Signaling through FcRγ-associated receptors on dendritic cells drives IL-33–dependent T_H2-type responses

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Background: Although allergic sensitization can be generated against various allergens, it is unknown how such a diversity of antigens is able to promote T_H2-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 T_H2-mediated inflammation, but the mechanism by which these diverse stimuli produce similar responses is unknown.
Objective: We sought to identify the DC signaling pathways used by T_H2 stimuli to promote T_H2-mediated inflammation.
Methods: C57BL/6, FcγRIII$^{-/-}$, FcRγ$^{-/-}$, and ST2$^{-/-}$ mice were sensitized and challenged with HDM, and inflammation was assessed based on results of flow cytometry and histology and cytokine production. Bone marrow–derived DCs from these strains were used in signaling and adoptive transfer experiments.
Results: Our findings indicate that 2 distinct T_H2 stimuli, ICs and HDM, use the FcRγ-associated receptors FcγRIII and Dectin-2, respectively, to promote T_H2-mediated lung 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 T_H2 cells, in DCs. Upregulation of IL-33 in DCs is dependent on FcRγ, Toll-like receptor 4, and phosphoinositide 3-kinase. Exogenous IL-33 is sufficient to restore the development of T_H2 responses in FcRγ-deficient mice. Finally, adoptive transfer of allergen-pulsed FcRγ$^{+/-}$ bone-marrow derived DCs restores the development of T_H2-type inflammation in FcRγ-deficient mice, demonstrating the necessity of this signaling pathway in DCs for allergen-induced inflammation.
Conclusion: These data identify a mechanism whereby T_H2 stimuli signal through FcRγ-associated receptors on DCs to upregulate IL-33 production and induce T_H2-mediated allergic airway inflammation. (J Allergy Clin Immunol 2014;■■■:■■■-■■■.)

Key words: *Dendritic cells, immune complexes, house dust mite, FcRγ, T_H2, allergic airway inflammation, lungs*

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 T_H2-type response.[2] Development of a T_H2-type response is dependent on respiratory dendritic cells (DCs) that produce specific cytokines, including IL-10 and IL-33, to promote skewing toward T_H2 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 they exhibit enzymatic activity. It has been suggested that allergens be divided into class I allergens, which have enzymatic activity (ie, dust mite and cockroach), and class II allergens, which do not have enzymatic activity (ie, pet dander).[7] Although allergic sensitization can be generated against various allergens, it is unknown how these diverse allergens can promote T_H2 skewing and differentiation leading to atopy.

Protein allergens that lack enzymatic activity are often modeled in mice by using the inert protein ovalbumin (OVA). Using this model allergen, we previously demonstrated that during secondary responses, OVA-specific IgG formed immune complexes (ICs) with OVA instilled in the airways. These ICs signaled through FcγRIII on DCs and, in the presence of a Toll-like receptor (TLR) 4 stimuli, upregulated IL-33 expression and promoted T_H2-mediated lung inflammation.[8] Although ICs represented one mechanism by which homogeneous protein allergens could promote T_H2-mediated allergic responses, it was unclear whether this model was relevant for other complex allergens, such as house dust mite (HDM), that are comprised of many immunogenic proteins in association with glycans and endotoxin. This allergen contains a wide variety of components that can contribute to its allergenicity, including cysteine proteases, chitinases, serine proteases, and others.[9] We recently found that HDM induced IL-33 expression in bone marrow–derived dendritic cells (BMDCs) in the absence of specific IgG, suggesting alternative pathways for the induction of T_H2 responses.[5] This study was designed to further clarify how diverse allergens are able to induce similar T_H2-type responses in the lungs.

In this study we determine that T_H2 stimuli (ie, ICs and HDM) signal through FcRγ-associated receptors on DCs to promote T_H2 responses in the lungs by increasing IL-33 expression. FcRγ is a common signaling component of multiple receptors, including

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Supported by National Institutes of Health (NIH) grants R21AI094408 (to A.I.S.), K08AI080948 (to N.A.B.), R01HL120952 (to N.A.B.), 5T32HL007605 (to C.L.H.), and 5T32HL007237 (to J.W.W.) and a Naomi Ragins-Goldsmith Fellowship from the University of Chicago (to M.Y.T.). The University of Chicago Cancer Center Core Facilities used are supported by National Institutes of Health grant P30CA14599.

Disclosure of potential conflict of interest: M. Y. Tjota has received travel support from the American Association of Immunologists. C. L. Hrusch has received research support from the National Institutes of Health and is employed by the University of Chicago. A. I. Sperling has received research support from the National Institutes of Health. The rest of the authors declare that they have no relevant conflicts of interest.

Received for publication February 17, 2014; revised April 25, 2014; accepted for publication June 9, 2014.

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0091-6749/$36.00

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http://dx.doi.org/10.1016/j.jaci.2014.06.013

Abbreviations used

BAL:	Bronchoalveolar lavage
BMDC:	Bone marrow–derived dendritic cell
cysLT:	Cysteinyl leukotriene
DC:	Dendritic cell
HDM:	House dust mite
IC:	Immune complex
OVA:	Ovalbumin
PI3K:	Phosphoinositide 3-kinase
Rag:	Recombination-activating gene
TLR:	Toll-like receptor
WT:	Wild-type

FcγRIII and Dectin-2, that have been shown to bind ICs and HDM, respectively.[10,11] Using these 2 different T_H2 stimuli, we demonstrate that ICs and HDM both induce expression of IL-33 in BMDCs in an FcRγ-, TLR4-, and phosphoinositide 3-kinase (PI3K)–dependent manner. HDM-induced allergic airway inflammation is FcRγ dependent, and this inflammatory response was restored in FcRγ$^{-/-}$ mice on administration of recombinant IL-33 during sensitization with HDM. Moreover, adoptive transfer of allergen-pulsed FcRγ$^{+/-}$ BMDCs is sufficient to restore development of T_H2-type inflammation in FcRγ$^{-/-}$ mice. These data identify a mechanism that can be engaged by different T_H2 stimuli to promote atopic asthma by activating DCs through FcRγ-associated receptors to induce IL-33 production and drive T_H2 responses.

METHODS

Mice

C57BL/6 mice (wild-type [WT] mice) were purchased from Harlan Laboratories (Indianapolis, Ind). B6.FcγRIII$^{-/-}$ and B6.FcRγ$^{-/-}$ mice were purchased from the Jackson Laboratories (Bar Harbor, Me). B6.ST2$^{-/-}$ mice were provided by Dr A. McKenzie (Medical Research Laboratory, University of Cambridge, Cambridge, United Kingdom).[12] B6.Dectin-2$^{-/-}$ mice were generated by Dr Y. Iwakura (Institute of Medical Science, Tokyo, Japan).[13] B6.TLR4$^{-/-}$ mice were provided by Dr C. Nagler (University of Chicago, Chicago, Ill). 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

HDM (XPB82D3A25; Greer Laboratories, Lenoir, NC), Grade V chicken egg OVA (Sigma-Aldrich, St Louis, Mo), 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, San Diego, Calif) 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. PI3K 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 μg of HDM, 500 ng of rIL-33, or both HDM and rIL-33) and day 7 (PBS or 100 μg of HDM) and then killed on day 11. For adoptive transfer experiments, mice were sensitized by instilling 1×10^6 treated BMDCs intratracheally into naive mice. On day 7, all the mice were challenged intratracheally with 100 μg of HDM and killed on day 11. By using the passive transfer model developed in our previous study,[8] 100 μL of α-OVA or α-BSA sera was injected intravenously into mice, followed by a 100-μg OVA intratracheal sensitization the next day; 1 week later, animals were challenged with 3 intratracheal OVA administrations. Airway inflammation was assessed as described in Tables E1 and E2 and the Methods section in this article's Online Repository at www.jacionline.org.

Statistical analysis

All statistical analyses were performed with GraphPad Prism software (GraphPad Software, La Jolla, Calif), and a P value less than .05 was considered significant. Experiments with 2 groups were analyzed by using an unpaired Student 2-tailed t test. Experiments with greater than 2 groups were analyzed with 1-way ANOVA and the *post hoc* Tukey test.

Cytokine analysis

For cytokine analysis, BMDCs were stimulated overnight, as described above. For the T cell restimulation assay, single-cell suspensions from the mediastinal lymph nodes were cultured with 25 μg/mL HDM at 2×10^5 cells per well for 48 hours. The plates were put through a freeze-thaw cycle to release intracellular cytokines before supernatants were collected and analyzed with the Multiplex bead array, according to the manufacturer's protocol (Millipore, Temecula, Calif).

RESULTS

HDM-mediated T_H2-type responses are dependent on FcRγ but not FcγRIII

Although we 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 T_H2 responses. We first demonstrated that an eosinophilic and CD4$^+$ T-cell response was only seen in WT mice that had received both an intratracheal sensitization and challenge with HDM a week apart; the mice were killed 4 days after the last challenge (see Fig E1 in this article's Online Repository at www.jacionline.org). No significant response was seen in mice that had received an HDM sensitization/PBS challenge, PBS sensitization/HDM challenge, or PBS sensitization/PBS challenge (see Fig E1). We then sensitized and challenged WT and FcγRIII$^{-/-}$ mice with HDM by using the protocol described above. Interestingly, we found no difference in the eosinophil or CD4$^+$ T-cell numbers in bronchoalveolar lavage (BAL) fluid between the 2 strains (Fig 1, *A*). Histologic examination of the lungs showed that both strains had thickening of the airway epithelium and inflammatory cell infiltrates around the airways (Fig 1, *B*). These results suggested that HDM used an FcγRIII-independent pathway to promote T_H2 inflammation in the lungs.

We had previously shown that glycans in HDM were recognized by the Dectin-2/FcRγ receptor complex, which led to increased T_H2-type responses through the production of cysteinyl leukotrienes (cysLTs), TNF-α, IL-6, IL-10, and IL-23.[14,15] Given that FcγRIII and Dectin-2 both signal through FcRγ, we hypothesized that FcRγ-containing receptors were used by T_H2 stimuli to induce T_H2 responses *in vivo*. It was previously shown that allergen-induced airway hyperresponsiveness and inflammation were FcRγ dependent by using an OVA sensitization and challenge model.[16] This led us to question whether HDM-mediated responses were also FcRγ dependent. Heterozygous (FcRγ$^{+/-}$) and knockout (FcRγ$^{-/-}$) littermates were compared

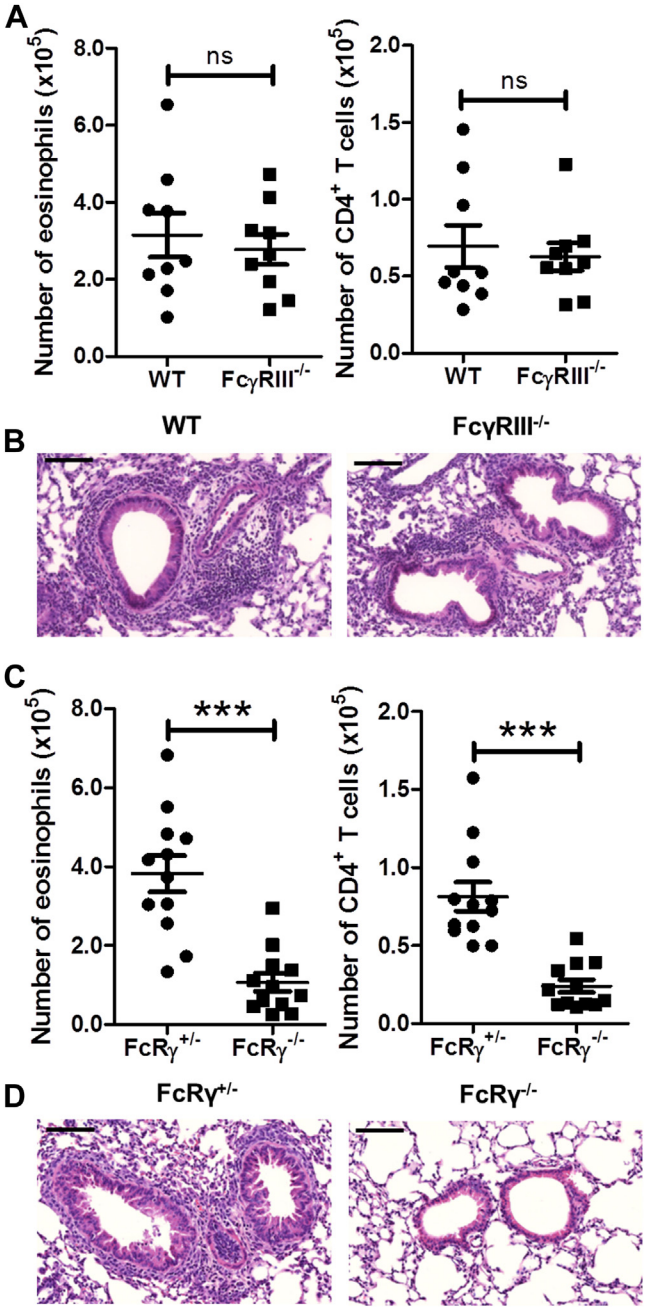

FIG 1. HDM-mediated inflammation is dependent on FcRγ but not FcγRIII. WT or FcγRIII$^{-/-}$ **(A** and **B)** and FcRγ$^{+/-}$ or FcRγ$^{-/-}$ **(C** and **D)** mice were sensitized on day 0 and challenged on day 7 with HDM. Fig 1, *A* and *C*, Airway inflammation was assessed on day 11 by determining eosinophil *(left panel)* and CD4^{+} T-cell *(right panel)* numbers in BAL fluid by using flow cytometry. Fig 1, *B* and *D*, Representative hematoxylin and eosin–stained sections of lung tissue from treated mice. *Black bars* = 100 μm. Data represent means ± SEMs. Data are combined from at least 3 independent experiments, with a total of at least 9 mice analyzed per group. ***$P < .001$. *ns*, Not significant.

after sensitization and challenge with HDM. Both eosinophil and CD4^{+} T-cell numbers were significantly decreased in the BAL fluid of FcRγ$^{-/-}$ mice, demonstrating that this receptor component was important for the induction of T_H2 responses to HDM (Fig 1, *C*). Furthermore, there was decreased inflammation in the airways of the FcRγ$^{-/-}$ mice, as evidenced by histology (Fig 1, *D*). These findings supported our hypothesis that FcRγ was part of a common signaling pathway that induced allergic airway inflammation.

IL-33 restores allergic airway inflammation in FcRγ$^{-/-}$ mice

Our previous work showed that HDM treatment of BMDCs induced expression of IL-33, suggesting that HDM-induced allergic airway inflammation might be dependent on the ST2/IL-33 pathway.[5] To determine whether HDM sensitization required ST2, ST2$^{+/-}$ and ST2$^{-/-}$ littermates were sensitized and challenged with HDM. Compared with ST2$^{+/-}$ mice, ST2$^{-/-}$ mice had a significant reduction in eosinophil and CD4^{+} T-cell numbers in the BAL fluid, as well as decreased lung inflammation and mucus production, as shown by means of histology (Fig 2).

Because 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 FcRγ$^{-/-}$ mice. On day 0, FcRγ$^{-/-}$ mice received an intratracheal administration of PBS alone, HDM alone, rIL-33 alone, or HDM plus rIL33 combined. On day 7, all mice were challenged with HDM. The FcRγ$^{-/-}$ mice sensitized with PBS, HDM, or IL-33 alone did not have airway or lung inflammation after HDM challenge (Fig 3). Strikingly, the FcRγ$^{-/-}$ mice that were sensitized with HDM in combination with rIL-33 had increased eosinophilia and CD4^{+} T-cell numbers in the BAL fluid and significant thickening of the airway epithelium and lymphocytic infiltration after challenge with HDM (Fig 3). Thus the addition of IL-33 was sufficient to restore airway and lung inflammation in FcRγ$^{-/-}$ mice. These data suggested that signaling through FcRγ-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 FcRγ and Dectin-2 dependent

WT, FcγRIII$^{-/-}$, and FcRγ$^{-/-}$ BMDCs were generated and treated overnight with OVA, ICs, or HDM to investigate whether signaling through FcRγ was required for increased IL-33 expression and production in DCs. IC or HDM treatment of WT BMDCs upregulated MHC class II and CD86 expression more than OVA, suggesting that these stimuli induced greater activation of BMDCs (see Fig E2, *A*, in this article's Online Repository at www.jacionline.org). As we had shown in our earlier study, ICs induced IL-33 in an FcγRIII-dependent manner (Fig 4, *A*).[8] HDM-induced upregulation of IL-33 was FcγRIII independent, supporting our earlier findings that HDM-mediated T_H2 inflammation did not require FcγRIII. Interestingly, similar to the induction by ICs, increased expression of IL-33 after treatment with HDM was dependent on FcRγ (Fig 4, *A*). We determined that after treatment with HDM, there was no significant difference in the expression of MHC class II, CD86, or CD40 on WT and FcRγ$^{-/-}$ BMDCs (see Fig E2, *B*). Furthermore, our previous work suggested there might be cross-talk between FcRγ-associated receptors and TLR4, and indeed, we found that IL-33 upregulation after HDM treatment was TLR4 dependent (see Fig E3 in this article's Online Repository at www.jacionline.org). To determine whether IL-33

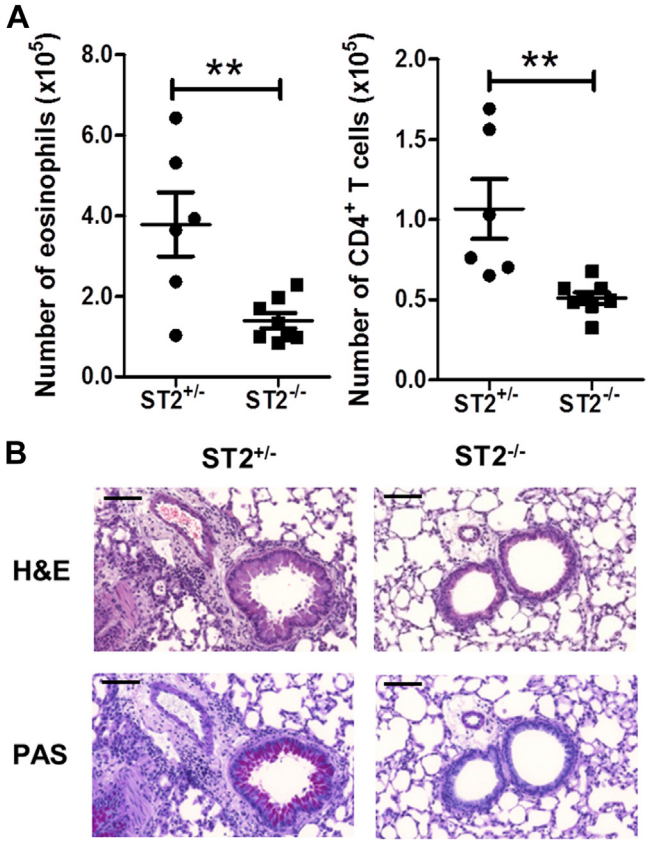

FIG 2. HDM-mediated T_H2-type responses are ST2 dependent. $ST2^{+/-}$ or $ST2^{-/-}$ mice were sensitized and challenged with HDM. **A,** Airway inflammation was assessed by determining eosinophil *(left panel)* and $CD4^+$ T-cell *(right panel)* numbers in BAL fluid. **B,** Representative hematoxylin and eosin *(H&E)*– and periodic acid–Schiff *(PAS)*–stained sections of lung tissue from treated mice. *Black bars* = 100 μm. Data represent means ± SEMs. Data are combined from at least 2 independent experiments, with a total of at least 6 mice analyzed per group. $^{**}P < .01$.

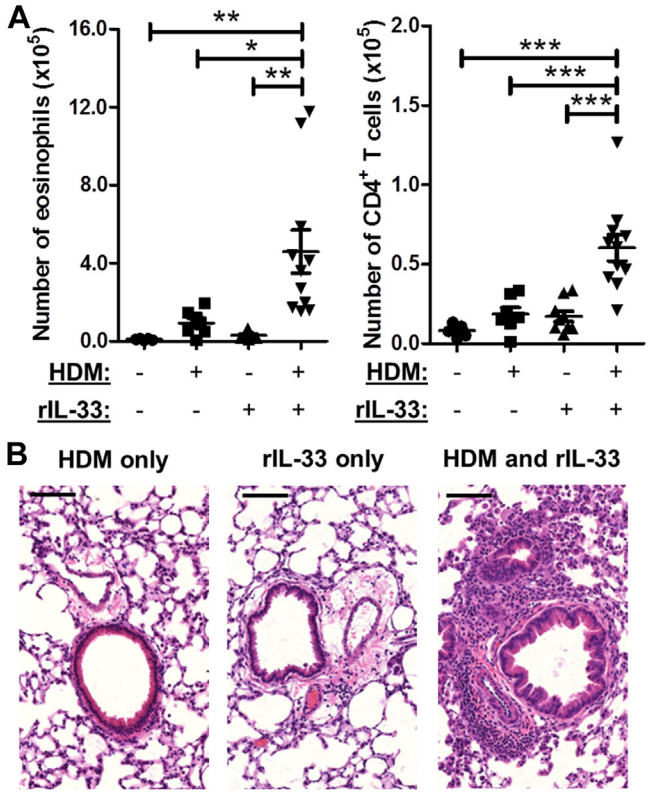

FIG 3. Administration of IL-33 during the sensitization phase restores airway inflammation in $FcR\gamma^{-/-}$ mice. $FcR\gamma^{-/-}$ mice were sensitized with PBS, HDM, rIL-33, or HDM and rIL-33 on day 0. On day 7, all mice received an intratracheal challenge of HDM. **A,** Airway inflammation was assessed on day 11 by determining eosinophil *(left panel)* and $CD4^+$ T-cell *(right panel)* numbers in BAL fluid. **B,** Representative hematoxylin and eosin *(H&E)*–stained sections of lung tissue from treated mice. *Black bars* = 100 μm. Data represent means ± SEMs. Data are combined from 3 independent experiments, with a total of at least 5 mice analyzed per group. $^{*}P < .05$, $^{**}P < .01$, and $^{***}P < .001$.

upregulation was downstream of the Dectin-2/FcRγ receptor complex, Dectin-$2^{-/-}$ BMDCs were treated with OVA, ICs, or HDM. In the absence of Dectin-2, there was no significant increase in *IL33* mRNA expression after treatment with HDM (Fig 4, *B*). From these findings, we determined that IC signaling through FcγRIII or HDM signaling through Dectin-2 used the common signaling component FcRγ to promote IL-33 expression.

Because both ICs and HDM upregulated IL-33 in an FcRγ-dependent manner but used different coreceptors, we investigated whether they engaged the same downstream signaling molecules for IL-33 induction. Previously, we found that downstream of FcRγ signaling on BMDCs, cysLT upregulation was Syk dependent, whereas IL-10 upregulation was PI3K dependent.[14,16] In addition, work on mast cells had demonstrated that IL-33 upregulation on FcεRI ligation was PI3K dependent.[17] WT BMDCs were treated with either ICs or HDM in the presence of a PI3K inhibitor (Ly294002) or Syk inhibitor (piceatannol) to investigate which pathway led to IL-33 upregulation. Interestingly, compared with the control group that received no inhibitors, IC- and HDM-induced IL-33 production was PI3K dependent. However, the Syk inhibitor did not reduce the IC- and HDM-induced IL-33 production found in the control groups (Fig 4, *C* and *D*). Taken together, these data identified a common pathway through FcRγ and PI3K, which was used by different T_H2 stimuli to induce production of IL-33 in DCs, which is distinct from the pathway used to produce cysLTs.

FcRγ-sufficient BMDCs reconstitute HDM-mediated inflammation in $FcR\gamma^{-/-}$ mice

In our studies on IC-mediated T_H2 inflammation, we demonstrated that T cells were necessary and sufficient for development of allergic airway inflammation.[8] WT or recombination-activating gene $(Rag)^{-/-}$ mice were sensitized and challenged with HDM, as described above, to determine whether T cells were playing a role in our HDM allergen model. Notably, there was a significant decrease in the number of total cells and eosinophils in the BAL fluid of $Rag^{-/-}$ mice (see Fig E4, *A*, in this article's Online Repository at www.jacionline.org). This inflammatory response was restored in $Rag^{-/-}$ mice that had received an adoptive transfer of nylon wool–nonadherent cells (see Fig E4, *B*). These data demonstrate that T cells are necessary for HDM-mediated allergic airway inflammation.

Development of T_H2 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

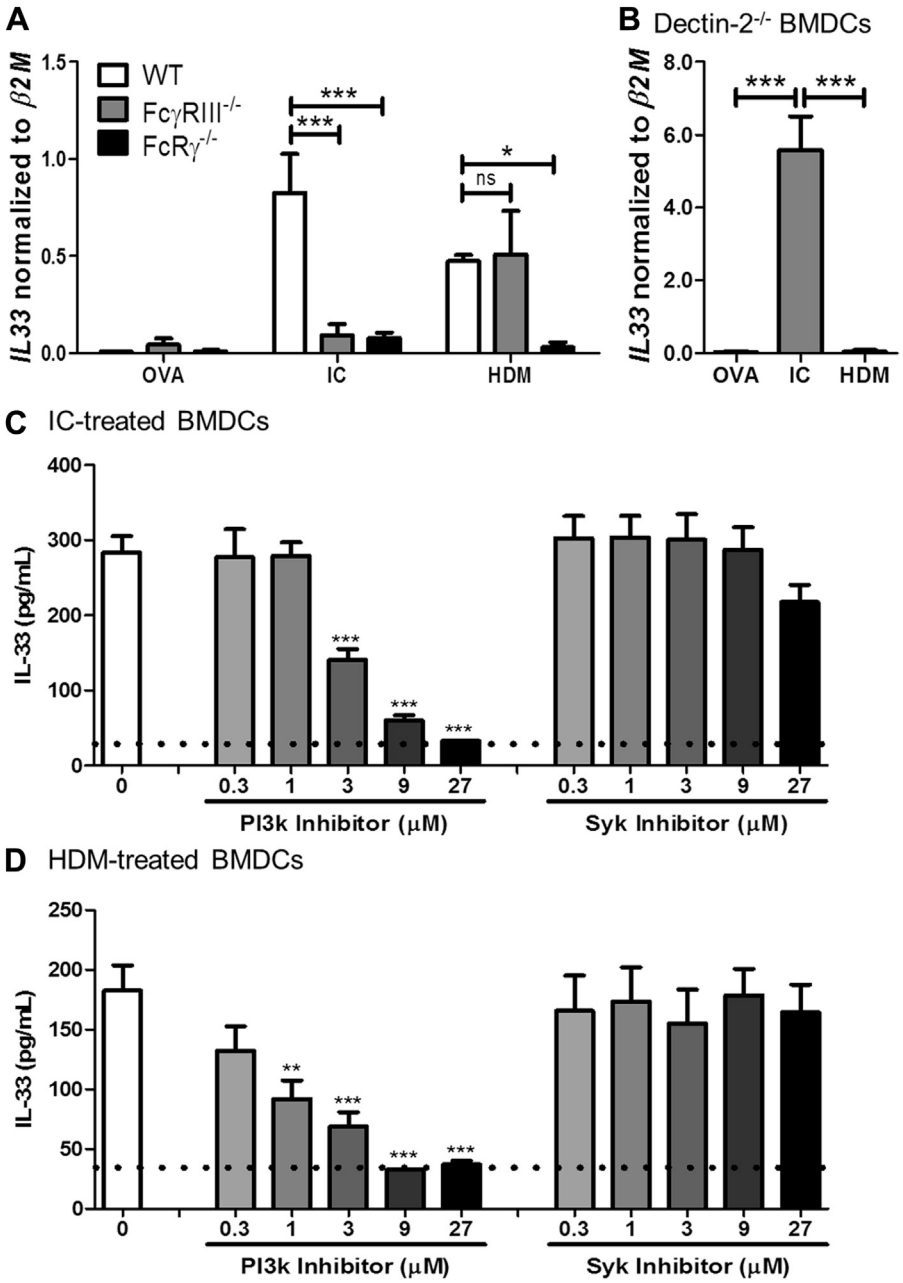

FIG 4. IL-33 upregulation in DCs is FcRγ and PI3K dependent. **A** and **B,** BMDCs were generated from WT, FcγRIII$^{-/-}$, and FcRγ$^{-/-}$ mice (Fig 4, *A*) or Dectin-2$^{-/-}$ mice (Fig 4, *B*) and treated with OVA, ICs, or HDM overnight before assessing for *IL33* mRNA expression normalized to β_2-microglobulin mRNA *(β2M)* expression. **C** and **D,** IL-33 protein expression was determined in WT BMDCs stimulated with ICs (Fig 4, *C*) or HDM (Fig 4, *D*) and treated with PI3K inhibitor (Ly294002) or Syk inhibitor (piceatannol). Data represent means ± SEMs from 3 independent culture sets. *$P < .05$, **$P < .01$, and ***$P < .001$. *ns*, Not significant.

whether the lack of inflammation in the FcRγ$^{-/-}$ mice was due to a deficient response from lung DCs. To address this question, allergen-pulsed FcRγ$^{-/-}$ or FcRγ$^{+/-}$ BMDCs were adoptively transferred into naive FcRγ$^{-/-}$ mice (Fig 5, *A*). FcRγ$^{-/-}$ and FcRγ$^{+/-}$ BMDCs were stimulated overnight with HDM before 1×10^6 BMDCs were transferred intratracheally into naive FcRγ$^{-/-}$ mice. On day 7, all the mice received an intratracheal challenge of HDM before being killed on day 11. The mice that received the FcRγ$^{+/-}$ BMDCs had significantly increased eosinophil and CD4$^+$ T-cell numbers in the BAL fluid, as well as increased inflammation, as seen on histology (Fig 5, *B*). 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 naive CD4$^+$ T cells (CD44$^-$CD62L$^+$) in the mediastinal lymph nodes of mice that had received FcRγ$^{+/-}$ BMDCs (Fig 5, *C*). Moreover, the draining lymph node cells from mice that had received FcRγ$^{+/-}$ BMDCs produced significantly more IL-4, IL-5, and IL-13 on restimulation with HDM (Fig 5, *D*). Together, these data demonstrated that signaling through FcRγ-associated receptors on DCs during

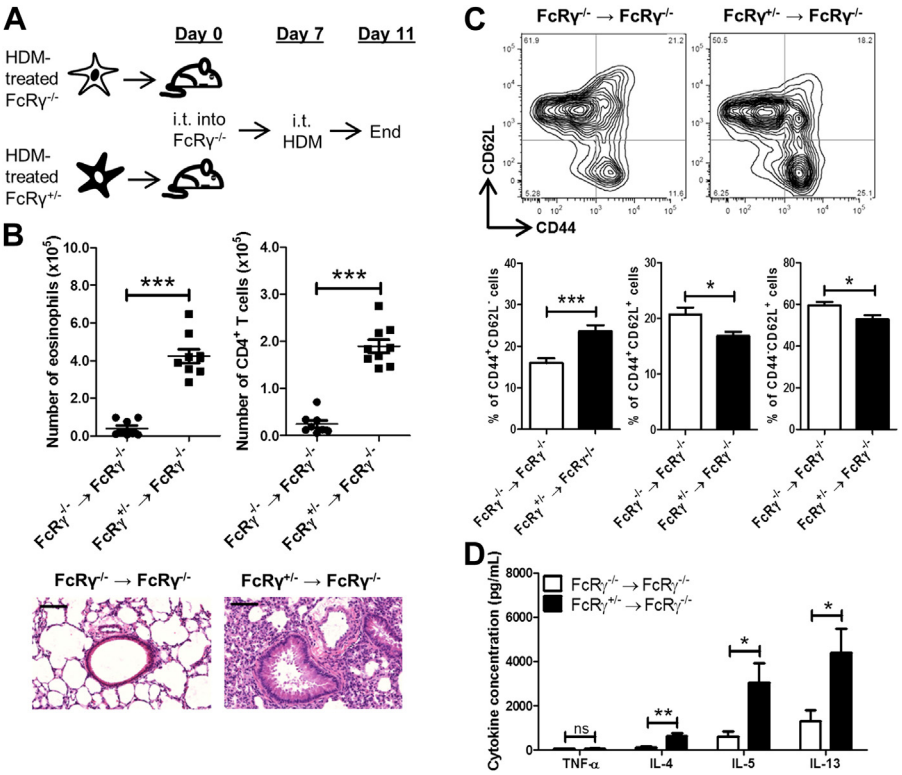

FIG 5. FcRγ$^{+/-}$ DCs are sufficient to reconstitute HDM responses in FcRγ$^{-/-}$ mice. **A,** FcRγ$^{+/-}$ or FcRγ$^{-/-}$ BMDCs were generated and treated overnight with HDM before being instilled intratracheally into naive FcRγ$^{-/-}$ mice. On day 7, mice were challenged intratracheally with HDM before being killed 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 fluid. Representative hematoxylin and eosin sections of lung tissue from the treated mice are shown. *Black bars* = 100 μm. **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 means ± SEMs. Data are combined from 2 independent experiments, with a total of at least 8 mice analyzed per group. *P < .05, **P < .01, and ***P < .001. *ns*, Not significant.

sensitization was sufficient to restore effective priming of T cells in the draining lymph node, which in turn led to the development of T_H2-type inflammation.

DISCUSSION

The focus of this study was to understand how 2 structurally and biologically diverse allergens were able to induce similar T_H2-type responses in the lungs. Using ICs and HDM, we determined that they both signaled through FcRγ-associated receptors, TLR4, and PI3K to upregulate IL-33 production in DCs. We further demonstrated that signaling through FcRγ-associated receptors on DCs was sufficient to restore T_H2 responses in FcRγ-deficient mice. These data identify a mechanism that can be engaged by different T_H2 stimuli to promote atopic asthma by activating DCs through FcRγ-associated receptors to induce IL-33 production and drive T_H2 responses.

Our results demonstrate that hematopoietic cells expressing FcRγ-associated receptors are able to upregulate IL-33, suggesting that this pathway might contribute to the pathogenesis of T_H2-mediated diseases. In human subjects 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 IL-33 levels in the sera and tissues of patients with asthma or allergies.[19-22] However, which human hematopoietic cells can act as a source of IL-33 during allergic airway inflammation has not been as well characterized. Our studies point to cells that express FcRγ-associated receptors, which in human subjects include DCs, monocytes, macrophages, mast cells, natural killer cells, and basophils.[23] An association between FcRγ and asthma was recently identified by Hinds et al[24] in a genome-wide association study of self-reported asthma for rs2070902 in the FcRγ locus *(FCER1G)*.[24] Although further investigations need to be carried out to clarify whether IL-33 upregulation in human cells is also downstream of FcRγ-associated receptors, taken together with our findings, these studies suggest that binding of allergens to FcRγ-associated receptors induces IL-33 production, which might 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, whereas 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 FcRγ-associated receptors. Allergens like HDM that contain glycans might enhance the

T_H2 response through direct binding of its components to FcRγ-associated receptors, such as Dectin-2. On the other hand, a homogeneous protein allergen like OVA cannot be directly recognized by an FcRγ-associated receptor, but in the presence of antigen-specific IgG generated during a primary response, it can form ICs that can ligate and activate FcγRIII. We have further demonstrated that the IC-mediated T_H2 inflammation model works with other homogeneous proteins, such as BSA. An eosinophilic and $CD4^+$ 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 T_H2 inflammation (see Fig E5 in this article's Online Repository at www.jacionline.org). 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 human subjects circulating allergen-specific ICs have been detected in allergic subjects, but whether they contribute to development of T_H2 responses in human subjects is still unclear.[25-27] Translation of our findings into human studies can help to elucidate whether this dichotomy occurs in human subjects as well.

Interestingly, our study suggests that FcRγ-associated receptors and TLR4 act in concert 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 FcRγ-associated receptor and TLR4.[8] This finding is consistent with other studies showing that activation of TLR4 led to the rapid upregulation of PI3K and that IgG-IC–elicited cytokines were dependent on TLR4 and FcγRIII association and signaling in peritoneal macrophages.[28,29] It is not clear whether TLR4 associates with other FcRγ-associated receptors, but our findings suggest a requirement for both signals to upregulate IL-33 on HDM treatment.

In addition, we demonstrated that Dectin-2 mediated HDM-induced IL-33 production from BMDCs. Canonical signaling downstream of Dectin-2/FcRγ 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] *Microsporum audouinii*, and *Trichophyton rubrum*.[31] Although Dectin-2/FcRγ signaling is known to activate Syk and generate nuclear factor κB–dependent cytokines through the adaptor protein caspase recruitment domain family, member 9, here we demonstrate a novel Syk-independent function.[14] Overall, our findings identify a pathway downstream of Dectin-2, FcRγ, and TLR4 activation, which promotes the production of IL-33 that can contribute to the development of T_H2 responses.

DCs express a wide variety of receptors that associate and signal through FcRγ, including FcγRI, FcγRIII, FcεRI, Dectin-2, activating immunoglobulin-like receptors (leukocyte immunoglobulin-like receptor or immunoglobulin-like transcript), DC activating receptor, and paired immunoglobulin-like receptor A.[23,32] More importantly, several studies have demonstrated or suggested that expression of these receptors on DCs can promote and exacerbate T_H2-type responses and allergic airway inflammation, including FcγRI,[11,33] FcγRIII,[8] FcεRI,[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 might have broader implications for other allergens that use FcRγ-associated receptors to promote and exacerbate allergic responses.

Asthma is a heterogeneous disease, and a more comprehensive view of how allergens are activating immune cells to promote T_H2-type responses must be taken into account to develop more effective therapeutics. Several clinical trials have investigated the potential use of anticytokine therapy against IL-1, IL-4, IL-5, IL-9, IL-10, IL-12, IL-13, IL-18, and TNF-α; however, there has been limited success with these approaches.[38,39] Taken together with our previous studies, FcRγ signaling on DCs upregulates cysLTs, IL-6, IL-10, IL-23, TNF-α, 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 FcRγ, and it suggests that a more comprehensive strategy for inhibiting the entire cascade might be more effective than many of the anticytokine therapeutic strategies that were previously used.

For laboratory support, we thank D. C. Decker and J. Casaos. We also thank A. McKenzie, Y. Iwakura, and C. Nagler for generously providing mice used in this study.

Key messages

- **This study identifies a mechanism through which allergens are able to promote allergic airway inflammation by activating FcRγ-associated receptors in concert with TLR4 on DCs.**
- **Ligation of FcRγ-associated receptors on DCs induces production of IL-33, which leads to development of T_H2 responses *in vivo*.**

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METHODS

Assessment of airway inflammation

For histology, the right middle lobe was removed from mice after BAL and fixed by means of immersion in 10% formalin. Fixed sections were stained with hematoxylin and eosin or periodic acid–Schiff 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 approximately 3 mL. For fluorescence-activated cell sorting analysis, 5×10^5 cells were resuspended in 100 μL of fluorescence-activated cell sorting buffer (PBS containing 0.1% sodium azide and 1% BSA) and blocked with 20 μL 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 $SSC^{hi}CCR3^{+}Gr1^{-}$, whereas $CD4^{+}$ T cells were gated as $SSC^{lo}CD3^{+}CD4^{+}CD8^{-}$. Flow cytometric analysis was performed on an LSRFortessa (BD Biosciences, San Jose, Calif), and the data were analyzed with FlowJo software (Tree Star, Ashland, Ore).

Nylon wool–nonadherent 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, Warrington, Pa) were equilibrated, after which cell suspensions were loaded onto the column. Nonadherent cells were eluted, and 1.0×10^7 cells were administered intravenously into naive mice 2 days before sensitization with HDM. They were then challenged with HDM a week later and killed 4 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 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 medium with 150 U/mL collagenase I (Invitrogen, Carlsbad, Calif). 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 BMDCs

BMDCs were generated as previously described.[E1] Bone marrow was flushed from the femurs and tibias of mice, and the cells were cultured in 10% complete Dulbecco modified Eagle medium supplemented with 20 ng/mL GM-CSF (Shenandoah Biotechnology, Warwick, Pa). On days 3 and 6, medium was replenished with 20 ng/mL GM-CSF. On day 8, the suspension cells were harvested, and 5×10^5 BMDCs were cultured in 24-well plates with OVA (viability, 91.9% ± 3.0%), OVA-IC (viability, 93.1% ± 3.1%), or HDM (viability, 91.7% ± 2.1%) at a concentration of 25 μg/mL.

Quantitative PCR

RNA was isolated from cells by using an RNeasy Micro Kit (Qiagen, Hilden, Germany), according to the manufacturer's protocol and quantified by using NanoDrop (Thermo Scientific, Uppsala, Sweden). cDNA synthesis was done by using the Superscript III Reverse Transcriptase kit (Invitrogen), according to the manufacturer's protocol. cDNA samples were amplified with the Power SYBR Green PCR master mix (Applied Biosystems, Foster City, Calif) and run on an ABI 7300 Cycler (Bio-Rad Laboratories, Hercules, Calif). Primer sequences used are available in Table E2.

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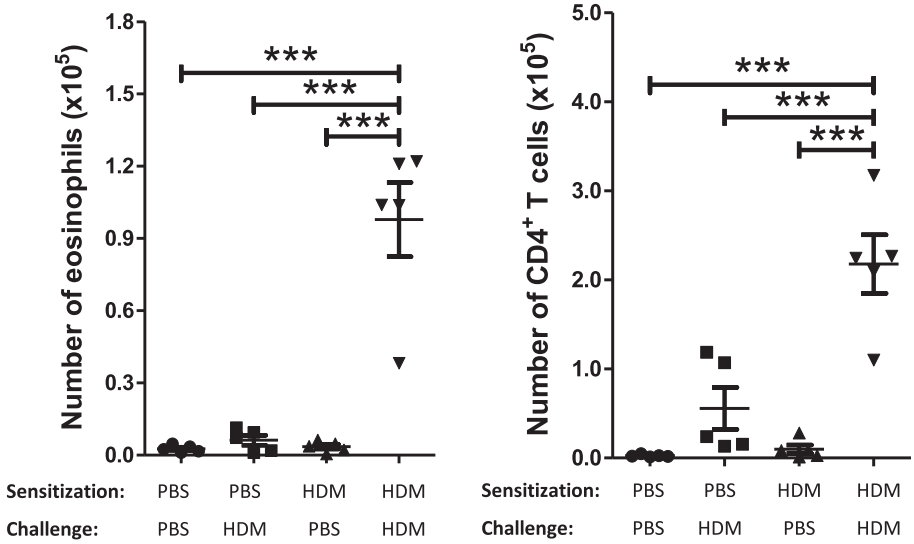

FIG E1. HDM-mediated T_H2 inflammation requires both sensitization and challenge with HDM. WT mice were sensitized on day 0 with PBS or HDM and challenged on day 7 with PBS or HDM, as indicated. On day 11, the mice were killed. Airway inflammation was assessed by determining eosinophil *(left panel)* and $CD4^+$ T-cell *(right panel)* numbers in BAL fluid by means of flow cytometry. Data represent means ± SEMs. ***$P < .001$.

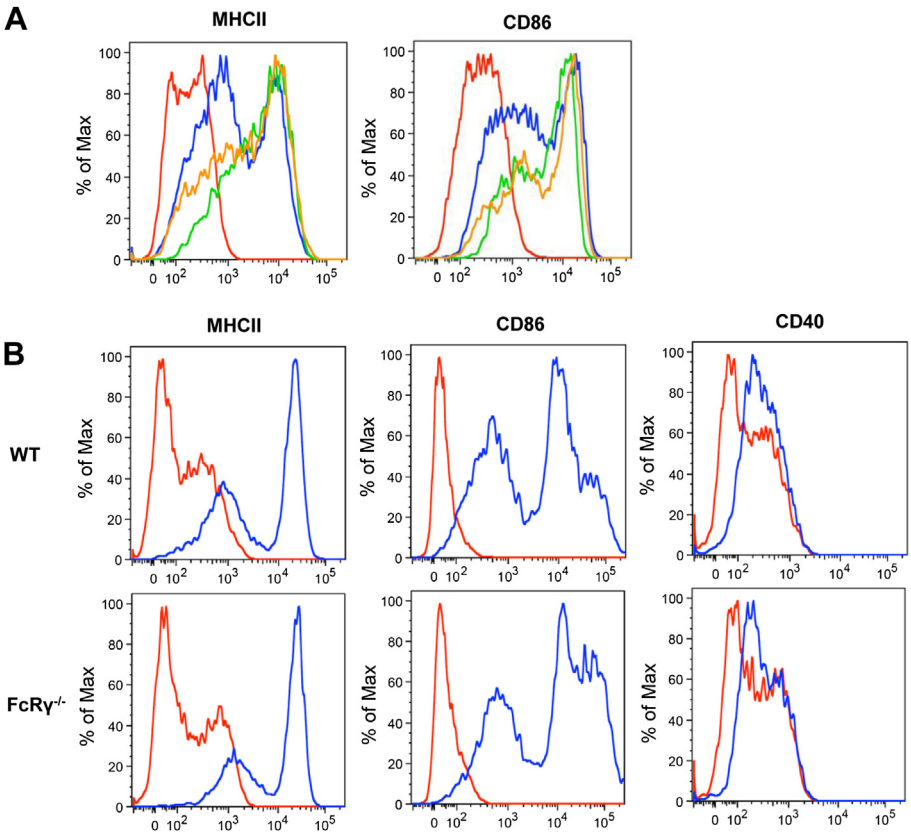

FIG E2. Analysis of activation markers on BMDCs. **A,** WT BMDCs were untreated *(red)* or treated with OVA *(blue)*, OVA-IC *(green)*, or HDM *(orange)* overnight. Expression of MHC class II and CD86 were assessed on $CD11c^{+}CD11b^{+}$ cells. **B,** WT or $FcR\gamma^{-/-}$ BMDCs were treated overnight with HDM, and expression levels of MHC class II, CD86, and CD40 were assessed on $CD11c^{+}CD11b^{+}$ cells. Fluorescence minus one values *(red)* are shown compared with those in the HDM-treated groups *(blue)*. Data are representative of 3 independent culture sets.

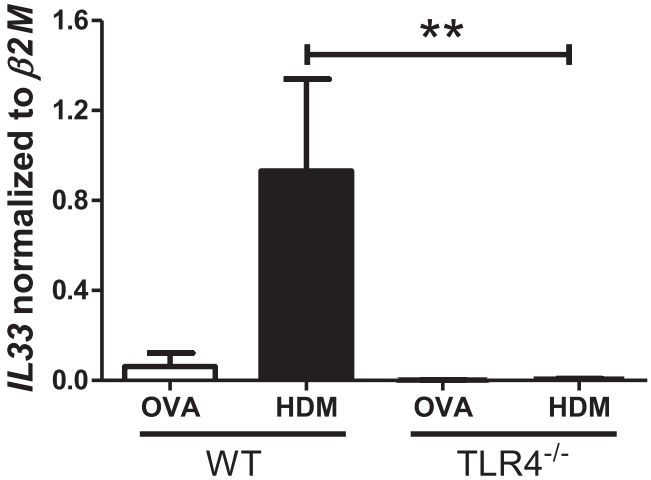

FIG E3. HDM-induced IL-33 upregulation in BMDCs is TLR4 dependent. BMDCs were generated from WT and TLR4$^{-/-}$ mice and treated with OVA or HDM overnight before assessing for *IL33* mRNA expression normalized to β_2-microglobulin mRNA *(β2M)* expression. Data represent means ± SEMs from at least 3 independent culture sets. $^{**}P < .01$.

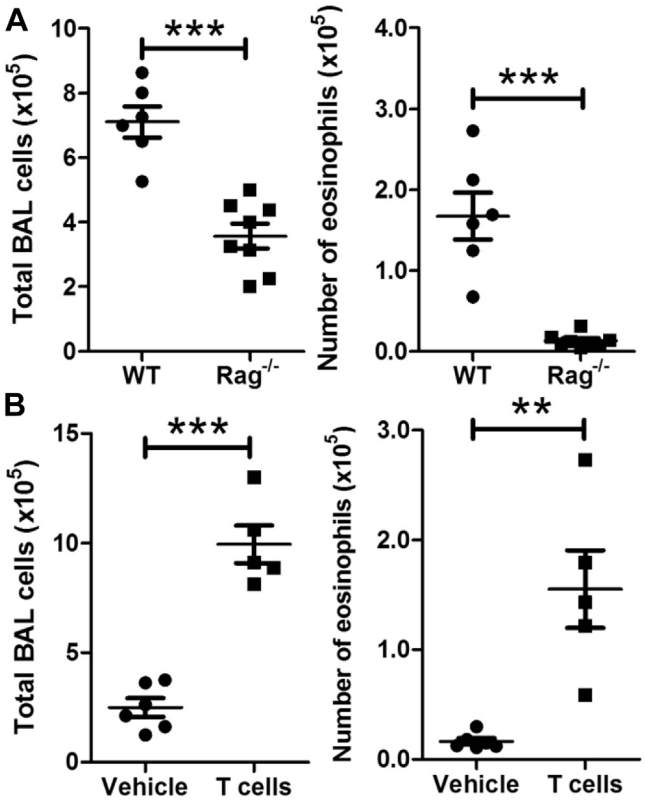

FIG E4. HDM-mediated T_H2 inflammation is dependent on T cells. **A,** WT or $Rag^{-/-}$ mice were sensitized and challenged with HDM. **B,** Naive $Rag^{-/-}$ mice received vehicle control or nylon wool–nonadherent T cells intravenously 2 days before being sensitized and challenged with HDM. Airway inflammation was assessed by determining total cell *(left panel)* and eosinophil *(right panel)* numbers in BAL fluid. Data represent means ± SEMs. $^{**}P < .01$ and $^{***}P < .001$.

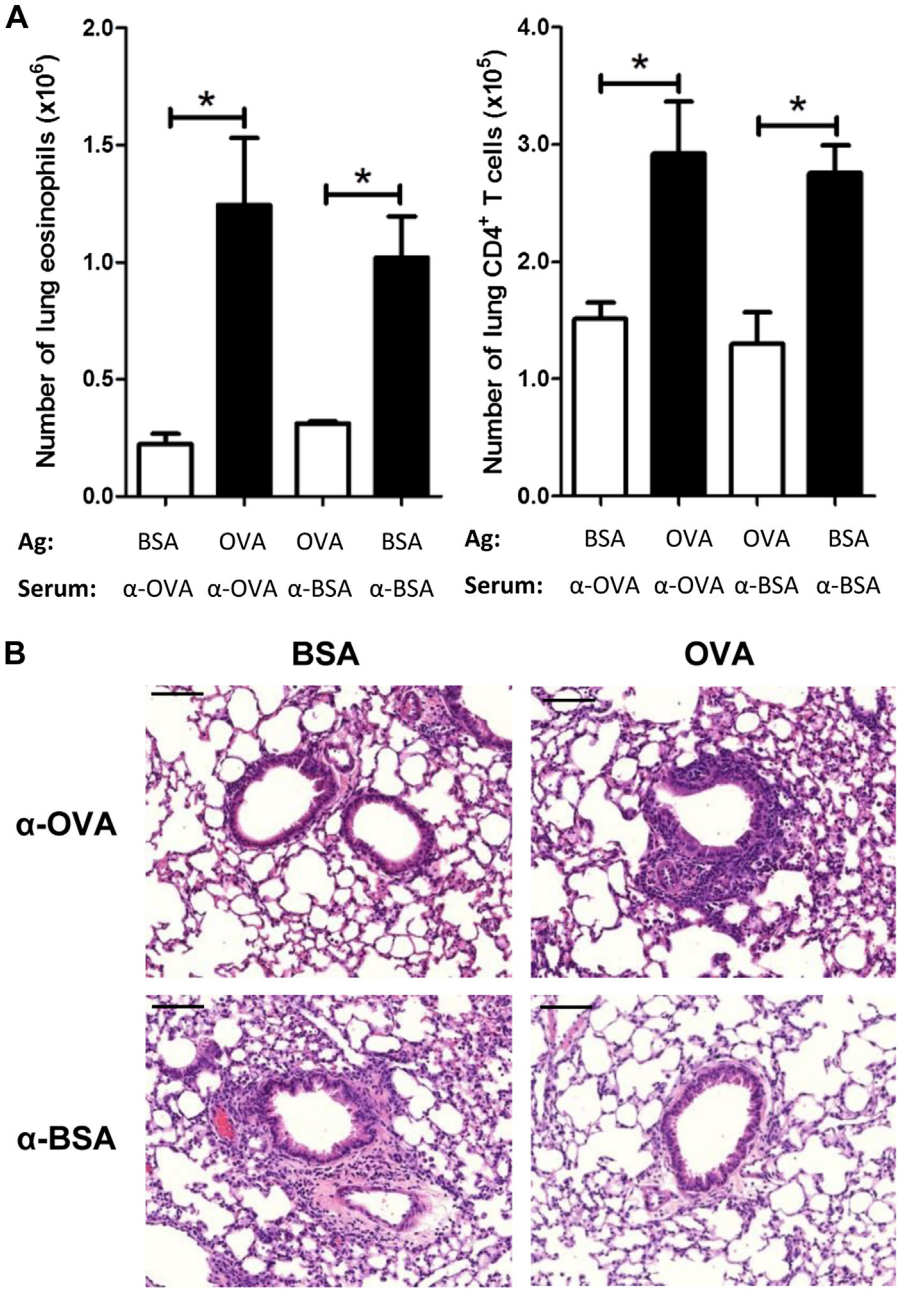

FIG E5. IC-mediated T_H2 inflammation is antigen specific. α-OVA or α-BSA serum was administered intravenously to naive mice. On day 1, mice were challenged intratracheally with OVA or BSA, as indicated in the graphs under antigen *(Ag)*. On days 8, 9, and 10, mice that received α-OVA were challenged with OVA, and mice that received α-BSA were challenged with BSA. On day 11, the mice were killed. **A,** Airway inflammation was assessed by determining eosinophil *(left panel)* and $CD4^+$ T-cell *(right panel)* numbers in lungs by using flow cytometry. **B,** Representative hematoxylin and eosin–stained sections of lung tissue from treated mice. *Black bars* = 100 μm. Data represent means ± SEMs. $*P < .05$.

TABLE E1. Flow cytometry antibodies

Specificity	Clone	Company	Dilution
CCR3	83101	R&D Systems, Minneapolis, Minn	1 μg/mL
CD3	145-2C11	eBioscience, San Diego, Calif	1 μg/mL
CD4	RM4-5	eBioscience	125 ng/mL
CD8	53-6.7	eBioscience	250 ng/mL
CD44	IM7	eBioscience	500 ng/mL
CD62L	MEL-14	eBioscience	250 ng/mL
Ly6C/G (Gr1)	1A8	BD PharMingen, San Jose, Calif	1 μg/mL

TABLE E2. Quantitative PCR primer sequences

	Primer sequence
IL33 forward	5′-GCTGCGTCTGTTGACACATT-3′
IL33 reverse	5′-CACCTGGTCTTGCTCTTGGT-3′
B2M forward	5′-CATACGCCTGCAGAGTTAAGCA-3′
B2M reverse	5′-GATCACATGTCCGATCCCAGTAG-3′

B2M, β_2-Microglobulin gene.