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RIP2 Promotes House Dust Mite-induced Allergic Airway Inflammation

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Abstract

House dust mites are one of the most significant environmental allergens in the establishment of the so-called “Atopic March”. It is known that the immune response to HDM is Th2 dominant, but the innate mechanisms leading to HDM-induced type 2 responses are still not completely understood. A number of innate immune receptors have been implicated in the response to HDM including Toll-Like Receptors, C-type Lectin Receptors, and Protease Activated Receptors. NOD2 is a member of the NOD-Like Receptor family which has been reported to be involved in the establishment of type 2 immunity and in blocking respiratory tolerance. NOD2 mediates its effects through its downstream effector kinase, RIP2. It has not been shown if RIP2 is involved in the innate response to HDM and in the resulting generation of type 2 immunity. Furthermore, the role of RIP2 in modulating allergic airway inflammation has been controversial. In this study, we show that RIP2 is activated in airway epithelial cells in response to HDM and is important for the production of CCL2. Using a murine HDM asthma model, we demonstrate that lung pathology, local airway inflammation, inflammatory cytokines, HDM-specific IgG₁ antibody production, and HDM-specific Th2 responses are all reduced in RIP2 KO mice compared to WT animals. These data illustrate that RIP2 can be activated by a relevant allergic stimulus and that such activation can contribute to allergic airway inflammation. These findings also suggest that RIP2 inhibitors might have some efficacy in downregulating the inflammatory response in type 2-dominated diseases.

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Authorship

M.H.M. performed a number of the experiments, collected some of the data, and contributed to the writing of the manuscript.

M.G.S. performed a number of the experiments, collected some of the data, and contributed to the writing of the manuscript.

K.P.A. performed and optimized a number of the pilot experiments and contributed to the writing of the manuscript.

L.P.S. performed a number of the experiments and collected some of the data.

J.S. performed all statistical analysis on the project in consultation with J.T.T-A.

J.T.T-A was responsible for the conception and design of the study. She also performed some of the experiments and collected some of the data, contributed to the writing of the manuscript, approved the final version of the manuscript to be submitted, and was involved in the interpretation of the data.

Conflict of Interest Disclosure

The authors declare no conflicts of interest.

Summary Sentence:

RIP2 is involved in the HDM-induced production of CCL2 in airway epithelial cells and is important for the progression of HDM-induced allergic airway inflammation.

Keywords

NOD2; allergic asthma; airway epithelium; dust mite; Th2

Introduction

The progressive development of allergic disorders beginning from atopic dermatitis in infancy, followed by allergic rhinitis, and concluding with the development of allergic asthma (the so-called “Atopic March”) has long been recognized. Sensitization to indoor allergens is one of the leading risk factors for the development and severity of allergic asthma (1–4). Of these indoor allergens, house dust mite (HDM) is one of the most prevalent and most consistently associated with the development of allergic diseases, with greater than 50% of asthmatics showing sensitization to this allergen (5–7). The mainstay treatments for asthma include inhaled corticosteroids and beta-agonists, which treat symptomatology but not the asthmatic disease course itself (8). Approved biologicals for specific types of asthma (eosinophilic) or uncontrolled asthma include anti-cytokine, anti-cytokine receptor, or anti-IgE therapy, which also work through symptom management (8). Although there are available disease modifying treatments such as subcutaneous immunotherapy (SCIT, or allergy shots), these have the disadvantage of a long treatment period (3–5 years) and carry the risk for serious adverse side-effects (9). In 2017, sublingual immunotherapy (SLIT) for HDM was approved by the FDA for use in the United States (10). SLIT is also disease-modifying and, compared to SCIT, carries a lower risk for anaphylaxis. However, SLIT is similar to SCIT in the need for a long treatment duration (years). Consequently, there remains a need for novel therapies that could potentially modify the adaptive immune response to HDM and reduce the resulting pathology in HDM-mediated allergic disorders. To this end, numerous studies have focused on dissecting the innate immune response to HDM [reviewed in (11)], with the hope of finding new receptors or innate molecules that can be targeted to modulate the adaptive immune response to HDM and alleviate HDM-associated allergy and allergic asthma.

Type 2 adaptive immune responses are characterized by the T helper cell 2 (Th2) mediated secretion of the prototypical type 2 cytokines: interleukin-4 (IL-4), IL-5, and IL-13. These key cytokines initiate a cascade of events that are critical for the control and expulsion of parasites under a host protective setting, but which are also associated with allergies and asthma in the pathologic setting (12). The actions of these cytokines on various cell types are responsible for the typical asthmatic hallmarks of airway narrowing and airway hyperresponsiveness, mucus overproduction, and goblet cell and smooth muscle hyperplasia. Orchestrating the development of type 2 immunity involves the contributions of multiple cell types including epithelial cells, dendritic cells (DCs), group 2 innate lymphoid cells (ILC2s), and mast cells or basophils, which all work in concert (both directly and indirectly) to

promote the development of Th2 cells (which can then amplify and sustain the response) (13, 14).

NOD2 is a pattern recognition receptor which has been implicated in the development of Th2 immunity. NOD2 recognizes the bacterial peptidoglycan fragment muramyl dipeptide (MDP), and utilizes the dual-specificity kinase Receptor Interacting Protein 2 (RIP2) (capable of phosphorylating Serine/Threonine, and Tyrosine residues) to transduce signals resulting in activation of NF- κ B, MAPK, and type I interferon pathways (15–19). Work performed by other groups has shown that immunization of mice with the prototypical antigen ovalbumin (OVA), co-administered with the NOD2 agonist MDP, skews the adaptive immune response to Th2 immunity (20). This is evidenced by increased IL-4 and IL-5 ova-specific T cell responses and increased type 2 associated ova-specific IgG₁ levels in the serum, responses that are reduced in the setting of NOD2 deficiency. Similar findings were observed when these experiments were repeated with RIP2-deficient mice (20). It was, therefore, expected that loss or inhibition of this downstream effector kinase would reduce type 2 responses and likely improve the disease course in an allergic asthma model. Indeed, intratracheal delivery of siRNA against RIP2 was shown to improve ova-induced allergic airway inflammation (21). However, conflicting reports indicating that RIP2 deficient mice develop ovalbumin-induced allergic airway disease to the same extent as wild-type animals has put the importance of RIP2 in the pathogenesis of such diseases in question (22). In this study, we examine whether RIP2 is involved in the response to house dust mite, a more physiologically relevant airway allergen. Following an HDM-induced allergic airway model, RIP2 KO mice demonstrated attenuated airway inflammation as well as reduced HDM-specific innate and adaptive responses, suggesting that targeting this kinase may pose some therapeutic benefit in HDM-mediated allergic disease.

Materials and Methods

Mice.

C57BL/6J mice (000664) and RIP2 KO mice (007017) were obtained from Jackson Laboratories and were bred and housed in the Animal Facility at the UCF Health Science Campus at Lake Nona, an AALAC-accredited rodent barrier facility with a strong record of being a specific pathogen-free environment. Both male and female mice ranging from 6–10 weeks were used for the experiments. Although mice of different strains were not co-housed long term, C57BL/6 mice were temporarily co-housed with RIP2 KO mice during recovery from allergen challenge. Both littermates and non-littermates of each strain were used. All animal procedures were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee of the University of Central Florida and using a reviewed and approved animal protocol. The numbers of animals of each strain used per experiment are indicated within the figure legends.

House Dust Mite Asthma Model.

Lyophilized *Dermatophagoides farinae* (house dust mite, HDM) was obtained from Greer Laboratories (XPB81D3A2.5, Lot no. 140120–85) (Lenoir, NC), resuspended in sterile PBS, and aliquoted and frozen until use. Forty microliters of a 1.5mg/ml HDM mixture was

delivered intratracheally (i.t.) on day 0. Twenty-five microliters of a 0.5mg/ml HDM mixture was delivered i.t. from days 7–11. All intratracheal instillations were performed with animals under isoflurane anesthesia and using an otoscope, endotracheal tube, guide wire, and gas-tight syringe for delivery of HDM. Depending on the experiment, animals were euthanized at day 1, day 7, or day 14 for collection of tissues.

Cell lines, Antibodies, and Western Blot.

Calu3 cells (HTB55), and A549 cells (CCL185) were obtained from ATCC (Manassas, VA) and cultured as recommended. For generation of CRISPR/Cas9 RIP2 KO lines, the following target sequences were cloned into lentiCRISPRv2 (52961): F –5'-CACCGAGATCATACTGCTCGGTG-3', R –5'AAACCACCGAGCACGTATGATCTC-3' using the protocols by the Zhang Lab available on the Addgene website (Cambridge, MA). A universal negative sequence was used as a control (Sigma, St. Louis, MO). HEK 293 cells were transfected with psPAX2 (12260) and pMD2.G (12259), along with the modified lentiCRISPRv2 mentioned above (all from Addgene, Cambridge, MA). Calu3 cells were transduced with virus containing 10µg/ml polybrene (EMD Millipore, Burlington, MA) for 48 hrs before selection with 1:5000 Puromycin (Invivogen, San Diego, CA). Cell lysates were harvested using cell lysis buffer [50 mM Tris (pH7.5), 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 1 mM EGTA, 1 mM β-glycerophosphate, 1mM PMSF, 1 mM NaVO₄, 10 nM Calyculin A, protease inhibitor cocktail (Sigma, St. Louis, MO)]. RIP2 was immunoprecipitated (IP) using H-300 antibody (Santa Cruz Biotechnologies, Dallas, TX) and Protein G sepharose beads (Invitrogen, Waltham, MA). IPs were washed at least 4 times before Western blotting. Phosphotyrosine was detected using an anti-phosphotyrosine antibody (P-Tyr-100) from Cell Signaling (Danver, MA). Western blotting was performed using nitrocellulose membranes (Bio-Rad, Hercules, CA).

Generation of Primary Murine Tracheal Epithelial Cells (MTECs).

MTECs were generated as previously described (23). Briefly, tracheal epithelial cells were isolated by enzymatic treatment and seeded onto native basement membrane coated transwell filters. They were monitored until they reached confluency, after which they were grown on an air-liquid interface (ALI) using serum-free media. These ALI cultures were grown an additional 7–10 days or until signs of a well-differentiated epithelium were apparent (cilia beating). Maturity of cultures was further confirmed by staining for tight junctions using anti-ZO-1 (Cell Signaling, Danvers, MA) and the presence of cilia using anti-acetylated tubulin (Sigma, St. Louis, MO).

Histopathological scoring.

Mice were euthanized and lung tissue was harvested for histology. Formalin fixed lung tissue was sent to AML labs (St. Augustine, FL) for paraffin embedding, sectioning, and Hematoxylin & Eosin (H&E) and Periodic acid-Schiff (PAS) staining. H&E and PAS stained sections were scored blindly using a modified histopathological scoring system for these mice (24). Modifications included combining scores for bronchoarterial inflammation, pulmonary vein inflammation, amuscular blood vessel inflammation, interalveolar space inflammation, and pleural inflammation into one Inflammatory Index score. For bronchoarterial inflammation, amuscular blood vessel inflammation, and interalveolar space

inflammation, both incidence and severity of each feature was scored from 0–2, with 2 signifying that most of the tissue was involved or the greatest amount of inflammation was present for the feature assessed. For pulmonary vein inflammation and pleural inflammation, only the severity of each feature was scored from 0–2. These scores were all combined into one Inflammatory Index score (maximum of 16). Mucus production was scored based on the incidence and severity of PAS staining throughout the section (0–2 for each feature for a maximum score of 4). Sections were additionally stained with Masson's trichrome to assess collagen deposition (Abcam, Cambridge, MA). Trichrome stained slides were scored blindly using a previously published scoring system (25). Peribronchial and perivascular trichrome staining was scored together from 0–3, and parenchymal trichrome staining was scored from 0–3. In each case, 3 represents the highest staining intensity and greatest amount of involvement of the airway tissues. This was combined into one score for collagen deposition (maximum of 6).

Flow Cytometry.

Bronchoalveolar lavage (BAL) was performed using 2 washes of 500 μ L of 1xPBS containing 0.06mM EDTA. A portion of the primary right lung lobe was homogenized using a Lung Dissociation Kit and a GentleMacs homogenizer (Miltenyi, Auburn, CA). An equal portion (by weight) was collected for each mouse. This anatomic location was selected based on the distribution of intratracheally delivered test dye. One million cells of either the lung or BAL were stained using a cocktail of the following antibodies: PE anti-mouse Siglec F (E50–2440, BD Biosciences, San Jose, CA), PerCP anti-mouse CD11b (M1/70, Biolegend, San Diego, CA), PE-Cy7 anti-mouse Ly-6G (1A8, Biolegend, San Diego, CA), APC anti-mouse CD11c (N418, eBioscience/ThermoFisher, Waltham, MA), APC-Cy7 anti-mouse CD45 (30-F11, Biolegend, San Diego, CA), FITC anti-mouse CD3 (145–2C11, Biolegend, San Diego, CA), and FITC anti-mouse B220 (RA3–6B2, Biolegend, San Diego, CA). Gating strategies used were similar to those published by others (26) and are shown in Supplemental Figure 1. Briefly, lung or BAL cells were gated for singlets. Of the singlets, an FSC vs CD45 plot was used to further gate CD45⁺ cells. A plot of CD11c versus Siglec-F gated on CD45⁺ cells was used to discriminate alveolar macrophages (CD11c⁺Siglec-F⁺) from eosinophils (CD11c⁺Siglec-F⁺). A plot of CD11b versus Ly-6G gated on CD45⁺ cells was used to identify neutrophils (CD11b⁺Ly-6G⁺). A plot of SSC versus CD3 and B220 gated on CD45⁺ cells was used to identify lymphocytes (CD3⁺ and B220⁺).

For intracellular cytokine staining, lungs were collected and homogenized as described above. Two million lung cells were stimulated for 6 hours with 5 ng/ml PMA and 0.5 μ g/ml of ionomycin in the presence of protein transport inhibitors (GolgiPlug and GolgiStop, BD Biosciences, San Jose, CA). For cell surface staining, APC-Cy7 anti-mouse CD45 (30-F11, Biolegend, San Diego, CA) and FITC anti-mouse CD4 (GK1.5, Biolegend, San Diego, CA) were used. The cells were fixed and permeabilized using a Fixation/Permeabilization kit (BD Biosciences, San Jose, CA), and stained intracellularly using PerCP/Cy5.5 anti-mouse IL-4 (11B11, Biolegend, San Diego, CA), PE anti-mouse IL-5 (TRFK5, Biolegend, San Diego, CA), and APC anti-mouse IL-17a (TC11–18H10.1, Biolegend, San Diego, CA).

For ILC2 staining, the following antibodies were used: FITC anti-mouse lineage (CD3, Gr-1, CD11b, B220, Ter-119) (Biolegend, San Diego, CA), FITC anti-mouse TCR β (H57-597, Biolegend, San Diego, CA), FITC anti-mouse TCR $\delta\gamma$ (eBioGL3, eBioscience/ThermoFisher, Waltham, MA), FITC anti-mouse CD11c (N418, Biolegend, San Diego, CA), FITC anti-mouse NK1.1 (PK136, Biolegend, San Diego, CA), APC anti-mouse CD45 (30-F11, Biolegend, San Diego, CA), APC/Cy7 anti-mouse KLRG1 (2F1, Biolegend, San Diego, CA), PerCP anti-mouse Thy1.2 (53-2.1, Biolegend, San Diego, CA), PE anti-mouse ST2 (RMST2-2, eBioscience/ThermoFisher, Waltham, MA), and PE-Cy7 Sca-1 (E13-161.7, Biolegend, San Diego, CA). The gating strategy is shown in Supplemental Figure 3.

All samples were acquired on a 6-color Novocyte cytometer (ACEA Biosciences, San Diego, CA) and analyzed using the NovoExpress software.

Lung homogenate cytokine and chemokine analysis.

A portion of the primary right lung lobe was homogenized in T-PER buffer containing protease inhibitors (ThermoFisher, Waltham, MA). Samples were analyzed for protein concentration via Bradford Assay (BioRad, Hercules, CA) and adjusted to 0.5mg/ml in Legendplex assay buffer (Biolegend, San Diego, CA) prior to performing a 13-plex bead-based immunoassay following the manufacturer's instructions (Legendplex Th panel and Legendplex Pro-inflammatory Chemokine panel, Biolegend, San Diego, CA). Samples were acquired on a 6-color Novocyte cytometer (ACEA Biosciences, San Diego, CA) and analyzed using the provided Legendplex software (Vigenetech, Carlisle, MA).

Serum antibody analysis.

Murine anti-HDM IgG₁, IgG_{2a}, IgE, and total IgG ELISA kits were obtained from Chondrex, Inc. (Redmont, WA) and used as directed by the manufacturer. A 1:10 dilution of serum in assay diluent was used. The assays were developed using a TMB substrate and stopped using 1M phosphoric acid (Chondrex Inc, Redmont, WA). The absorbance was read at 450nm with 570nm background reading subtracted using a Cytation 5 Cell Imaging Multi-Mode Reader with the accompanying Gen5 software (Biotek, Winooski, VT).

ELISPOT Assay.

Ninety-six well, flat-bottom plates (Nunc MaxiSorp, ThermoFisher, Waltham, MA) were coated overnight at 4°C with cytokine-specific capture antibodies diluted in 1xPBS. Plates were blocked with 1% BSA in PBS for 1 hour at room temperature and washed with 1xPBS prior to plating cells and antigens. One hundred microliters of media, HDM extract (ALK Abello-USA, Round Rock, TX) at a final concentration of 100 PNU/ml, or ConA (Sigma, St. Louis, MO) at a final concentration of 2 μ g/ml, was used as a stimulus. To this, 100 μ L of 1 $\times$ 10⁶ RBC-lysed splenocytes (isolated on day 14 of regimen) or lung draining lymph nodes (isolated on day 7 of regimen) were added. Th2 responses were assessed after 48 hours of incubation at 37°C. Plates were washed with 1xPBS and 1xPBS- 0.0025% Tween (PBS-T). Biotinylated detection antibodies, were diluted in 1xPBS-1% BSA-0.0025% Tween, added to the plates, and incubated at 4°C overnight. After washing, gold anti-biotin antibody (GABA) diluted 1:35 in Dilution Buffer T (U-CyTech, Utrecht, Netherlands) was used as a tertiary reagent, added to the plates, and incubated at 37°C for 2 hours. Tertiary reagent was

washed off and a 1:1 mixture of Activator I and Activator II (U-CyTech, Utrecht, Netherlands) was added to plates at room temperature for assay development until spots were apparent (~45mins). Development was stopped by rinsing with water. The following clones of capture and detection antibodies were used: IL-4: 11B11 and BVD6–24G2-biotin (Biolegend, San Diego, CA), IL-5: TRFK5 and TRFK-4 biotin (Biolegend, San Diego, CA). For IL-13, a mouse IL-13 development kit was used (R&D, Minneapolis, MN), IL-17: TC11–18H10 and TC11–8H4 – biotin (Biolegend, San Diego, CA). Image analysis and spot count were performed using the Cytation 5 Cell Imaging Multi-Mode Reader with the accompanying Gen5 software (Biotek, Winooski, VT).

RNA extraction and Quantitative RT-PCR.

Calu3 cells or MTECs were harvested 4hr after stimulation with 250 PNU/ml of HDME. Lung was harvested 24hrs after HDM administration (40µl of a 1.5mg/ml HDM in PBS). RNA was extracted using a RNeasy Plus Mini kit (Qiagen, Germantown, MD) following the manufacturer's instructions. Reverse transcription was carried out using the QuantiTect Reverse Transcription kit (Qiagen, Germantown, MD) following the manufacturer's instructions. Real time PCR was performed using a SYBR Green Master Mix and the CFX96 Touch™ Real-Time PCR Detection System (BioRad, Hercules, CA). The following mouse (m) and human (h) primers were used: mCCL2 F 5' CTGCTGTTTCACAGTTGCCG 3', mCCL2 R 5' GCACAGACCTCTCTCTTGAGC 3', mGAPDH F 5' TGCCCCCATGTTTGTGATG 3', mGAPDH R 5' TGTGGTCATGAGCCCTTCC 3', hCCL2 F 5' CACCTTCATTCCCCAAGGGC 3', hCCL2 R 5' TCGGAGTTTGGGTTTGCTTG 3', hGAPDH F 5' TTAAAGCAGCCCTGGTGAC 3', hGAPDH R 5' CTCTGCTCCTCTGTTCGAC 3'. Data are presented as fold change calculated using the comparative Ct method.

Statistical Analysis.

Statistical analysis was performed by Julia Soulakova using SAS®9.4 (27). Significance level was fixed at the 10% level for each response measured. Figure legends indicate specific tests used for analysis of each dataset, number of animals per group, and number of times the experiment was repeated.

Results

RIP2 KO mice have reduced lung pathology compared to WT mice when subjected to a house dust mite asthma model.

To reconcile conflicting reports assessing the importance of RIP2 in type 2 dominated inflammatory diseases (21, 22), we chose to use a more physiologically relevant model of asthma. To this end, we subjected WT and RIP2 KO mice to repeated intratracheal administration of house dust mite (Fig 1A) (28). At day 14 of this regimen, mice were euthanized and their lungs fixed and harvested for histological examination. Paraffin-embedded sections were stained using H&E, PAS, and Trichrome stains, and scored by a blinded observer. Using a combination of parameters encompassing bronchoarterial inflammation, amuscular blood vessel inflammation, inter-alveolar space inflammation, pleural inflammation, and pulmonary vein inflammation, we report an inflammatory index

(maximum score of 16) and show that RIP2 KO mice develop significantly less inflammation in the lungs on this model compared to their WT counterparts (Fig 1B, arrows indicate some bronchoarterial inflammation within the section; and corresponding graph in Fig 1C). In addition, RIP2 KO mice again show significantly improved histopathological scores compared to WT mice when looking at mucus production via PAS staining (magenta stain in PAS sections in Fig 1B, and corresponding graph in Fig 1D), lumen narrowing and epithelial layer thickening (Fig 1B, 1E, 1F), and collagen deposition via trichrome staining (blue stain in Trichrome sections in Fig 1B and corresponding graph in Fig 1G). That is to say, HDM-treated RIP2 KO mice show less mucus production, epithelial thickening, and collagen deposition, but greater lumen area, or less narrowing, when compared to WT mice. These data illustrate that by multiple histopathological parameters, mice lacking RIP2 have improved lung pathology when subjected to a house dust mite asthma model.

RIP2 KO mice have decreased numbers of eosinophils recruited to the airway.

To assess whether the decreased pathology observed in the absence of RIP2 corresponded to a reduction in recruitment of inflammatory cells to the allergic airway, we harvested bronchoalveolar lavage (BAL) cells and stained for lymphocyte, neutrophil, alveolar macrophage, and eosinophil markers, and subjected these to flow cytometric analysis. As shown in Fig 2A, when both WT and RIP2 KO mice were subjected to an HDM asthma model, a significantly reduced number of total cells, eosinophils, and neutrophils were recovered in the lavage fluid of HDM-treated RIP2 KO mice compared to HDM-treated WT animals. Although HDM treatment resulted in a significant increase in lymphocyte and alveolar macrophage numbers in the BAL (p value for overall effect induced by HDM treatment indicated within the lymphocyte and alveolar macrophage graphs in Fig 2A), this effect was similar for WT and RIP2 KO groups. A portion of the lung was also dissociated and stained for inflammatory cells. Similar to what was observed with BAL cells, HDM-treated RIP2 KO animals had a significantly decreased number of eosinophils within the lung tissue when compared to HDM-treated WT animals. Although HDM treatment resulted in a significant effect when looking at the percentage of CD45 cells, lymphocytes, alveolar macrophages, and neutrophils recruited into the lung (p value for overall effect induced by HDM treatment indicated within each graph in Fig 2B), this effect was similar for WT and RIP2 KO groups.

RIP2 contributes to the generation of HDM-specific Th2 and Th17 immunity.

The HDM asthma model has been shown to be an inducer of both Th2 and Th17 immunity. In order to determine whether RIP2 is involved in the generation of HDM-specific Th2 and Th17 responses, both ELISPOT assays and intracellular cytokine staining assays for measurement of antigen-specific, cytokine secreting cells were performed. WT and RIP2 KO mice were untreated or subjected to a HDM asthma model. On day 14, mice were euthanized and splenocytes were collected. WT animals subjected to the HDM asthma regimen displayed a significant increase in the numbers of HDM-specific IL-4, IL-5, IL-13, and IL-17 secreting splenocytes in response to re-stimulation with HDM extract (Fig 3A). These responses were significantly diminished in HDM-treated mice lacking RIP2 (Fig 3A). In addition, day 7 lung draining lymph node cells were also collected for ELISPOT analysis. Similar to the response observed with day 14 splenocytes, lung draining lymph node cells

from HDM-treated RIP2 KO mice showed a significant reduction in the numbers of IL-4, IL-5, and IL-17 producing cells in response to re-stimulation with HDM extract when compared to HDM-treated WT animals (Fig 3B). We also performed intracellular cytokine staining at day 14 of the regimen using dissociated lung cells from untreated and HDM-treated WT and RIP2 KO mice. Consistent with the ELISPOT data, we observed an increase in the percentage of CD4⁺IL-4⁺, CD4⁺IL-5⁺, and CD4⁺IL-17⁺ cells in WT mice subjected to the HDM regimen (Fig 3C). This was significantly reduced in HDM-treated mice lacking RIP2 (Fig 3C). Lastly, we performed a bead-based multiplex assay using lung homogenates from WT and RIP2 KO mice that were untreated or subjected to a HDM asthma model (Supplemental Fig 2). These data show that there was a significant induction of IL-4, IL-5, IL-9, IL-6, IL-10 and TNF- α in WT animals treated with HDM. Loss of RIP2 resulted in a significant decrease in IL-4, IL-5, and TNF- α in response to HDM treatment (Supplemental Fig 2). Taken together, these data demonstrate that mice lacking RIP2 have a defect in the generation of HDM-specific Th2 and Th17 immunity and a reduction in local levels of HDM-induced Th2-associated cytokines.

Loss of RIP2 does not affect expansion of group 2 innate lymphoid cells (ILC2s) in the lung in response to HDM.

ILC2s are an innate immune cell population which have been shown to contribute to allergic inflammation and generation of Th2 immunity (29). ILC2s rapidly expand in response to allergens and can be an early source of the Th2-associated cytokines IL-5 and IL-13, which they secrete in response to allergen associated proteases (30). Given that they are also potent in inducing eosinophil migration (31, 32), we sought to determine if loss of RIP2 results in a differential expansion of ILC2s in the lung, which could then lead to the observed defects in eosinophil recruitment in RIP2 KO animals. Lung cells were obtained from day 14 untreated or HDM-treated WT and RIP2 KO mice and stained with ILC2 markers for flow cytometric analysis (see Supplemental Fig 3 for gating strategy). Figure 4 shows a significant increase in the numbers of ILC2 cells upon HDM treatment when classified by ST2 (Fig 4A), Sca-1 (Fig 4B), KLRG1 (Fig 4C), or all 3 markers (Fig 4D) (p values for the overall effect of HDM regimen are shown within each figure). However, the HDM treatment affected both WT and RIP2 KO mice equally and no differences in ILC2 numbers were observed between HDM-treated WT and RIP2 KO animals.

Loss of RIP2 results in defective generation of dust mite-specific IgG₁ antibody responses.

The HDM asthma model has been previously characterized as being Th2 dominant. The Th1 dominant cytokine IFN- γ is known to promote class switching to antibodies of the IgG_{2a} isotype, while the Th2 dominant IL-4 cytokine promotes class switching to primarily IgG₁ and IgE (33). Both WT and RIP2 KO HDM-treated animals showed an increase in the levels of dust mite-specific IgE, IgG₁, and total IgG antibodies when compared to controls (Fig 5A, 5B, and 5C). No induction of HDM-specific antibodies of the IgG_{2a} subclass was observed (all below detection limit of assay). RIP2 KO HDM-treated animals showed a significant decrease in the levels of dust mite-specific IgG₁ antibodies compared to the WT HDM-treated group (Fig 5B). When total HDM-specific IgG levels were tested, RIP2 KO HDM-treated animals also showed a significant decrease in the levels of dust mite-specific total IgG antibodies compared to the WT HDM-treated group (Fig 5C). Overall, these data

suggest a potential defect in class switching to the Th2-associated IgG₁ isotype in RIP2 KO mice in the setting of a HDM allergic airway model.

RIP2 is activated in airway epithelial cells in response to HDM and promotes the expression of inflammatory chemokines.

Given the importance of the airway epithelium in the establishment of Th2 immunity, we sought to determine whether RIP2 is involved in the response to HDM within this compartment. To this end, we stimulated 2 different human airway epithelial cell lines, Calu3 and A549, with house dust mite extract, and determined RIP2 tyrosine phosphorylation via Western Blot. We have previously shown that tyrosine phosphorylation of RIP2 can be used as a surrogate for RIP2 activity (34). In Fig 6A we demonstrate that stimulation of both of these cell lines with dust mite extracts leads to tyrosine phosphorylation of RIP2 in a time-dependent manner. To determine whether this observed RIP2 activation in response to an allergen is important for the resulting inflammatory response within airway epithelial cells, we engineered Calu3 cells deficient in RIP2 via CRISPR/Cas9 gene-targeting. We show that Calu3 cells that lack RIP2 have decreased expression of CCL2 in response to stimulation with dust mite extract compared to their WT counterparts (Fig 6B). In order to study the role of RIP2 in a system that more closely resembles the *in vivo* airway, we generated primary murine tracheal epithelial cell (MTEC) cultures from WT and RIP2 deficient mice. MTEC cultures from both WT and RIP2 KO mice show features of a well-defined airway including proper barrier formation assessed by staining for the tight junction protein ZO-1 and the presence of cilia as assessed by staining for acetylated tubulin or as visualized from H&E staining of paraffin-embedded MTEC cultures (Fig 6C). Similar to the RIP2 deficient Calu3 human airway epithelial cell lines, stimulation of RIP2 deficient MTECs with HDME resulted in decreased expression as well as production of CCL2 when compared to WT MTEC cultures stimulated with HDME (Fig 6C). In addition, lung homogenates and lung RNA were obtained from untreated or 24 hr HDM-treated WT and RIP2 KO mice. Quantitative RT-PCR and bead-based immunoassays show a similar defect in the expression and production of CCL2 in HDM-treated RIP2 KO mice when compared to HDM-treated WT mice (Fig 6D). Defective production of KC, TARC, and MIP-1 β was also observed in RIP2 KO mice treated with HDM (Supplemental Fig 4). Altogether, this data suggests that RIP2 can be activated in airway epithelial cells upon stimulation with house dust mite and that loss of RIP2 leads to a consistent defect in CCL2 production in response to HDM in airway epithelial cells and lung tissue.

Discussion

Type 2 immunity can be induced by stimuli as varied as alum (35), insect venom (36), helminth antigens (37, 38), and environmental allergens (39). Polarization to type 2 immunity is a complex process which is unique from the development of other types of effector immunity in the sense that multiple cell types and cellular interactions (direct and indirect) are required to completely “license” DCs and provide the appropriate cytokine milieu for proper differentiation of CD4⁺ Th2 cells. As such, there has been much interest in identification of the receptors and proteins on DCs, accessory cells, epithelial cells, and stromal cells, which are important for the generation of type 2 immunity. Some examples of

these include TLR4 in airway epithelial cells(40), Dectin-2 in DCs(41), and NOD1 and NOD2 on DCs and stromal cells(20, 42).

Despite NOD1, NOD2, or RIP2 not being generally regarded as susceptibility genes for allergic or asthmatic disease, a number of reports suggest an association between polymorphisms in NOD1, NOD2, and RIP2 genes and allergic or asthmatic status(43–47). In addition, other studies have reported upregulation of these proteins in the setting of allergy or asthma. In our model, disease is attenuated in RIP2 KO mice but not abrogated. This could be due to a number of factors. First, the contributions of NOD1/2 and RIP2 to overall Th2 development when compared to that contributed by other receptors or genetic factors (such as MHC) may be limited. Second, the influence of NOD proteins in shaping adaptive immunity may be multifold. Even in the initial studies reporting the influence of NOD1 and NOD2 on Th2 immunity, it was also noted that Th1 immunity was affected as well(20). In addition, multiple reports exist describing the importance of NOD2 in shaping Th17 responses(48). Likely, these outcomes may be dependent on co-engagement of other pattern recognition receptors on DCs or the generation of other Th polarizing stimuli. Nonetheless, mouse models clearly demonstrate that sole engagement of the NOD1 and NOD2 receptors within the DC and stromal compartments are sufficient for the development of Th2 immunity(20, 42). Given that both NOD proteins utilize the kinase RIP2 for many, if not most, of their effector functions, it would stand to reason that not only would RIP2 be likely involved in the generation of type 2 immunity, but being downstream of both receptors and having a clearly druggable enzymatic activity, may serve as a more attractive therapeutic target.

In this study, we demonstrate that RIP2 can be directly activated in airway epithelial cells upon exposure to house dust mite protein extracts. In human airway epithelial cell lines, primary murine tracheal epithelial cells, and murine lung tissue from HDM-treated mice, we demonstrate that loss of RIP2 results in a consistent defect in CCL2 production. CCL2 (MCP-1) is a potent chemoattractant for monocytes, but in addition, is also known for its role in polarizing CD4 T cells to type 2 immunity (49–51). Indeed, although we observed no reduction in the numbers of alveolar macrophages in the airway in the absence of RIP2, we did not further dissect monocyte recruitment, particularly those of the inflammatory type (Ly6C^{hi}). We did observe defects in eosinophil recruitment, dust mite-specific IL-4 and IL-5 production, and dust mite-specific IgG₁ antibody production, pointing to a defect in skewing to type 2 immunity. Although we cannot rule out the contribution of CCL2 from other sources (monocytes/macrophages), we did observe a significant defect in the expression of CCL2 in lung tissue from HDM-treated RIP2 KO mice when compared to HDM-treated WT mice (Fig 6C). Additionally, we also observed a reduction in the HDM-induced production of TARC (CCL17) in lung tissue from HDM-treated RIP2 KO animals compared to controls (Supplemental figure 4). TARC is a chemokine which serves as a potent chemoattractant for Th2 cells as it binds CCR4 preferentially expressed on this subset (52–54). An early defect in production of this chemokine could potentially contribute to the reduced numbers of Th2 cells observed within the lung and lung draining lymph node in HDM-treated animals in the absence of RIP2 (Fig 3). Altogether, the data suggest a model in which RIP2 is activated in the airway epithelium in response to HDM, upregulating the expression of the Th2 polarizing chemokine CCL2 and promoting the production of the Th2 recruiting chemokine

TARC. Although possibly not the sole mechanism for the observed effects, the actions of RIP2 within the epithelial compartment may contribute to the generation / polarization of pathologic HDM-specific Th2 responses. This, potentially combined with enhanced recruitment of Th2 cells, would, in turn, promote airway eosinophilia, lung inflammation, mucus overproduction, and increases in HDM-specific antibodies. Further work will need to be done to conclusively show the importance of RIP2-induced, airway epithelial-derived factors. Additional studies such as bone marrow chimera experiments will need to be performed in the future to establish the importance of RIP2 signaling in epithelial/stromal compartments versus hematopoietic compartments.

Although this work confirms the involvement of RIP2 in promoting a type 2 response and in contributing to allergic airway inflammation using a clinically relevant allergen, a number of questions additionally arise. What is the nature of the agonist promoting RIP2 activation in dust mite? Given that so far, only NOD1 and NOD2 have been demonstrated to be *bona fide* upstream receptors for activation of RIP2, the most obvious scenario would be the presence of contaminating bacterial peptidoglycan fragments within this mixture. Another intriguing possibility would be the existence of an alternative means to activate RIP2 independent of NOD1 or NOD2, a pathway that is triggered by exposure of epithelial cells to HDM. Indeed, ongoing work in our laboratory has identified additional receptors upstream of RIP2 which can lead to RIP2 activation independent of NOD2 (manuscript in preparation). Whether or not these same receptors are engaged upon HDM exposure is something that will still need to be tested.

Lastly, although our overall findings support the potential use of RIP2 targeted therapies in type 2 dominated diseases, how efficacious these will ultimately be, is still uncertain. Previous work, as well as our own, have shown that RIP2 possesses both kinase-dependent and kinase-independent effects (17, 55). We have previously demonstrated the efficacy of pharmacologically targeting RIP2 in a murine model of inflammatory bowel disease and in an MDP-induced peritonitis model (55), and since our discovery of RIP2 as a dual-specificity kinase (34), many other such kinase inhibitors have been discovered and developed (56–58). Whether or not the kinase-dependent actions of RIP2 are equally important in type 2 disorders such as allergic asthma to justify the use of such inhibitors is an interesting question which still remains to be addressed.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

ALI	air-liquid interface
BAL	bronchoalveolar lavage
CCL2	Chemokine (C-C motif) ligand 2; also known as MCP-1
CCL17	Chemokine (C-C motif) ligand 17; also known as TARC
CD45	cluster of differentiation 45
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
DC	dendritic cell
Df	Dermatophagoides farinae
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid, or egtazic acid
ELISA	enzyme-linked immunosorbent assay
ELISPOT	enzyme-linked immunospot
H&E	hematoxylin and eosin
HDM	house dust mite
HDME	house dust mite extract
HEK	human embryonic kidney
IFN	interferon
Ig	immunoglobulin
IL	interleukin
ILC2	group 2 innate lymphoid cells
KO	knock-out
MAPK	mitogen-activated protein kinase
MCP-1	monocyte chemoattractant protein-1; also known as CCL2
MDP	muramyl dipeptide
MTEC	murine tracheal epithelial cells
NaCl	sodium chloride
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells

NOD	nucleotide oligomerization domain
PAS	periodic acid schiff
PMA	Phorbol 12-myristate 13-acetate
PMSF	phenylmethane sulfonyl fluoride
PNU	protein nitrogen units
RIP2	Receptor Interacting Protein 2
SCIT	subcutaneous immunotherapy
SFU	spot-forming units
siRNA	small interfering ribonucleic acid
SLIT	sublingual immunotherapy
TARC	Thymus- and activation-regulated chemokine; also known as CCL17
Th	helper T cell
TLR	Toll-like Receptor
T-PER	tissue protein extraction reagent
WT	wild-type
ZO-1	zona occludens protein 1

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Figure 1. RIP2 KO mice have reduced lung pathology compared to WT mice when subjected to a house dust mite asthma model.

A.) WT and RIP2 KO mice were left untreated or subjected to a house dust mite model of asthma. On day 14, lungs were harvested, fixed in 10% formalin, and embedded in paraffin. B.) Consecutive sections were stained with either H&E, PAS, or Trichrome. Severity of lung pathology was scored based on C.) inflammatory index, D.) mucus production, E.) lumen narrowing, F.) epithelial thickness, and G.) collagen deposition by a blinded observer. Statistical analysis was performed using a two-way ANOVA followed by a two-tailed t-test for all parameters except inflammatory index and mucus production. For inflammatory index, the normality assumption was not supported with data for the control groups. For

mucus production, the control group for RIP2 KO had no variance, preventing statistical analysis using this dataset (n.t., not tested). Therefore, for these groups, Wilcoxon tests were used. Data in graphs represent means $\pm$ SEM. Data are aggregated from more than 3 independent experiments using n=10–20 mice /control group and n=15–26 mice / HDM treatment group. WT, wild-type; Ctrl, control; HDM, *D. farinae* house dust mite. ** = $p < 0.05$, *** = $p < 0.01$, **** = $p < 0.001$

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Figure 2. RIP2 KO mice have decreased numbers of eosinophils recruited to the airway. WT and RIP2 KO mice were left untreated or subjected to a house dust mite model of asthma. On day 14, bronchoalveolar lavage (BAL) cells or dissociated lung cells were isolated, stained for immune cell markers, and subjected to flow cytometric analysis. A.) The total number of cells in the BAL for each population of cells is shown. B.) The percentage of cells in the lung for each population of cells is shown. Statistical analysis was performed using a two-way ANOVA followed by a two-tailed t-test for each parameter. In cases where no interaction was observed (effect of the HDM regimen was found to be similar for WT and RIP2 KO mice), the p value for the overall effect of HDM administration is indicated within the graph and no further post-hoc tests were performed. Data in graphs represent means $\pm$ SEM. Data are aggregated from more than 3 independent experiments using n=10–13 mice / control group and n=13–14 mice / HDM treatment group for BAL cells, and n=7 mice / control group and n=11 mice / HDM treatment group for lung cells. WT, wild-type; Ctrl, control; HDM, *D. farinae* house dust mite. ** = $p < 0.05$, *** = $p < 0.01$, **** = $p < 0.001$

Figure 3. RIP2 contributes to the generation of HDM-specific Th2 and Th17 immunity.

WT and RIP2 KO mice were left untreated or subjected to a house dust mite model of asthma. A.) Day 14 splenocytes or B.) day 7 lung draining lymph nodes were collected and an ELISPOT assay was performed using house dust mite extracts as the recall antigen. Numbers of cytokine secreting cells in response to re-stimulation with dust mite extract are shown. C.) Intracellular cytokine staining (ICS) was performed using PMA + ionomycin restimulation of d14 lung cells. The percentage of lung cells which are CD4⁺ and also IL-4⁺, IL-5⁺, or IL-17⁺ are shown. Statistical analysis was performed using Wilcoxon tests for all comparisons. Data in graphs represent means $\pm$ SEM. Data are aggregated from at least 3 independent experiments (except for ICS which was aggregated from 2 independent experiments). For d14 ELISPOT experiments using splenocytes, n=11 mice /control group and n=22–27 mice / HDM treatment group were used. For d7 ELISPOT experiments using draining lymph node cells, n=10 mice /control group and n=12 mice / HDM treatment group were used. For ICS experiments using lung cells, n=5–6 mice /control group and n=5 mice / HDM treatment group were used. WT, wild-type; Ctrl, control; HDM, *D. farinae* house dust mite; SFU, spot-forming units. * = $p < 0.1$, ** = $p < 0.05$, *** = $p < 0.01$, **** = $p < 0.001$

Figure 4. Loss of RIP2 does not affect expansion of group 2 innate lymphoid cells in the lung in response to HDM.

WT and RIP2 KO mice were left untreated or subjected to a house dust mite model of asthma. On day 14, dissociated lung cells were isolated, stained for ILC2 markers, and subjected to flow cytometric analysis. ILC2 cells were identified as a Lin⁻CD45⁺Thy1.2⁺ population which were also A.) ST2⁺, B.) Sca-1⁺, C.) KLRG1⁺, or D.) KLRG1⁺Sca-1⁺ST2⁺. Statistical analysis was performed using a two-way ANOVA. In all cases, no interaction was observed (effect of the HDM regimen was found to be similar for WT and RIP2 KO mice). Therefore, no further testing was performed. The p value for the overall effect of HDM administration is indicated within each graph. Data in graphs represent means $\pm$ SEM. Data are aggregated from 2 independent experiments using n=6 mice/control group and n=7 mice / HDM treatment group. WT, wild-type; Ctrl, control; HDM, *D. farinae* house dust mite. **** = $p < 0.001$

Figure 5. Loss of RIP2 results in defective generation of HDM-specific IgG₁ antibody responses.

WT and RIP2 KO mice were left untreated or subjected to a house dust mite model of asthma. On day 14, serum was collected and dust mite-specific antibody isotypes and subclasses were determined using a direct ELISA. Shown are levels of A.) IgE, B.) IgG₁, and C.) total IgG anti-HDM antibodies. Anti-HDM antibodies of the IgG2a subclass were also tested but were below detection and are, therefore, not shown. No variance in control groups (all zero values) prevented statistical testing (n.t., not tested). However, given that all values for the treatment groups were positive, we concluded (without statistical testing), that the HDM regimen was effective for both genotypes. For statistical analysis between WT and RIP2 KO HDM treatment groups, a Wilcoxon test was performed. Data in graphs represent means $\pm$ SEM. Data are aggregated from more than 3 independent experiments using $n=13-17$ mice /control group and $n=24$ mice / HDM treatment group. WT, wild-type; Ctrl, control; HDM, *D. farinae* house dust mite. ** = $p < 0.05$, **** = $p < 0.001$

Figure 6. RIP2 is activated in airway epithelial cells in response to HDM extract and is important for the production of CCL2.

A.) Calu3 and A549 human airway epithelial cells were treated with 250 PNU/ml of *D. farinae* house dust mite extract. RIP2 was immunoprecipitated and Western Blotting was performed to determine tyrosine phosphorylation of RIP2. Relative densities of the bands are shown below each Western Blot. B.) CRISPR/Cas9 targeting was used to generate RIP2 deficient Calu3 cells. Control or RIP2 deficient Calu3 cells were stimulated with dust mite extract for 4hrs and expression of CCL2 was determined. A Wilcoxon rank-sums test was performed to assess: treatment effect among WT mice, treatment effect among RIP2 KO mice, and genotype effect among HDM-treated mice. Data in graphs represent means $\pm$ SEM using cells harvested at 5 independent occasions (n=5). Confirmation of the loss of

RIP2 is shown via Western Blot above the CCL2 expression data. C.) Murine tracheal epithelial cells (MTECs) were generated from WT and RIP2 KO mice. MTECs were grown on native basement membrane coated Transwell filters until they showed features of a fully differentiated airway (cilia beating). MTECs were fixed with 10% buffered formalin, embedded in paraffin, and sectioned for H&E staining. A separate set was prepared for immunofluorescence staining for ZO-1 and acetylated tubulin. MTECs from WT or RIP2 KO mice were stimulated with dust mite extract for 4hrs and expression and production of CCL2 was determined. Data in graphs represent means $\pm$ SEM using MTECs generated on 3 independent occasions (n=3). Small sample size prevented statistical testing. D.) WT or RIP2 KO mice were administered HDM acutely (24 hrs), and lung tissue was collected for RNA extraction to assess CCL2 expression and preparation of homogenates to assess CCL2 production. Statistical analysis was performed using a Wilcoxon rank-sums test. Data in graphs represent means $\pm$ SEM. Data are aggregated from 3 independent experiments using n=9 mice /control group and n=10–11 mice / HDM treatment group for gene expression studies, and n=8 mice /control group and n=8 mice / HDM treatment group for lung homogenates. WT, wild-type; Ctrl, control; HDM, *D. farinae* house dust mite; PNU, protein nitrogen units. ** = $p < 0.05$, *** = $p < 0.01$, **** = $p < 0.001$