

Group 2 innate lymphoid cells facilitate sensitization to local, but not systemic, T_H2-inducing allergen exposures

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Background: Allergic inflammation involves the sensitization of naive CD4⁺ T cells to allergens, resulting in a T_H2-skewed inflammatory response. Although antigen presentation by dendritic cells to T cells in the lymph node is crucial for T_H2 cell development, the innate signals that initiate adaptive type 2 inflammation and the role of group 2 innate lymphoid cells (ILC2s) are poorly understood.

Objective: We sought to investigate the influence of ILC2s and the route of priming on the development of an adaptive type 2 immune response to lung allergens.

Methods: Wild-type and ILC2-deficient mice were exposed intranasally or systemically to the T_H2-inducing antigens house dust mite or ovalbumin in a model of allergic airway inflammation or the T_H17-inducing bacterial antigen *Saccharopolyspora rectivirgula* in a model of hypersensitivity pneumonitis. The formation of an adaptive immune response was evaluated based on serum antibody titers and production of T cell-derived cytokines (IL-4, IL-5, IL-13 and IL-17A).

Results: We find that lung ILC2s play a critical role in priming the adaptive type 2 immune response to inhaled allergens, including the recruitment of eosinophils, T_H2 cytokine production and serum IgE levels. Surprisingly, systemic priming with ovalbumin, with or without adjuvants, circumvents the requirement for ILC2s in inducing T_H2-driven lung inflammation. ILC2s were also found to be dispensable for the sensitization to T_H1- or T_H17-inducing antigens.

Conclusion: These data highlight a critical role for ILC2s in the development of adaptive type 2 responses to local, but not

systemic, antigen exposure. (*J Allergy Clin Immunol* 2014;133:1142-8.)

Key words: Innate lymphocytes, allergy, asthma, house dust mite, T_H2, IgE

Allergic airway inflammation (AAI)/asthma is a chronic inflammatory disease characterized by a T_H2-biased immune response, antibody class-switching to IgE, and reversible airflow obstruction. Its prevalence has increased by 12% between 2001 and 2009, and it currently affects 8.2% of the population in the United States alone, resulting in an annual economic burden of \$56 billion.¹ The most prevalent therapeutic strategies were developed in the 1970s and include the use of inhaled corticosteroids to dampen inflammation and long-acting β -agonists to induce smooth muscle cell relaxation.² Although effective, these drugs do not target the underlying immune dysregulation that leads to disease. Similarly, in developing new therapeutics, the current emphasis has been on biologics targeting downstream effectors of allergic responses, primarily antibodies directed at IgE and T_H2 cytokines. These new treatment options have shown only marginal clinical success, and the data suggest that targeting the effector response of allergic inflammation might not represent an optimal approach.³ In summary, there is a current unmet need in the treatment of allergic airway disease. It is likely that a greater understanding of the mechanisms involved in the initial priming and sensitization phase could unveil novel therapeutic targets.

House dust mite (HDM) is one of the most common indoor allergens, and up to 85% of asthmatic patients respond to this allergen. Inhaled HDM acts on airway epithelial cells in a Toll-like receptor 4-dependent manner, inducing the release of cytokine danger signals, including IL-25, IL-33, thymic stromal lymphopoietin (TSLP), and GM-CSF.⁴⁻⁶ Understanding how allergens are able to promote naive CD4⁺ T-cell polarization to a T_H2 phenotype has been hampered by the lack of a clearly defined innate cell type that produces T_H2-associated cytokines (eg, IL-4, IL-5, and IL-13) during the sensitization phase. Studies with *Rag2*^{-/-} mice, which lack T and B cells, identified a novel innate cell type that produced IL-5 and IL-13 in response to IL-25.⁷ Several groups have since characterized populations of innate lymphoid cells that act as robust sources of IL-5 and IL-13, but not IL-4, in various tissues, including the spleen, adipose tissue, lymph nodes, lungs, and nasal mucosa.⁸⁻¹⁴ Although initially referred to as nuocytes, natural helper cells, or innate helper type 2 cells, they are now more simply referred to as group 2 innate lymphoid cells (ILC2s) based on their unique expression profile of cell-surface antigens (CD45⁺Lin⁻CD127⁺CD25⁺CD90.2⁺ST2⁺Sca1⁺) and their production of the type 2 cytokines IL-5 and IL-13 in response

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Abbreviations used

AAI: Allergic airway inflammation
BAL: Bronchoalveolar lavage
BMT: Bone marrow transplant
DC: Dendritic cell
HDM: House dust mite
HP: Hypersensitivity pneumonitis
ILC2: Group 2 innate lymphoid cell
OVA: Ovalbumin
PE: Phycoerythrin
ROR α : Retinoic acid receptor–related orphan receptor α
TSLP: Thymic stromal lymphopoietin
WT: Wild-type

to epithelium–derived IL-25, IL-33, and TSLP. ILC2s also require the transcription factors retinoic acid receptor–related orphan receptor α (ROR α),^{15,16} GATA-3,^{17,18} and T-cell factor 1¹⁹ for their specification and maturation. Previously, we showed that ROR α -deficient mice have an impaired innate response to protease allergens in the lung.¹⁵ However, the selective role of ILC2s in the priming of different CD4⁺ T_H subsets and the development of adaptive immune responses in the lung has yet to be examined. Using distinct models of adaptive immunity induction, we demonstrate a specific and highly selective role for ILC2s in promoting T_H2, but not T_H1 or T_H17, lung inflammation in response to inhaled antigens.

METHODS

Mice and bone marrow transplantation

C57BL/6J, B6.SJL-*Ptprca*^a *Pepc*^b/BoyJ (Ly5.1), and B6.C3(Cg)-*Rora*^{sg/sg} mice were purchased from the Jackson Laboratories (Bar Harbor, Me) and were bred and maintained in a specific pathogen-free environment at the Biomedical Research Centre. Pups from *Rora*^{sg/+} breeders were genotyped using DNA obtained from ear clips with primer sequences and protocols from the JAX online database. Bone marrow cells were collected from wild-type (WT) and *Rora*^{sg/sg} littermates, and 1×10^7 bone marrow cells were injected intravenously into lethally irradiated Ly5.1 recipient mice (900 rads in split doses). Peripheral blood chimerism was verified 8 weeks after transplantation by means of saphenous vein bleeds, and all mice used had greater than 90% hematopoietic cells originating from the Ly 5.2 donor mice. All protocols were approved by the Animal Care Committee of the University of British Columbia.

Antibodies and flow cytometry

Staining and antibody dilutions were prepared in PBS containing 2% FCS, 2 mmol/L EDTA, and 0.05% sodium azide. Samples were first blocked in buffer containing 5 μ g/mL anti-CD16/32 (2.4G2) to block nonspecific antibody binding. Alexa Fluor 450–conjugated CD3e (145-2C11), CD11c (N418) CD11b (M1/70), CD19 (1D3), CD45R/B220 (RA3-6B2), NK1.1 (PK136), Gr1 (RB6-8C5), and Ter119 (TER-119); phycoerythrin (PE)–conjugated CD25 (PC61.5); PE-Cy7–conjugated CD3e, B220, and stem cell antigen 1 (D7); allophycocyanin–conjugated IL-5; allophycocyanin–effluor780–conjugated B220; and effluor650–conjugated CD90.2 (53-2.1) were purchased from eBioscience (San Diego, Calif). PE-conjugated Siglec-F (E50-2440), PerCP-Cy5.5–conjugated CD45.2 (104), and V500–conjugated CD45 (30-F11) were purchased from BD Biosciences (San Jose, Calif). Fluorescein isothiocyanate–conjugated anti-neutrophil (7/4) was purchased from Abcam (Cambridge, Mass). Fluorescein isothiocyanate–conjugated ST2 (DJ8) was purchased from MD Bioproducts (St Paul, Minn). Pacific Blue–conjugated CD45 (I3/2) and Alexa Fluor 647–conjugated CD11c (N418) and CD45.1 (Ly5.1) were produced in house. Intracellular cytokine

staining was performed with the Cytotfix/Cytoperm kit (BD Biosciences), and viable cells were identified by using the efluor-450, 506, or 780 fixable viability dye (eBioscience). Samples were acquired on a BD LSRII, and data analysis was performed with FlowJo software (TreeStar, San Carlos, Calif).

Lung explant culture

Mice were sacrificed by means of CO₂ asphyxiation, and lungs were inflated with 1.5 mL of Dulbecco modified Eagle medium containing 10% FCS, 2-mercaptoethanol, penicillin/streptomycin, and 1% low-melting-point agarose kept at 37°C and cooled on ice. Lungs were sliced with a razor into approximately 0.5-mm-thick sections and placed in 2 mL of culture media stimulated with either PBS or 50 μ g/mL HDM antigen for 18 hours. Golgi-Plug (BD Biosciences) was added to the explant cultures 6 hours before collection, and a single-cell suspension was made by passing the lung slices through a 70- μ m cell strainer. Lung ILC2s were identified as Lin[–]CD45⁺CD90.2⁺CD25⁺Sca1⁺ST2⁺ viable cells and gated for IL-5 expression based on fluorescence minus one controls. Secreted IL-5 collected in the cell-free supernatant was quantified by means of ELISA with purified and biotin antibody pairs to IL-5 (TRFK5 and TRFK4) from eBioscience.

Induction and assessment of AAI

HDM (*Dermatophagoides pteronyssinus*) extract was obtained from Greer Laboratories (Lenoir, NC; containing 0.034 μ g of *Der p* 1 and 0.095 EU/ μ g of total protein), and disease was induced, as previously described.²⁰ Mice were treated intranasally with endotoxin-free PBS or HDM on days 0, 1, and 2 with 25 μ g of total protein and on days 14, 15, 16, and 17 with 5 μ g, and mice were sacrificed 24 hours after the final challenge.

For ovalbumin (OVA)/alum–induced AAI, mice were injected intraperitoneally with 50 μ g of chicken OVA (grade III; Sigma, St Louis, Mo) adsorbed to 650 μ g of aluminum hydroxide (Sigma) on days 0 and 7. OVA contained less than 0.125 EU/100 ng of protein, as measured using the PYROGENT Gel Clot assay (Lonza, Walkersville, Md). Mice were treated intranasally on days 21, 22, 23, 25, and 27 with 50 μ g of OVA, and mice were sacrificed on day 28.

Hypersensitivity pneumonitis (HP) was induced, as previously described.²¹ *Saccharopolyspora rectivirgula* was obtained from the ATCC (29034). Bacterial cultures were grown in Trypticase Soy Broth at 55°C before being spun down and washed with endotoxin-free water. Bacterial cells were lysed and lyophilized before being resuspended to 4 mg/mL in PBS for use. Mice were exposed intranasally to 40 μ L (160 μ g) of *S rectivirgula* antigen 3 times a week for 3 weeks. Mice were sacrificed 4 days after the last intranasal exposure.

Mice were sacrificed by means of intraperitoneal injection of 2,2,2-tribromoethanol (Avertin, Sigma). Bronchoalveolar lavage (BAL) fluid was collected by means of 3 aspirations with 1 mL of sterile saline, followed by blood collection through cardiac puncture. BAL cells were enumerated and differentiated by means of flow cytometry with antibodies to CD3e, CD11c, CD45, CD45R/B220, Siglec-F, and 7/4.

Detection of antigen-specific IgG₁, IgG_{2a}, and IgE

ELISA for total serum IgE was performed according to the manufacturer's instructions (BD Biosciences). For detection of antigen-specific IgE, IgG₁ and IgG_{2a}, plates were coated with 25 μ g/mL *S rectivirgula* or HDM antigen in carbonate buffer (0.1 mol/L sodium carbonate, pH 9.5). Serum was diluted in assay diluent (PBS with 3% BSA) and added to the plates for 2 hours at 37°C. Antigen-specific IgGs were detected with horseradish peroxidase–conjugated antibodies to mouse IgG₁ or IgG_{2a}, biotin-conjugated anti-mouse IgE (R35-72), streptavidin–horseradish peroxidase, and TMB substrate (BD Pharmingen, San Jose, Calif).

Immune analysis

Lungs were minced and digested with 200 U/mL collagenase IV (Sigma) for 1 hour at 37°C, a single-cell suspension was obtained by passing the tissue through a 70- μ m cell strainer, red blood cells were lysed, and leukocytes were

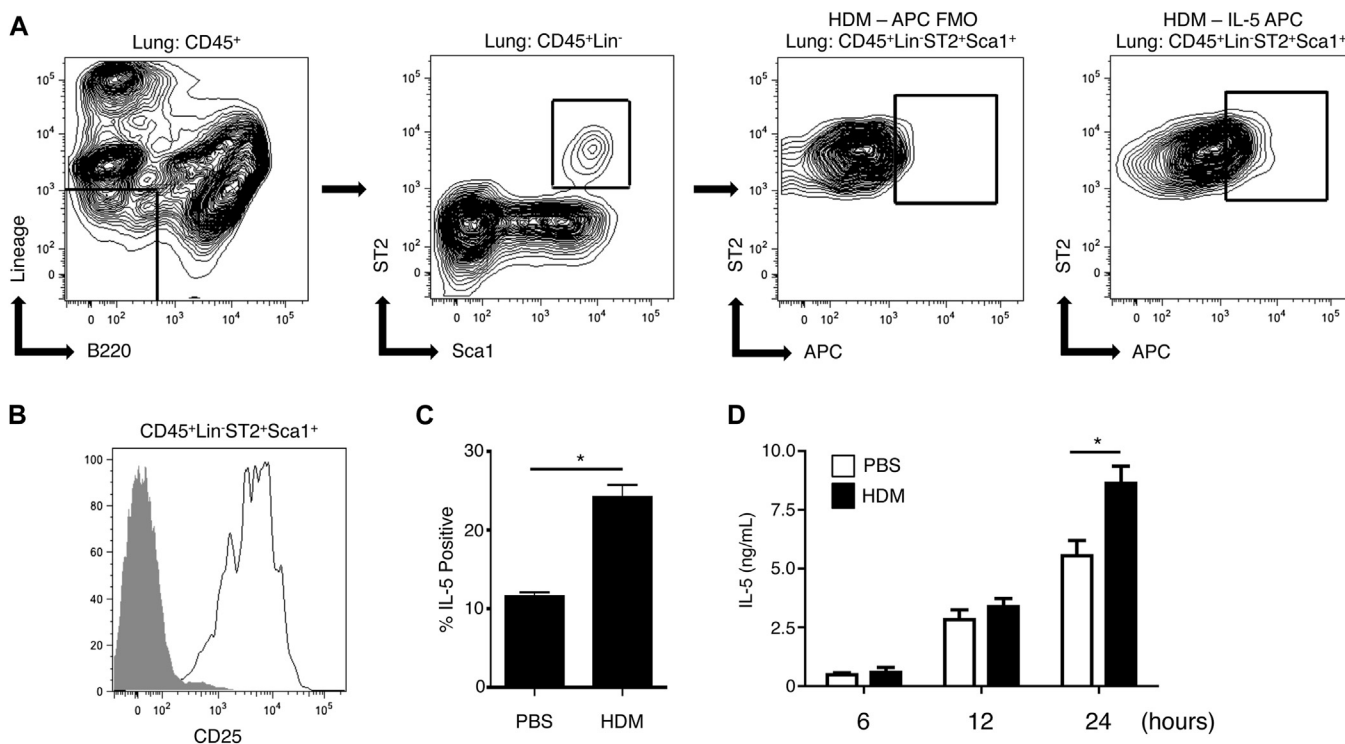


FIG 1. HDM induces lung ILC2 production of IL-5. **A**, Lung explants from WT mice were stimulated with HDM or PBS, followed by intracellular staining of ILC2s for IL-5 production compared with fluorescence minus one (FMO) controls. **B**, Expression of CD25 by lung ILC2s. **C**, Percentage of IL-5⁺ ILC2s from lung explants stimulated with HDM or PBS. **D**, IL-5 released in the supernatant of PBS- or HDM-stimulated lung explants was measured by ELISA at 6, 12, and 24 hours. APC, Allophycocyanin.

enriched by means of Percoll separation. Leukocytes were counted and stimulated at 4 to 8×10^6 cells/mL in culture media (Dulbecco modified Eagle medium with 10% FCS, penicillin/streptomycin, $50 \mu\text{mol/L}$ 2-mercaptoethanol, and 25 mmol/L HEPES) with the indicated concentration of HDM or *S rectivirgula* antigens for 72 hours. Cell-free supernatants were collected, and cytokine levels were measured by means of ELISA with purified and biotin antibody pairs to IL-13 (eBio13A, eBio1316H) or IL-17A (eBio17CK15A5, eBio17B7) from eBioscience.

RNA isolation and quantitative RT-PCR

Lungs were homogenized in Trizol (Life Technologies, Carlsbad, Calif) by using a Qiagen TissueLyser II (Valencia, Calif). Total RNA was extracted and reversed transcribed with a high-capacity cDNA kit (Life Technologies), and quantitative RT-PCR was performed with Sybr green (KAPA Biosystems, Woburn, Mass). The primers used for quantitative PCR are listed in the Methods section in this article's Online Repository at www.jacionline.org. Reactions were carried out in an ABI 7900 real-time PCR machine (Life Technologies), and values are expressed relative to *Actb*.

Histology

Lung tissue was fixed in 10% buffered formalin overnight and embedded in paraffin. Sections ($5 \mu\text{m}$ thick) were stained with hematoxylin and eosin. A histologic disease score from 0 to 4 was attributed based on severity for peribronchial, perivascular, and parenchymal immune cell infiltration, resulting in a maximum score of 12.

Statistics

Results are presented as means $\pm$ SEMs. The Student *t* test was used to determine statistical significance.

RESULTS

HDM induces lung ILC2 activation and IL-5 secretion

To test whether HDM could elicit ILC2-dependent production of T_H2 cytokines, we used lung explant cultures, which maintain the lung microenvironment and epithelium-leukocyte interactions. Lung explants were stimulated with HDM antigen *in vitro*, and ILC2 production of intracellular IL-5 was measured by flow cytometry. Lung ILC2s were identified based on their unique expression of cell-surface markers: positively reacting with the panhematopoietic marker CD45, stem cell antigen 1, and IL-33 receptor (ST2; Fig 1, A) and lack of expression of common leukocyte proteins (CD3, CD11b, B220, Gr1, NK1.1, and Ter119). Gated ILC2s were also found to express IL-2 receptor α (CD25; Fig 1, B). HDM treatment of WT explants led to a rapid induction of IL-5 synthesis compared with that seen in saline controls (Fig 1, C and D), suggesting that these cells could serve as downstream effectors in response to a common human allergen.

ILC2s facilitate HDM-induced AAI

To examine the role of ILC2s in the development of allergic asthma, we used a HDM mouse model of AAI (Fig 2, A). In this model repeated intranasal exposure of HDM antigen induces several hallmark features of clinical asthma, including airway and lung parenchymal eosinophilia; a T_H2 -biased immune response with high levels of IL-4, IL-5, and IL-13 secretion; and antibody class-switching to IgE.⁶

Although ILC2s are known to produce IL-5 in response to protease antigens in the lung,¹⁰ their role in regulating the

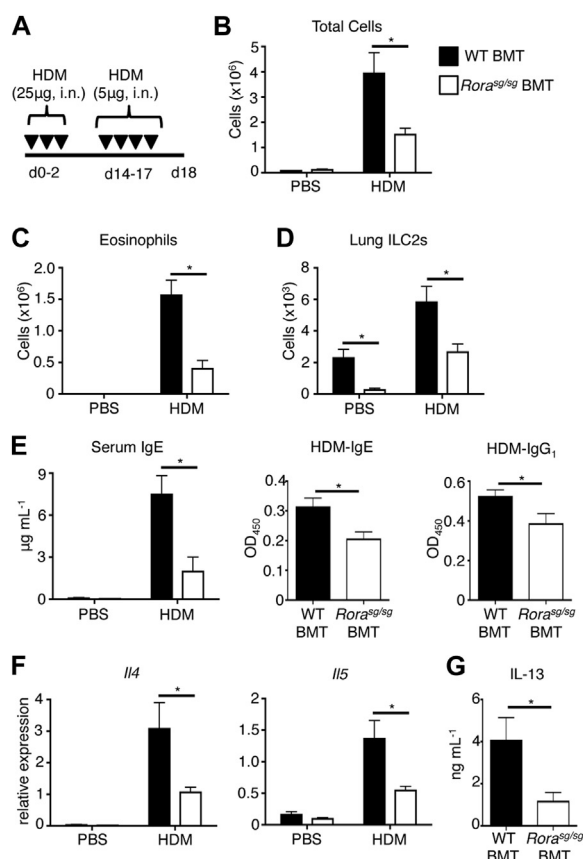


FIG 2. HDM-induced AAI in WT and ILC2-deficient mice. **A**, Schematic of HDM disease model. **B** and **C**, Total cells (Fig 2, **B**) and eosinophils (Fig 2, **C**) collected in BAL samples of WT or ILC2-deficient mice. **D**, Total CD45.2⁺ lung ILC2s from PBS- and HDM-exposed WT or ILC2-deficient mice. **E**, Serum total IgE and HDM-specific IgE and IgG₁ levels were quantified by ELISA. **F**, Transcript levels of *Il4* and *Il5* from lung RNA relative to *Actb*. **G**, Lung leukocytes were isolated and restimulated *ex vivo* with 50 µg/mL HDM for 72 hours. IL-13 levels were quantified by ELISA. Data are representative of 3 independent experiments (Fig 2, **B** and **C** and **E-G**; *n* = 4-8) or combined from 2 independent experiments (Fig 2, **D**; *n* = 4-5). Error bars are means ± SEMs. **P* < .05. *i.n.*, Intranasal.

development of an adaptive immune response to physiologically relevant antigens is still unknown. To address this issue, we generated mice selectively lacking ILC2s by transplanting WT mice (CD45.1) with bone marrow from *Rora*^{sg/sg} mice (CD45.2) to produce hematopoietic chimeras. These chimeric mice have a very selective defect in ILC2 production (although rare, residual, radioresistant CD45.1⁺ ILC2s can be detected in transplanted chimeras; see Fig E1 in this article's Online Repository at www.jacionline.org). Indeed, RORα expression is tightly regulated in the hematopoietic compartment, with high expression found only on ILC2s and T_H17 cells but not Th2 cells (see Fig E2, **A**, in this article's Online Repository at www.jacionline.org). Additionally, we found that isolated CD4⁺ T cells from WT or *Rora*^{sg/sg} bone marrow transplanted (BMT) mice displayed no difference in their ability to polarize to T_H2 cells, as marked by equal production of the T_H2 cytokines IL-5 and IL-13, as well as equal expression of the transcription factor *Gata3* (see Fig E2, **B** and **C**). When exposed to a HDM model of AAI, *Rora*^{sg/sg} chimeras exhibited a significant impairment in T_H2 response induction, with a marked reduction in leukocyte infiltration,

particularly eosinophils, into the airways 18 days after initial HDM exposure, whereas challenge with saline (PBS) resulted in very low levels of airway infiltrate comprised almost exclusively of macrophages (Fig 2, **B** and **C**). Additionally, HDM exposure resulted in a significant expansion of ILC2s in the lungs of WT BMT mice and a low level expansion in *Rora*^{sg/sg} BMT mice (Fig 2, **D**). ILC2-deficient mice also exhibited significantly reduced levels of both total serum IgE and HDM antigen-specific IgE and IgG₁ (Fig 2, **E**) and significantly reduced expression of lung transcripts for the T_H2-associated cytokines *Il4* and *Il5* (Fig 2, **F**). Isolated lung leukocytes were restimulated *ex vivo* with HDM antigen to evaluate the activity of T_H2 cells in the inflamed lung tissue. ILC2-deficient mice displayed a strikingly reduced recall response, as indicated by decreased antigen-induced production of IL-13 (Fig 2, **G**).

Histologically, we found that loss of ILC2s also resulted in a significant reduction in lung histopathology, with reduced leukocyte infiltration into the lung tissue, particularly in the peribronchiolar and perivascular space (Fig 3, **A** and **B**). Consistent with the significant reduction in BAL eosinophil numbers in ILC2-deficient mice, the frequency of tissue-infiltrating parenchymal eosinophils was also reduced, as quantified by the evaluation of the eosinophil-specific mRNA *Prg2* (encoding major basic protein 1) in the lung (Fig 3, **C**). Thus our results suggest that RORα-dependent ILC2s are critical for the development of allergic lung inflammation.

ILC2s are dispensable for the development of T_H2 responses to systemically delivered antigens

Although these data suggest that ILC2s are essential for the induction of T_H2 cell responses to physiologically relevant antigens when delivered locally through the natural intranasal route, they do not address whether there is a similar requirement for these cells in priming the adaptive immune system to systemically administered antigens in the presence of adjuvants. To test this, we used the well-described OVA/alum model of AAI (Fig 4, **A**). In this model mice are primed intraperitoneally (systemically) with OVA and alum, followed by intranasal challenge (local) with OVA. In contrast to our results with intranasal HDM antigen, we found that ILC2s were completely dispensable for the induction of AAI in this model. ILC2-deficient mice had similar numbers of airway-infiltrating leukocytes and displayed normal recruitment of eosinophils into the airways and lung tissue (Fig 4, **B**, **C**, and **E**). Likewise, we detected no significant differences in serum IgE levels or *Il5* expression (Fig 4, **D** and **E**), suggesting normal development of an adaptive T_H2 response.

Systemic delivery of antigens without adjuvant elicits ILC2-independent T_H2 responses

Alum acts as a strong T_H2-inducing adjuvant that causes release of various danger signals, such as uric acid, that could bypass ILC2s and act on resident macrophages and dendritic cells (DCs). To examine whether ILC2s are required for systemic antigen priming of allergic responses in the absence of external adjuvant, we primed *Rora*^{sg/sg} chimeras intraperitoneally with OVA in the absence of alum, followed by intranasal challenges with OVA (Fig 5, **A**). Surprisingly, ILC2-deficient mice had equivalent levels of leukocyte and eosinophilic infiltrates in the airways and tissues and exhibited no difference in IgE levels or *Il5* expression

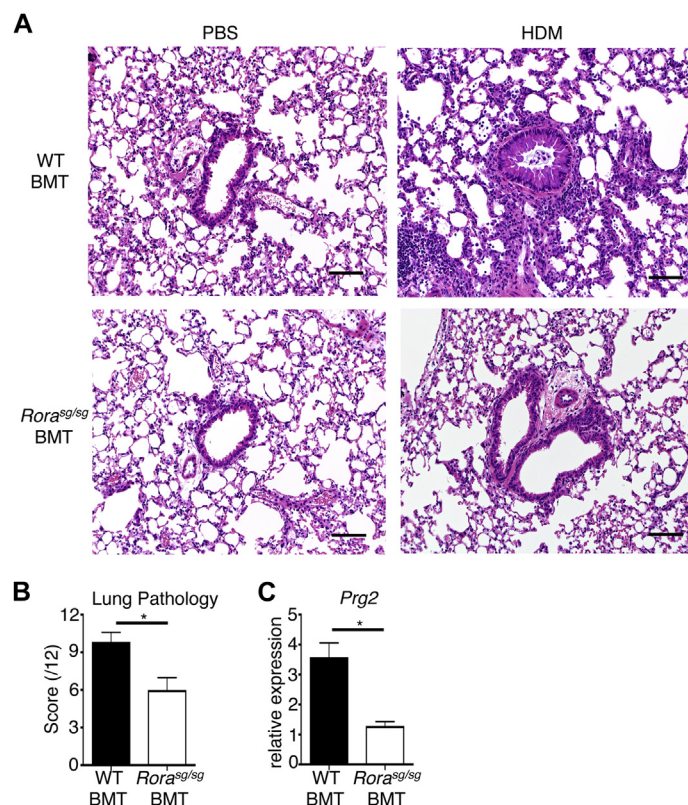


FIG 3. Airway inflammation in HDM-exposed WT and ILC2-deficient mice. **A**, Hematoxylin and eosin staining of PBS- or HDM-treated WT and ILC2-deficient mice. Scale bar = 100 μ m. **B**, Lung sections were scored for peribronchial, perivascular, and parenchymal infiltration (a score of 0-4 was given for each parameter, for a maximum score of 12). **C**, Infiltrating lung eosinophils were quantified by *Prg2* transcript levels in total lung RNA. Error bars are means $\pm$ SEMs. * P < .05.

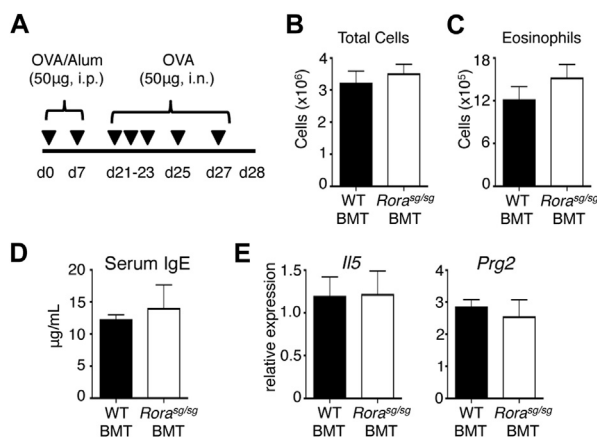


FIG 4. OVA/alum-induced AAI in WT and ILC2-deficient mice. **A**, Schematic of disease model for OVA/alum-induced AAI. **B** and **C**, Total cells (Fig 4, B) and eosinophils (Fig 4, C) collected in BAL samples of WT or ILC2-deficient mice. **D**, Serum total IgE levels were quantified by ELISA. **E**, Transcript levels of *Il5* and *Prg2* from lung RNA relative to *Actb*. Data are representative of 2 independent experiments (n = 1-7). Error bars are means $\pm$ SEMs. *i.n.*, Intranasal; *i.p.*, intraperitoneal.

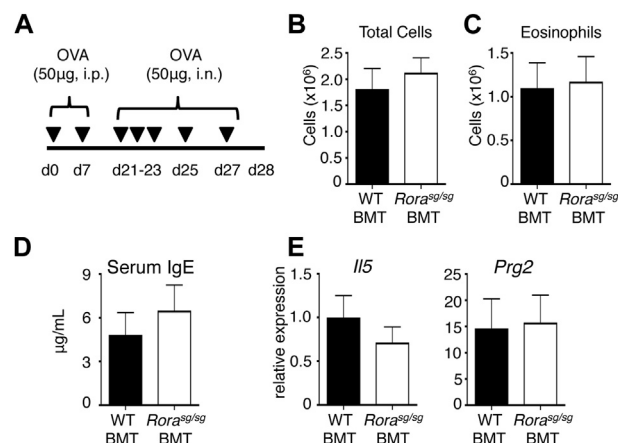


FIG 5. OVA-induced AAI in WT and ILC2-deficient mice. **A**, Schematic of disease model for OVA-induced AAI. **B** and **C**, Total cells (Fig 5, B) and eosinophils (Fig 5, C) collected in BAL samples of WT or ILC2-deficient mice. **D**, Serum total IgE levels were quantified by ELISA. **E**, Transcript levels of *Il5* and *Prg2* from lung RNA relative to *Actb*. Error bars are means $\pm$ SEMs (n = 4 per group). *i.n.*, Intranasal; *i.p.*, intraperitoneal.

(Fig 5, B-E). This suggests that systemic antigen delivery, irrespective of the addition of an exogenous adjuvant, is able to induce an ILC2-independent allergic airway inflammatory response.

ILC2s are dispensable for local priming of T_H1/T_H17 responses

Although we find ILC2s are essential for T_H2 sensitization to locally delivered antigens in the lung, it remains unclear whether

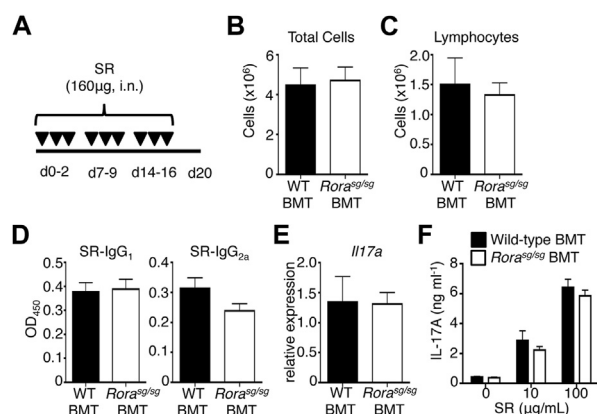


FIG 6. *S rectivirgula* (SR)-induced HP in WT and ILC2-deficient mice. **A**, Schematic of disease model for *S rectivirgula*-induced HP. **B** and **C**, Total cells (Fig 6, B) and lymphocytes (Fig 6, C) collected in BAL samples of WT or ILC2-deficient mice. **D**, Serum *S rectivirgula*-specific IgG₁ and IgG_{2a} levels were quantified by ELISA. **E**, Transcript levels of *I17a* from lung RNA relative to *Actb*. **F**, Lung leukocytes were isolated and restimulated with the indicated concentration of *S rectivirgula* for 72 hours. Secreted IL-17A was measured by ELISA. Error bars are means $\pm$ SEMs ($n = 4$ per group).

they play any positive or negative role in the development of T_H1/T_H17 responses. HP is a chronic lung inflammatory disease caused by repeated airborne exposure to predominantly organic antigens and is characterized by a T_H1/T_H17-biased immune response.²¹⁻²³ In human subjects exposure to *S rectivirgula*, a bacteria present in moldy hay, leads to farmer's lung (a subtype of HP), and this is recapitulated in mice after repeated intranasal exposure to this antigen (Fig 6, A).

WT and ILC2-deficient mice were treated as outlined in Fig 6, A, and clinical features of HP were assessed on day 20 after induction. We found that loss of ILC2s had no effect on the ability to mount an adaptive T_H1/T_H17 response to bacterial *S rectivirgula* antigens. ILC2-deficient mice had similar levels of alveolar infiltrate with no difference in alveolar lymphocyte accumulation (Fig 6, B and C). Unlike allergic asthma, which is characterized by high serum IgE and IgG₁ levels typical of a T_H2 immune response, HP induces production of antigen-specific IgG_{2a}. ILC2-deficient mice had normal *S rectivirgula*-specific IgG_{2a} and IgG₁ levels (Fig 6, D). There was also no difference in T_H17 responses in the lung, as measured based on *I17a* transcript levels and IL-17A production from restimulated lung cultures (Fig 6, E and F). We conclude that ILC2s are largely dispensable for T_H1/T_H17 inflammatory responses.

DISCUSSION

The initiation of T_H2 responses and the innate cell types responsible for the production of cytokines before the polarization of naive CD4⁺ T cells to a T_H2 phenotype is not well understood and is an important area of investigation. Lung ILC2s represent a recently characterized leukocyte population that reside in the naive lung and rapidly produce the T_H2-associated cytokines IL-5 and IL-13 in response to inhaled proteases.¹⁵ Here we have formally examined their role in initiation of an adaptive immune response in the lung to T_H1/T_H17 and T_H2 antigens (summarized in Fig E3 in this article's Online Repository at www.jacionline.org). For these studies, we used *Rora*^{sg/sg} bone marrow chimeras as mice that selectively lack ILC2s. *Rora*^{sg/sg}

mice carry a mutation in the ligand-binding domain of ROR α that leads to severe neurologic defects but, within the hematopoietic compartment, also results in an extremely selective defect in ILC2 production.^{15,16} Using these ILC2-deficient mice, we have shown that ILC2s represent a critical relay between HDM-induced epithelial barrier damage and the subsequent generation of an adaptive T_H2 response. Interestingly, we found that although allergen exposure elicits a robust expansion of ILC2s in the lungs of WT mice, it also induces a modest but significant increase in ILC2 numbers in *Rora*^{sg/sg} BMT mice. This might result from functional compensation by other transcription factors (GATA-3, T-cell factor 1, and Gfi1^{17-19,24}), which are known to be important for ILC2 development in situations in which large amounts of factors associated with ILC2 proliferation and survival, such as IL-25, IL-33, and TSLP are produced. Although we have found ILC2s to be important for the formation of an adaptive T_H2 response, they do not produce the prototypical T_H2-inducing cytokine IL-4 and instead produce large amounts of IL-5 and IL-13, suggesting a role for one or both of these cytokines in the ILC2-mediated T_H2 response. ILC2s have been shown to be potent inducers of eosinophil migration, either through their production of IL-5 or potentially through production of eosinophil chemotactic agents, such as eotaxin-1 or eotaxin-2. Eosinophils have been found to induce DC maturation through release of their granule protein eosinophil-derived neurotoxin, which can act as an agonist of DC Toll-like receptor 2 receptors.²⁵ In addition, ILC2 production of IL-13 could also stimulate lung-resident DCs and promote either their migration to the draining lymph node or their ability to promote T_H2 differentiation.²⁶

Human ILC2-equivalent cells have also been identified, and although they are found in healthy human lungs, they are enriched in the nasal polyps of patients with rhinosinusitis, suggesting their possible relevance in disease pathology.¹² Indeed, circulating and peripheral CD34⁺ progenitor cells have also been characterized that share several key features of ILC2s (eg, TSLP-mediated release of the type 2 cytokines IL-5 and IL-13) and are more abundant in sputum samples from allergic patients, highlighting their clinical importance.²⁷ Finally, genome-wide association studies have also found polymorphisms in the *RORA* locus to be associated with greater risk of asthma (along with *IL33* and *IL13*), suggesting a potential link between ILC2s and disease.²⁸ In summary, our data with *Rora*^{sg/sg} bone marrow chimeras now provide a formal link between these previously detected innate cytokine-producing cells and the generation of adaptive allergic immune responses to local antigen stimulation.

To test whether these cells are also required in models of systemic exposure to allergens, we primed mice intraperitoneally with OVA as a model antigen. Surprisingly, we found that systemic administration of antigen, along with an alum adjuvant, obviated the requirement for ILC2s in the development of a robust T_H2 response. It is possible that the strong immune-stimulatory effects of alum, such as release of uric acid and stimulation of inflammatory monocytes,²⁹ overcame any requirement for ILC2s in the sensitization phase. To test this, we repeated the same model of systemic antigen delivery in the absence of adjuvant and again found that this too led to an ILC2-independent T_H2 response. Thus our data would argue that although ILC2s play a critical role in the initiation of local responses to antigen exposures, they are dispensable in responses to systemically delivered antigens, which likely short-circuits the need for DC-dependent antigen delivery to the draining lymph nodes. This finding also

highlights the important interplay between lung-resident ILC2s and the epithelium in shaping the innate and adaptive immune response to inhaled allergens. The lung epithelium is a potent producer of ILC2-activating cytokines, such as IL-25, IL-33, and TSLP, and HDM-induced experimental asthma is dependent on epithelium-derived signals.⁴

Although we found ILC2s to facilitate sensitization to the T_H2-inducing HDM antigen, they were completely dispensable for sensitization to T_H1/T_H17-inducing antigens in a murine model of HP. This was surprising because ROR α and its related transcription factor, ROR γ , are important for proper T_H17 differentiation,³⁰ and mice deficient in IL-17 or its receptor are protected from the development of HP.^{22,23} Although we found IL-17A production to be reduced in *Rora*^{sg/sg} BMT mice in both the HDM and OVA models of AAI (data not shown), we found no difference in IL-17A production in the T_H17 model of HP. Thus it is likely that *S rectivirgula* exposure is able to induce a strong T_H17-polarizing stimulus and that the ROR γ expression in ROR α -deficient T cells is sufficient for T_H17 development, whereas HDM and OVA produce a weak T_H17-inducing signal that requires synergistic activity of both ROR α and ROR γ for proper T_H17 differentiation. However, the ability of ILC2-deficient mice to mount a normal response in a T_H1/T_H17 model of lung inflammation does not preclude the potential role for other lung-resident innate lymphoid cell populations in modifying responses to bacterial antigens. However, our data rule out a significant role for ILC2s in this process.

In summary, our data highlight a pivotal role for ILC2s in the initiation of T_H2 responses to mucosal routes of antigen exposure. They also highlight these cells as potential targets for therapeutics aimed at blocking the initiation of allergic responses before the development of adaptive immunity.

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Key messages

- ILC2s facilitate sensitization to local, but not systemic, T_H2-inducing allergen exposures.
- ILC2s are dispensable for the development of an adaptive immune response to local bacterial antigens that induce T_H1/T_H17 responses.

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METHODS

In vitro T-cell polarization

CD4⁺ T cells were isolated from the spleens and lymph nodes of naive WT or *Rora*^{sg/sg} BMT mice by means of negative selection using RoboSep (STEMCELL Technologies, Vancouver, British Columbia, Canada). CD4⁺ cells (2.5×10^5) were cultured for 6 days in culture media (Iscove modified Dulbecco medium supplemented with 10% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin, and 150 μmol/L monothioglycerol) with 1 μg/mL each of plate-bound anti-CD3 (145-2C11) and anti-CD28 (37.51) in the

presence of neutral (10 ng/mL IL-2), T_H2 (10 ng/mL IL-2, 10 ng/mL IL-4, and 10 μg/mL anti-IFN-γ [XMG1.2]) or T_H17 (10 ng/mL IL-1β, 20 ng/mL IL-6, 10 ng/mL IL-23, 10 ng/mL TNF-α, 1 ng/mL TGF-β, 10 μg/mL anti-IL-4 [11B11], and 10 μg/mL anti-IFN-γ [XMG1.2]) conditions. Cell-free supernatants were collected at day 6 for cytokine measurements, and total RNA was extracted with Trizol for quantitative RT-PCR. Immature ILC2s were sort purified from C57Bl/6 bone marrow (CD45⁺Lin⁻CD127⁺CD25⁺ST2⁺) and cultured for 7 days in media supplemented with 10 ng/mL each of IL-7 and IL-33.

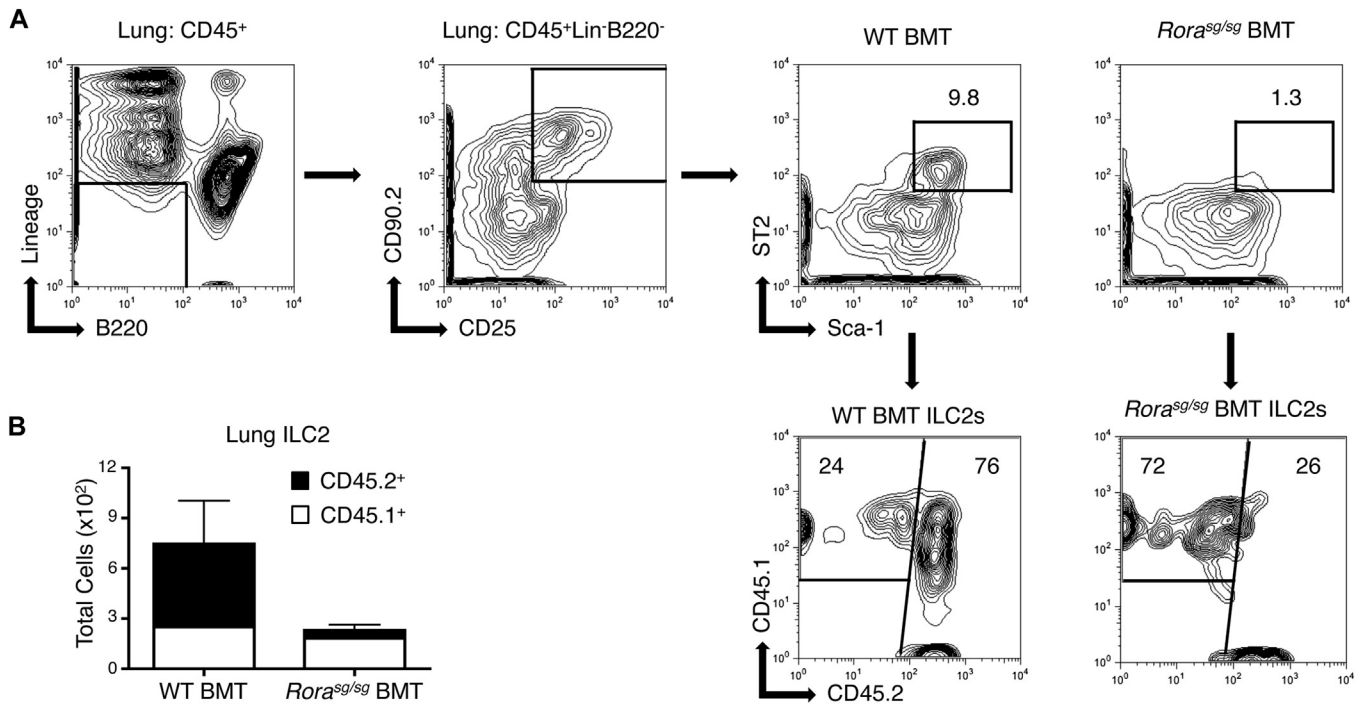


FIG E1. Reduced lung ILC2s in *Rora*^{sg/sg} BMT mice. **A**, Flow cytometric analysis of lung ILC2s from WT and *Rora*^{sg/sg} BMT mice. **B**, Quantification of lung CD45.2⁺ ILC2s from donor mice (WT or *Rora*^{sg/sg}) or residual radioresistant ILC2s (CD45.1⁺). Data are representative of 2 independent experiments. Error bars are means $\pm$ SEMs.

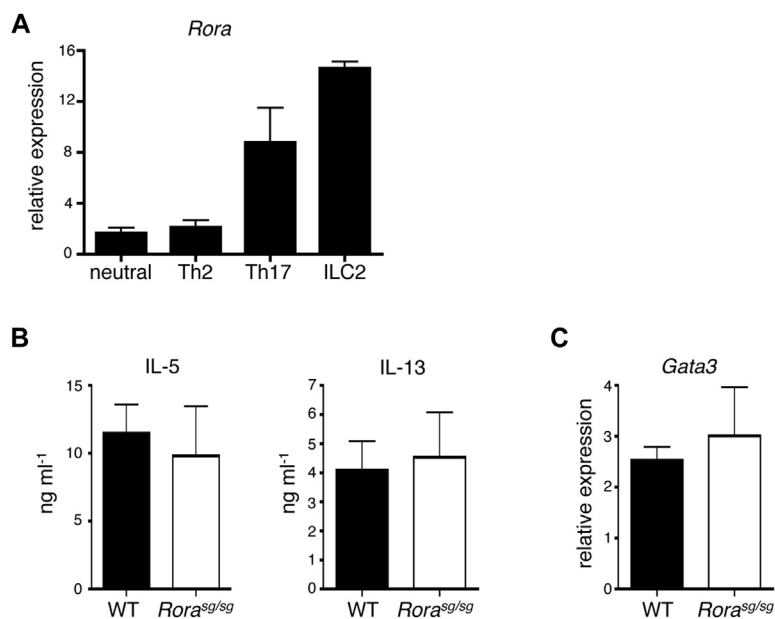


FIG E2. ROR α expression profile and function in T_H2 polarization. CD4⁺ T cells isolated from WT or *Rora*^{sg/sg} BMT mice were stimulated under neutral, T_H2- or T_H17-polarizing conditions for 6 days. **A**, Expression of *Rora* transcript from T-cell polarization cultures or purified ILC2s was measured by using quantitative PCR relative to *Actb*. **B**, Secreted IL-5 and IL-13 from T_H2 polarization conditions was quantified by means of ELISA. **C**, Expression of *Gata3* transcript from T_H2 polarization conditions was measured by using quantitative PCR relative to *Actb*. Data are representative of 2 independent experiments. Error bars are means $\pm$ SEMs.

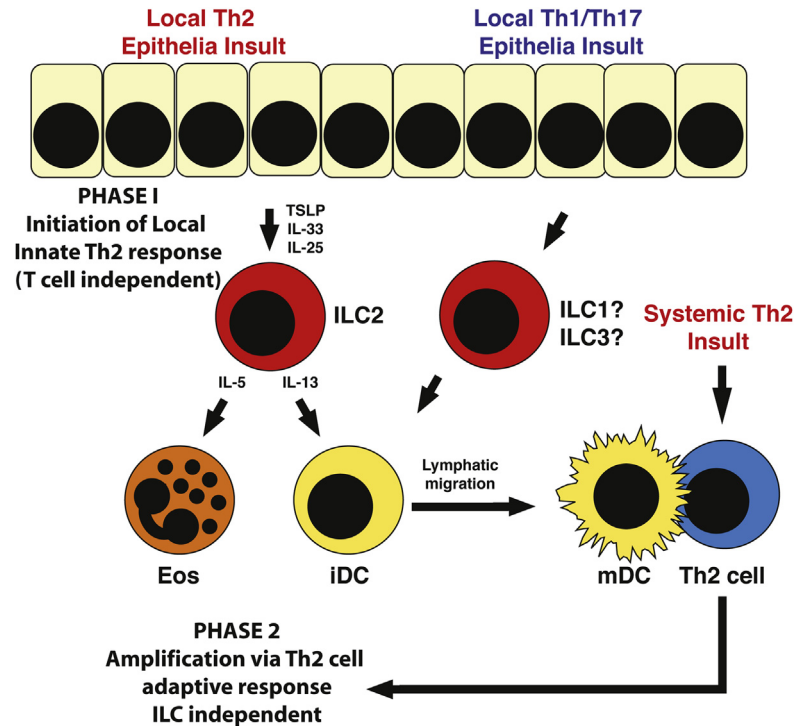


FIG E3. Model for ILC2 function in lung inflammation. Exposure of the lung epithelium to HDM antigens results in loss of barrier integrity and release of innate danger signals (TSLP, IL-25, and IL-33), which in turn activate lung-resident ILC2s to release IL-5 and IL-13. This results in eosinophil recruitment and activation of immature lung resident dendritic cells (iDC). Activated lung DCs pick up antigen for transport to the draining lymph nodes and activation of a Th2 adaptive response. Systemic immunization with allergen obviates the need to ILC2s to mount an adaptive Th2 response. Local exposure to bacterial Th1/Th17-inducing antigens (*S. rectivirgula*) activate a pathway independent of group 2 ILCs but might rely on other lung-resident innate lymphocyte populations, such as the IL-17A- and IL-22-producing ILC3s. Eos, Eosinophil; mDC, myeloid dendritic cell.

TABLE E1. Primer sequences

Primer	Sequence (5'-3')
<i>Actb</i> forward	GGCTGTATTCCCCTCCATCG
<i>Actb</i> reverse	CCAGTTGGTAACAATGCCATGT
<i>Il4</i> forward	TCGGCATTGGAACGAGGTC
<i>Il4</i> reverse	CAAGCATGGAGTTTCCCATG
<i>Il5</i> forward	GATGAGGCTTCCTGTCCCTACTC
<i>Il5</i> reverse	TCGCCACACTTCTCTTTTGG
<i>Il13</i> reverse	CCTGGCTCTTGCTTGCCTT
<i>Il13</i> forward	GGTCTTGTGTGATGTTGCTCA
<i>Il17a</i> forward	AGCAGCGATCATCCCTCAAAG
<i>Il17a</i> reverse	TCACAGAGGGATATCTATCAGGGTC
<i>Prg2</i> forward	ATGGGTGACTCTGGATGCAAG
<i>Prg2</i> reverse	GCGGACTGGATTCCGAAGT
<i>Rora</i> forward	AGACAGCTTGTACGCCGAG
<i>Rora</i> reverse	GGTCATCATGCAGTTCCGTCA
<i>Gata3</i> forward	CTCGGCCATTTCGTACATGGAA
<i>Gata3</i> reverse	GGATACCTCTGCACCGTAGC