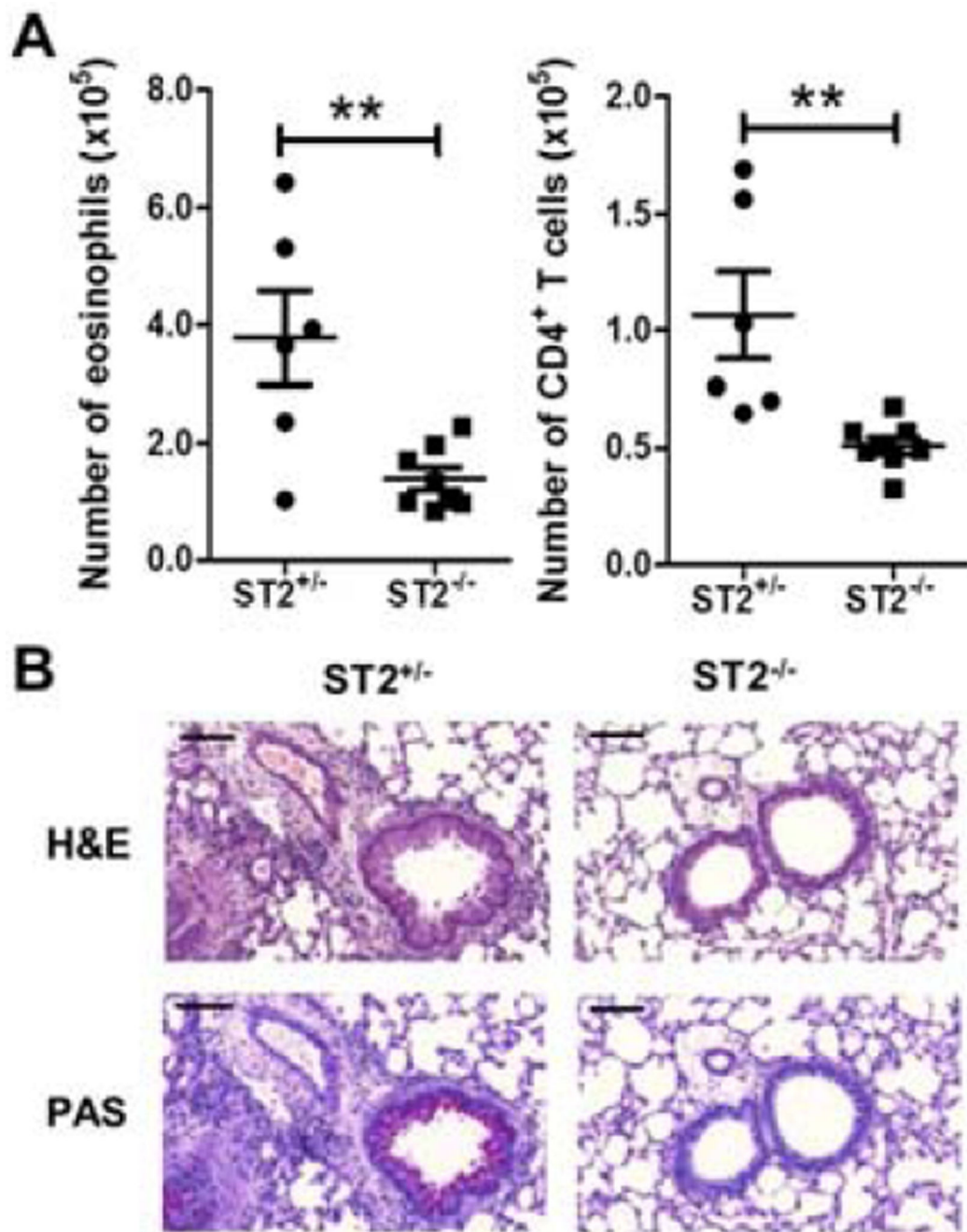
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**Key Message**

- This study identifies a mechanism through which allergens are able to promote allergic airway inflammation by activating FcR $\gamma$ -associated receptors in concert with TLR4 on DCs.
- Ligation of FcR $\gamma$ -associated receptors on DCs induces production of IL-33 that leads to the development of Th2 responses *in vivo*.



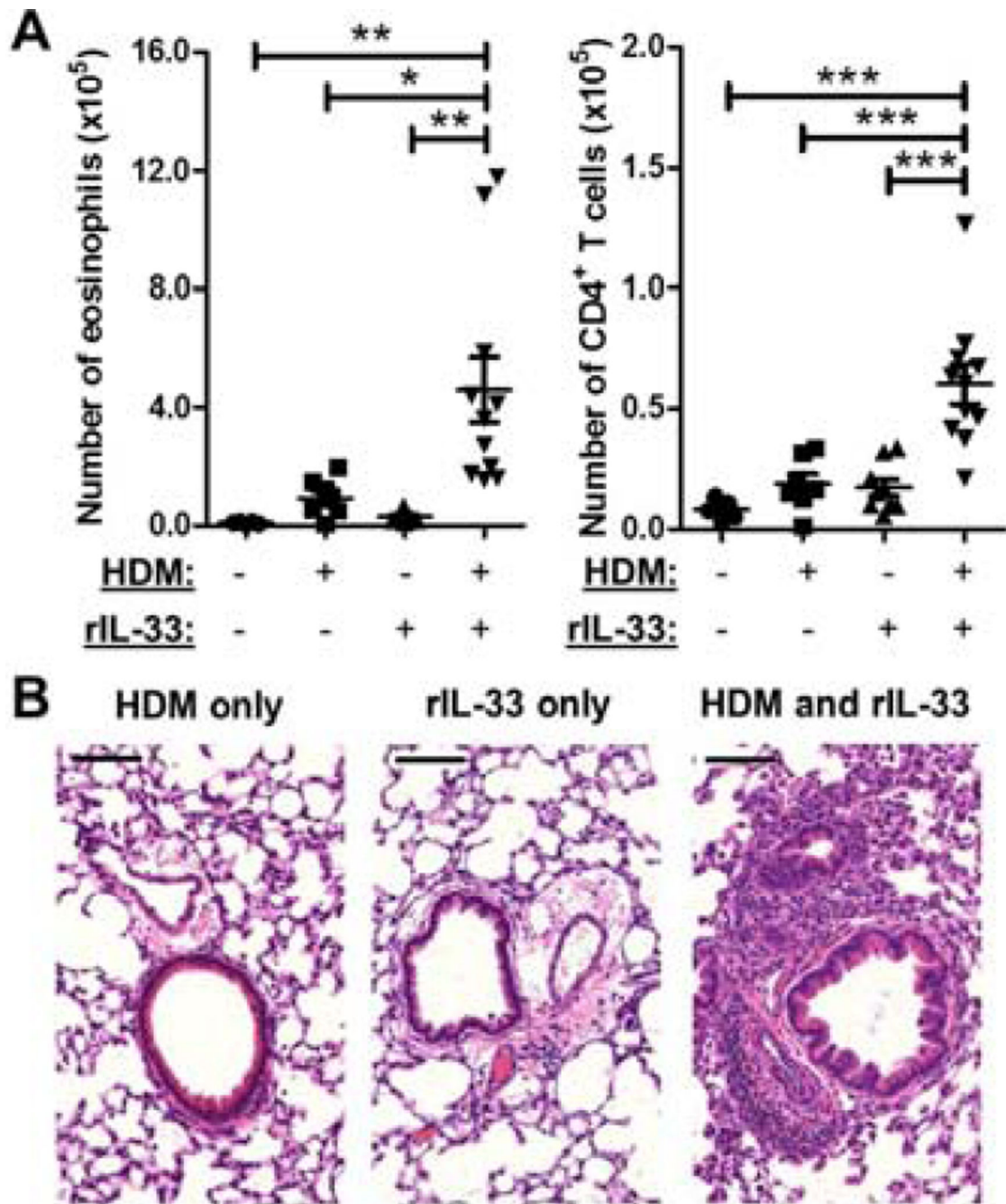
**Figure 1. HDM-mediated inflammation is dependent on Fc $\gamma$  but not Fc $\gamma$ RIII**  
 WT or Fc $\gamma$ RIII<sup>-/-</sup> (A and B) and FcR $\gamma$ <sup>+/-</sup> or FcR $\gamma$ <sup>-/-</sup> (C and D) mice were sensitized on day 0 and challenged on day 7 with HDM. Airway inflammation was assessed on day 11 by determining (A) and (C) the number of eosinophils (left panel), and CD4<sup>+</sup> T cells (right panel) in the BAL by flow cytometry. (B) and (D) Representative H&E sections of lung tissue from treated mice. Black bars = 100  $\mu$ m. Data represent the mean  $\pm$  SEM (\*\*\*,  $P < 0.001$ , ns = not significant). The data are combined from at least three independent experiments with a total of at least nine mice analyzed per group.



**Figure 2. HDM-mediated Th2-type responses are ST2 dependent**

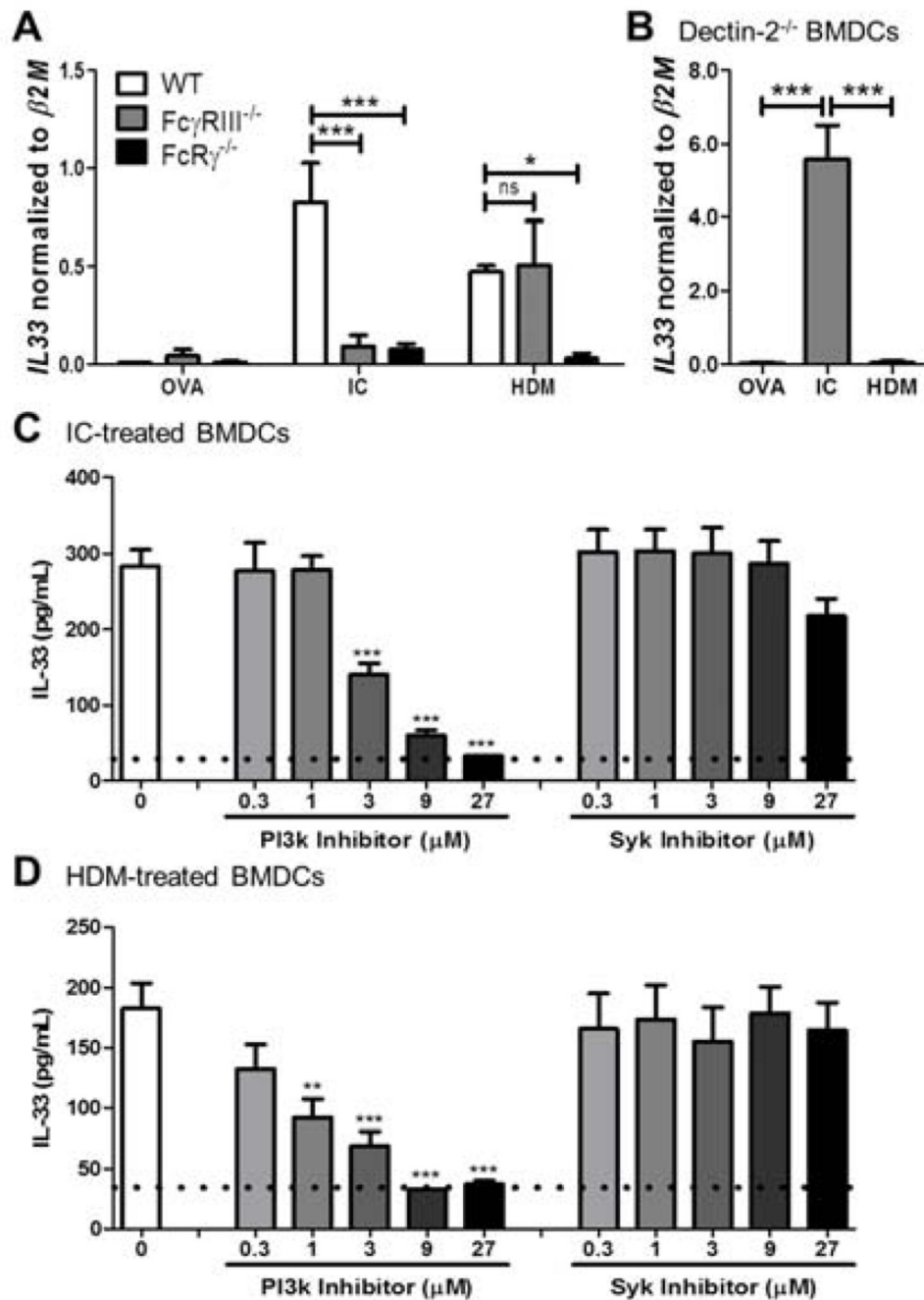
ST2<sup>+/-</sup> or ST2<sup>-/-</sup> mice were sensitized and challenged with HDM. (A) Airway inflammation was assessed by determining the number of eosinophils (left panel), and CD4<sup>+</sup> T cells (right panel) in the BAL. (B) Representative H&E and PAS sections of lung tissue from treated mice. Black bars = 100 μm. Data represent the mean ± SEM (\*\*, P < 0.01). The data are combined from at least two independent experiments with a total of at least six mice analyzed per group.



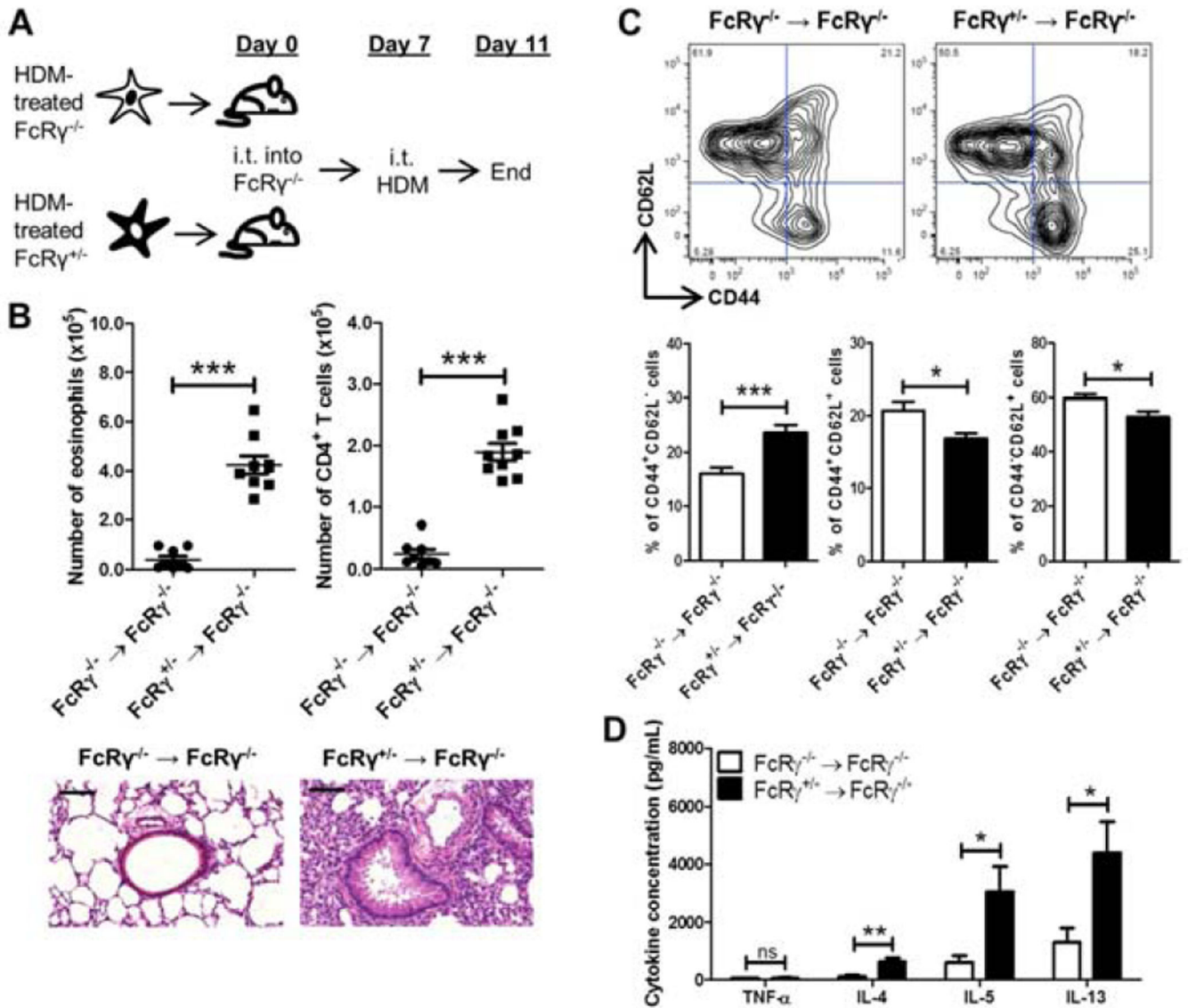


**Figure 3. Administration of IL-33 during the sensitization phase restores airway inflammation in  $FcR\gamma^{-/-}$  mice**

$FcR\gamma^{-/-}$  mice were sensitized with PBS, HDM, rIL-33, or HDM and rIL-33 on day 0. On day 7, all mice received an i.t. challenge of HDM. Airway inflammation was assessed on Day 11 by determining (A) the number of eosinophils (left panel), and CD4<sup>+</sup> T cells (right panel) in the BAL. (B) Representative H&E sections of lung tissue from treated mice. Black bars = 100  $\mu$ m. Data represent the mean  $\pm$  SEM (\*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ ). The data are combined from three independent experiments with a total of at least five mice analyzed per group.



**Figure 4. IL-33 upregulation in DCs is FcR $\gamma$  and PI3-kinase dependent**  
 BMDCs were generated from (A) WT, Fc $\gamma$ RIII<sup>-/-</sup>, and FcR $\gamma$ <sup>-/-</sup> mice or (B) Dectin-2<sup>-/-</sup> mice and treated with OVA, ICs, or HDM overnight before assessing for IL-33 mRNA expression normalized to  $\beta$ 2M mRNA expression. IL-33 protein expression was determined in WT BMDCs stimulated with (C) ICs or (D) HDM and treated with PI3-kinase inhibitor (Ly294002) or Syk inhibitor (piceatannol). Data represent the mean  $\pm$  SEM from three independent culture sets (\*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ ; ns = not significant).



**Figure 5.  $FcR\gamma^{-/-}$  DCs are sufficient to reconstitute HDM responses in  $FcR\gamma^{-/-}$  mice**  
 (A)  $FcR\gamma^{+/-}$  or  $FcR\gamma^{-/-}$  BMDCs were generated and treated overnight with HDM before being instilled i.t. into naïve  $FcR\gamma^{-/-}$  mice. On day 7 the mice were challenged i.t. with HDM before being sacrificed on day 11. (B) Airway inflammation was assessed by determining the number of eosinophils (left panel), and  $CD4^+$  T cells (right panel) in the BAL. Representative H&E sections of lung tissue from the treated mice. Black bars = 100  $\mu$ m. (C) Representative flow plots of draining lymph node cells gated on  $CD3^+CD4^+$  cells used to determine the percentage of  $CD44^+CD62L^-$ ,  $CD44^+CD62L^+$ , and  $CD44^-CD62L^-$  cells. (D) Amount of cytokine in culture supernatants from HDM-restimulated mediastinal lymph node cells. Data represent the mean  $\pm$  SEM (\*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ ; ns = not significant). The data are combined from two independent experiments with a total of at least eight mice analyzed per group.