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This information is current as of October 26, 2011

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J Immunol; Prepublished online 21 October 2011;

doi:10.4049/jimmunol.1102133

<http://www.jimmunol.org/content/early/2011/10/21/jimmunol.1102133>

Supplementary Data

<http://www.jimmunol.org/content/suppl/2011/10/21/jimmunol.1102133.DC1.html>

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The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
9650 Rockville Pike, Bethesda, MD 20814-3994.
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Print ISSN: 0022-1767 Online ISSN: 1550-6606.



Rapamycin Attenuates Airway Hyperreactivity, Goblet Cells, and IgE in Experimental Allergic Asthma

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The mammalian target of rapamycin (mTOR) signaling pathway integrates environmental cues, promotes cell growth/differentiation, and regulates immune responses. Although inhibition of mTOR with rapamycin has potent immunosuppressive activity, mixed effects have been reported in OVA-induced models of allergic asthma. We investigated the impact of two rapamycin treatment protocols on the major characteristics of allergic asthma induced by the clinically relevant allergen, house dust mite (HDM). In protocol 1, BALB/c mice were exposed to 10 intranasal HDM doses over a period of 24 d and treated with rapamycin simultaneously during the sensitization/exposure period. In protocol 2, rapamycin was administered after the mice had been sensitized to HDM (i.p. injection) and prior to initiation of two intranasal HDM challenges over 4 d. Airway hyperreactivity (AHR), IgE, inflammatory cells, cytokines, leukotrienes, goblet cells, and activated T cells were assessed. In protocol 1, rapamycin blocked HDM-induced increases in AHR, inflammatory cell counts, and IgE, as well as attenuated goblet cell metaplasia. In protocol 2, rapamycin blocked increases in AHR, IgE, and T cell activation and reduced goblet cell metaplasia, but it had no effect on inflammatory cell counts. Increases in IL-13 and leukotrienes were also blocked by rapamycin, although increases in IL-4 were unaffected. These data demonstrated that rapamycin can inhibit cardinal features of allergic asthma, including increases in AHR, IgE, and goblet cells, most likely as a result of its ability to reduce the production of two key mediators of asthma: IL-13 and leukotrienes. These findings highlight the importance of the mTOR pathway in allergic airway disease. *The Journal of Immunology*, 2011, 187: 000–000.

Asthma prevalence has increased substantially in recent years, especially in children (1–3). Allergic asthma is the most common form and is characterized by airway inflammation, airway hyperreactivity (AHR), goblet cell metaplasia, and increases in IgE and Th2 cytokines (1, 4, 5). Although glucocorticoids and bronchodilators are the mainstay of asthma treatment, these therapies are not effective in all asthmatics (1).

The discovery of the drug rapamycin (6, 7) has led to intense study of its target: the mammalian target of rapamycin (mTOR). mTOR is downstream of the PI3K-signaling cascade and signals via two complexes: mTOR complex (mTORC)1 and mTORC2 (8, 9). Activation of mTORC1, which is sensitive to rapamycin, leads to phosphorylation and activation of the p70 ribosomal S6 kinase 1 and, subsequently, S6 ribosomal protein (S6), which promotes ri-

bosomal protein synthesis (8). Although several reports indicated that mTORC2 is not inhibited by rapamycin, there is evidence showing that rapamycin can inhibit mTORC2 activity, depending on the specific cell type, duration, and dose of rapamycin treatment (10). mTOR is known to play a major role in regulating cell metabolism, growth/differentiation, and survival in many cell types (8, 11). Dysregulation of this pathway has been implicated in various diseases, including cancer and type 2 diabetes (9, 12, 13). Rapamycin is used as an immunosuppressant drug to prevent transplant rejection (14, 15); however, its effects on inflammation in OVA-induced models of asthma are mixed (16–18). In addition, studies in OVA models (16–18) did not address whether mTOR inhibition alters IL-13 and leukotrienes, which are important mediators of allergic asthma responses, including AHR and goblet cell metaplasia.

The goal of our study was to determine whether rapamycin would attenuate key characteristics of allergic asthma (AHR, inflammation, goblet cell metaplasia, IgE) and important mediators (IL-13 and cysteinyl leukotrienes) in a clinically relevant model induced by exposure to house dust mite (HDM). We hypothesized that inhibition of mTOR with rapamycin would attenuate allergic airway disease via reductions in these key mediators. To test this hypothesis, mice were either exposed to HDM and treated with rapamycin simultaneously or sensitized to HDM by systemic injection and then treated with rapamycin during subsequent intranasal (i.n.) HDM challenges. Multiple end points were assessed, including sensitization, AHR, inflammation, goblet cells, T cells, cytokines, and leukotrienes.

Materials and Methods

Animals

Animal protocols and procedures were approved by the Animal Care and Use Committee at the Cincinnati Children's Hospital Research Foundation (Cincinnati, OH). Six- to eight-week-old female BALB/c mice were

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Received for publication July 25, 2011. Accepted for publication September 23, 2011.

This work was supported by National Institutes of Health Grants U19A170235 (to G.K.K.H.) and HL097135 (to T.D.L.C. and G.K.K.H.); National Institute of Environmental Health Sciences Grant T32 ES010957 (to E.B.B.); and American Heart Association Established Investigator Grant 740069N (to T.D.L.C.).

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The online version of this article contains supplemental material.

Abbreviations used in this article: AHR, airway hyperreactivity; BALF, bronchoalveolar lavage fluid; CLCA3, chloride channel, calcium activated, family member 3; HDM, house dust mite; i.n., intranasal; mTOR, mammalian target of rapamycin; mTORC, mammalian target of rapamycin complex; Muc5AC, mucin 5AC; P-S6, phosphorylated S6 ribosomal protein; S6, S6 ribosomal protein.

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purchased from Charles River Laboratories (Wilmington, MA). The treatment protocols used in these studies are described below.

Protocol 1

Mice were exposed to 10 i.n. doses of HDM (50 µg in 20 µl saline; Greer Laboratories, Lenoir, NC) or saline (0.9% NaCl, 20 µl; control group) over 24 d (Fig. 1A). In a third study group, mice were exposed to HDM and treated with rapamycin. Rapamycin (4 mg/kg) (LC Laboratories, Woburn, MA) was administered by i.p. injection, six times a week starting with the first HDM exposure and continuing until 1 d after the last HDM exposure, for a total of 20 treatments.

Protocol 2

Mice were sensitized to HDM by three i.p. injections of HDM (50 µg in 100 µl saline) at 7-d intervals (Fig. 1B). Seven days after the last i.p. injection, two i.n. challenges of HDM (50 µg in 20 µl saline) were given 2 d apart. Another group was treated with rapamycin (4 mg/kg, i.p.), starting 1 d prior to the first i.n. HDM challenge and continued until 1 d after the second HDM challenge, for a total of six rapamycin treatments. Study groups were mice given i.p. saline + i.n. saline (saline/saline), i.p. HDM + i.n. saline (HDM/saline), i.p. HDM + i.n. HDM (HDM/HDM), and i.p. HDM + i.n. HDM + i.p. rapamycin (HDM/HDM+Rapa).

Airway hyperreactivity

AHR was studied in anesthetized mice 48 h after the last HDM challenge. Anesthesia was delivered by i.p. injection of ketamine/xylazine/acepromazine (4:1:1) solution (0.2 ml/animal). Changes in airway resistance to methacholine were assessed, as previously described (19). Briefly, a tracheostomy was performed, and the mouse was connected to a flexiVent system (SCIREQ, Montreal, QC, Canada). Airway resistance was measured after nebulization of 1× PBS (baseline) and then increasing doses of methacholine (6.25, 12.5, 25, and 50 mg/ml; acetyl-β-methylcholine chloride, Sigma, St. Louis, MO). After the flexiVent studies, mice were killed by cutting the thoracic aorta. Blood was collected, and serum was extracted for measurements of IgG1 and IgE.

Allergic sensitization

Total and HDM-specific IgG1 and IgE levels were measured in serum by ELISA to assess allergic sensitization, as previously described (20). Briefly, for total IgG1 and IgE, plates were coated overnight with 2 µg/ml anti-mouse IgG1 or IgE in PBS, respectively (Pharmingen, San Jose, CA). For HDM-specific IgG1 and IgE, plates were coated with 0.01% HDM in PBS. The following day, plates were blocked with 1% BSA in PBS for 1 h and then coated with samples and IgG1 or IgE standards. Biotin-anti-mouse IgG1 or IgE (2.0 µg/ml, Pharmingen) was used to capture IgG1 and IgE Abs, respectively. Streptavidin-HRP (1:100; R&D Systems, Minneapolis, MN) was added to detect biotin-labeled Abs, and the reaction was developed by tetramethylbenzidine substrate reagent set (1:1) (BD Biosciences, San Jose, CA).

Inflammatory cells and cytokines

Bronchoalveolar lavage fluid (BALF) was collected from mice to assess the inflammatory response, as previously described (19). Briefly, the lungs were lavaged via a tracheostomy with 1 ml 1× PBS containing BSA (1%) and EDTA (2 mM). The BALF was centrifuged (5000 rpm), and the supernatant was frozen at -80°C for measurements of cytokines and leukotrienes. Cell pellets were resuspended, and RBCs were lysed using RBC lysing buffer (Sigma). The remaining cells were resuspended in 1× PBS after centrifugation. Total inflammatory cells were counted using a hemacytometer, and cytospins were performed on the remaining cells for differential cell counts. Diff-Quick staining of cytospin slides (Shandon Lipshaw, Pittsburgh, PA) was performed. Three hundred cells were counted per slide, and the percentages of macrophages, lymphocytes, neutrophils, and eosinophils were calculated. Total differential inflammatory cell counts were also calculated. Cytokines in the BALF were measured using a multiplex biomarker panel (Chemokine Panel I) and Luminex xMAP technology (Millipore, Billerica, MA), following the manufacturer's instructions. Cysteinyl leukotriene (C₄, D₄, E₄) levels were measured by a commercial company (ElisaTech, Aurora, CO) using ELISA kits (Cayman, Ann Arbor, MI), according to the manufacturer's instructions.

Western blot analysis

Western blot analysis was performed on lung homogenates using the following Abs to assess the levels of phosphorylated S6 (P-S6, 1:1,000; Cell

Signaling, Danvers, MA), total S6 (1:1,000; Cell Signaling), chloride channel, calcium activated, family member 3 (CLCA3, 1:2,000; Abcam, Cambridge, MA) (21, 22), and β-tubulin (1:1,000; Cell Signaling). Secondary Abs included goat anti-rabbit and goat anti-mouse (1:10,000, Calbiochem). ECL Plus system was used for chemiluminescence detection (GE Healthcare). An LAS4000 imaging system and Multi Gauge 3 software (Fujifilm, Tokyo, Japan) were used to image and quantitate the chemiluminescent signal for each Western blot. Values were normalized to β-tubulin to control for protein loading and transfer efficiency.

Mucin 5AC immunohistochemistry

Lungs were inflated fixed at a constant pressure (25 cm H₂O) by tracheal installation of 4% paraformaldehyde, transferred to 70% ethanol after 24 h, and embedded in paraffin, as previously described (19). Immunostaining for mucin 5AC (Muc5AC) was performed on 5-µm paraffin-embedded sections. Slides were incubated with a primary Muc5AC mouse mAb (1:200; Thermo Scientific, Waltham, MA) overnight at 4°C, followed by a goat anti-mouse IgG1 secondary Ab (1:200; Southern Biotech, Birmingham, AL). Signal was detected using the diaminobenzidine method of detection (19). Digital images of Muc5AC immunostaining were obtained using a Zeiss Axioplan 2 microscope and camera (Carl Zeiss Microimaging, Thornwood, NY).

Quantitative real-time PCR analysis

RNA for quantitative real-time PCR was isolated using the SV Total RNA Isolation System (Promega, Madison, WI) and reverse transcribed into cDNA using an iScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA), following the manufacturer's instructions. cDNA samples were amplified with TaqMan, and a primer/probe set specific for Muc5AC was used (assay ID: Mm01276725_g1; Applied Biosystems, Carlsbad, CA). Quantification of gene expression was analyzed by a 7300 Real Time PCR System (Applied Biosystems) in triplicate, and expression levels were normalized to β-actin mRNA levels.

Flow cytometry for activated and regulatory T cells

The upper right lung lobe was minced and incubated at 37°C for 25–30 min in 2 ml RPMI 1640 containing Liberase DL (0.5 mg/ml; Roche Diagnostics, Indianapolis, IN) and DNase I (0.5 mg/ml; Sigma). Lung cells were passed through a 70-µm cell strainer, and the strainer was washed with 5 ml RPMI 1640 + DNase I media. Cells were centrifuged and resuspended in 2 ml RPMI 1640 before counting with a hemacytometer and confirming viability by trypan blue exclusion. Approximately 500,000 lung cells were transferred to a 96-well V-bottom plate on ice, centrifuged, and resuspended in 1× PBS containing FcBlock (2.4G2 mAb). To assess the levels of activated T cells, lung T cells were stained with CD3e-FITC, CD4-PE/Cy7, CD69-PE, or CD25-allophycocyanin (BioLegend, San Diego, CA). Live and dead cells were labeled with LIVE/DEAD Fixable Aqua Dead Cell Stain Kit, according to the manufacturer's instructions (Invitrogen by Life Technologies, Carlsbad, CA). To assess the levels of regulatory T cells, intracellular staining for Foxp3-PerCP5.5 was performed using the classic protocol and reagents from eBioscience (San Diego, CA). Acquisition was done on a FACS Canto III (Becton Dickinson, Mountain View, CA) and analyzed using FlowJo software (Tree Star, Ashland, OR).

Statistical analysis

Statistical analysis was performed with Prism 5 software (GraphPad Software, San Diego, CA). Unpaired *t* tests, one-way ANOVA with the Tukey post hoc test, and two-way ANOVA tests with the Bonferroni post hoc test were used to make comparisons. The *p* values < 0.05 were considered statistically significant.

Results

Protocol 1

Phosphorylated S6. After 10 treatments with i.n. HDM (Fig. 1A), Western blot analysis for P-S6 was performed on lung homogenates from saline controls, HDM, and HDM + rapamycin-treated mice to verify that the dose of rapamycin was sufficient to inhibit signaling downstream of mTOR. Mice exposed to HDM had a 2-fold increase in P-S6 levels compared with saline controls (Fig. 2). Rapamycin treatment blocked this increase.

AHR and goblet cell metaplasia. To determine whether rapamycin treatment reduced HDM-induced AHR, methacholine challenges were performed using a flexiVent system. Increases in AHR were

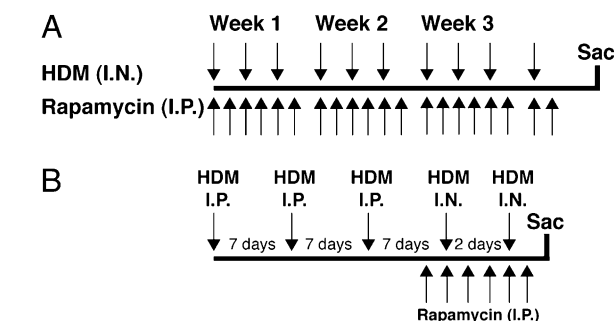


FIGURE 1. Study protocols. *A*, Protocol 1: mice were exposed to 10 i.n. HDM doses over 24 d. Rapamycin treatment by i.p. injection was started simultaneously with HDM exposure and continued 6 d/wk until 1 d after the last HDM exposure, for a total of 20 injections. *B*, Protocol 2: mice were sensitized to HDM by 3 i.p. injections before i.n. challenge with saline or HDM. Rapamycin treatment was started 6 d after the last i.p. injection and 1 d prior to i.n. HDM exposure.

observed in HDM-exposed mice at 25 mg/ml (4.4-fold) and 50 mg/ml (3.6-fold) methacholine compared with saline controls (Fig. 3A). AHR in HDM-exposed mice treated with rapamycin was similar to saline controls. Muc5AC immunostaining was performed to assess changes in goblet cells. Increased Muc5AC staining was observed in HDM-exposed mice (Fig. 3B) compared with saline controls. Increases in another goblet cell protein, CLCA3, were observed by Western blot in HDM-exposed mice (Fig. 3C). Rapamycin treatment reduced Muc5AC staining in the lungs and decreased CLCA3 protein levels by 80%.

Inflammatory cells and allergic sensitization. To determine the effects of rapamycin on allergic airway inflammation, total and differential inflammatory BALF cell counts were performed. Total BALF cell count increases with HDM exposure were prevented by rapamycin (data not shown). Specifically, increases in macrophages, neutrophils, and eosinophils were all prevented by rapamycin treatment (Fig. 4A). The percentage of macrophages, neutrophils, and eosinophils also changed with HDM exposure; however, there was not a significant change in the percentage of neutrophils and eosinophils after rapamycin treatment (Fig. 4A). To assess allergic sensitization, HDM-specific IgG1 and IgE levels

were measured in serum, and both were elevated in HDM-exposed mice (Fig. 4B). Rapamycin prevented these increases, so that HDM-specific IgG1 and IgE levels were similar to saline controls. Rapamycin also blocked increases in total IgG1 and IgE levels after HDM exposure (Supplemental Fig. 1).

Protocol 2

IgG1 and IgE. Data from protocol 1 demonstrated that administration of rapamycin simultaneously with HDM exposure blocked allergic sensitization and disease; however, the protective effect of rapamycin on these processes may be due to rapamycin preventing allergic sensitization. In protocol 2, allergic sensitization was first established by i.p. injection of HDM. Rapamycin treatment was initiated 6 d after the last i.p. injection of HDM and 1 d prior to i.n. HDM challenge (Fig. 1B). Control animals for these experiments were injected i.p. with either saline or HDM and then treated with i.n. saline (saline/saline or HDM/saline, respectively). HDM/saline mice showed increases in HDM-specific IgE and IgG1 compared with saline/saline controls (Fig. 5A, Supplemental Fig. 2). HDM/HDM mice had higher levels of HDM-specific IgE (7-fold) and IgG1 (1.25-fold) compared with HDM/saline control mice. In HDM/HDM+Rapa mice, HDM-specific IgE and IgG1 levels were similar to HDM/saline controls. Total serum IgG1 and IgE were also increased after HDM sensitization (Supplemental Fig. 2). Rapamycin attenuated the increase in total IgE but not total IgG1.

AHR and goblet cells. AHR was increased in HDM/HDM mice at 25 mg/ml (4.5-fold) and 50 mg/ml (5.3-fold) methacholine compared with HDM/saline controls (Fig. 5B). In HDM/HDM+Rapa mice, AHR was similar to both control groups (saline/saline, HDM/saline). To assess goblet cells, CLCA3 protein levels were measured by Western blot analysis. CLCA3 protein was not detectable in HDM/saline control mice, but it was readily detectable in lung homogenates from HDM/HDM mice. CLCA3 protein was reduced by 41% in HDM/HDM+Rapa mice compared with HDM/HDM mice (Fig. 5C). To further assess the goblet cell response, quantitative real-time RT-PCR was performed on lung RNA for the expression of Muc5AC. Muc5AC mRNA levels were normalized to β -actin mRNA. Muc5AC mRNA expression was increased 32-fold in HDM/HDM mice compared with saline/saline and HDM/saline controls. Rapamycin reduced Muc5AC mRNA expression by 84% (Fig. 5D). Abundant goblet cells were also detected in the airways of HDM/HDM mice by Muc5AC immunostaining, whereas no staining was detectable in HDM/saline controls (Fig. 5E). Muc5AC staining for goblet cells was reduced in HDM/HDM+Rapa mice.

Inflammatory cell counts. Total BALF macrophages, neutrophils, and eosinophils (Fig. 6A), as well as the percentage of eosinophils (Fig. 6B), were increased in HDM/HDM mice compared with HDM/saline controls and remained elevated in HDM/HDM+Rapa mice (Fig. 6).

T cells, cytokines, and leukotrienes. To examine the cellular and molecular mediators of the allergic response, T cells, cytokines, and leukotriene levels were assessed. The total number of CD69⁺ Foxp3⁺ CD4⁺ T cells was increased (1.7-fold) in the lungs of HDM/HDM mice compared with HDM/saline controls (Fig. 7A, Supplemental Fig. 3). CD69⁺ Foxp3⁺ T cell numbers were reduced in HDM/HDM+Rapa mice compared with HDM/HDM mice and were similar to HDM/saline controls. The total number of Foxp3⁺ CD25⁺ T regulatory cells in the lungs of HDM/HDM mice was increased (1.6-fold) compared with HDM/saline controls. Foxp3⁺ CD25⁺ T regulatory cell numbers in HDM/HDM+Rapa mice were reduced compared with HDM/HDM mice and were similar to HDM/saline controls (Fig. 7B, Supplemental Fig. 3). The ratio of Foxp3⁺ CD25⁺ T regulatory cells/CD25⁺ Foxp3⁺ T cells was lower

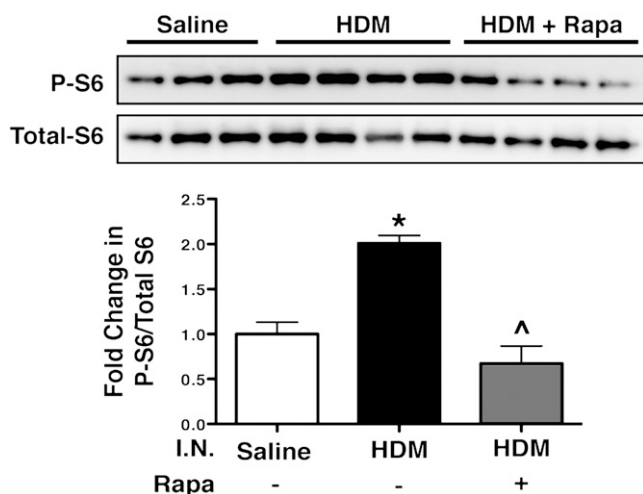


FIGURE 2. P-S6 levels corrected to total S6 levels in lung homogenates of mice exposed to i.n. HDM and treated with rapamycin. Western blot analysis of P-S6 protein, a downstream mediator of mTOR signaling, was increased after HDM exposure compared with saline controls. Rapamycin (Rapa) prevented this increase in P-S6 ($n = 3$ or 4 mice/group). * $p < 0.05$, versus saline; ^ $p < 0.05$, versus HDM.

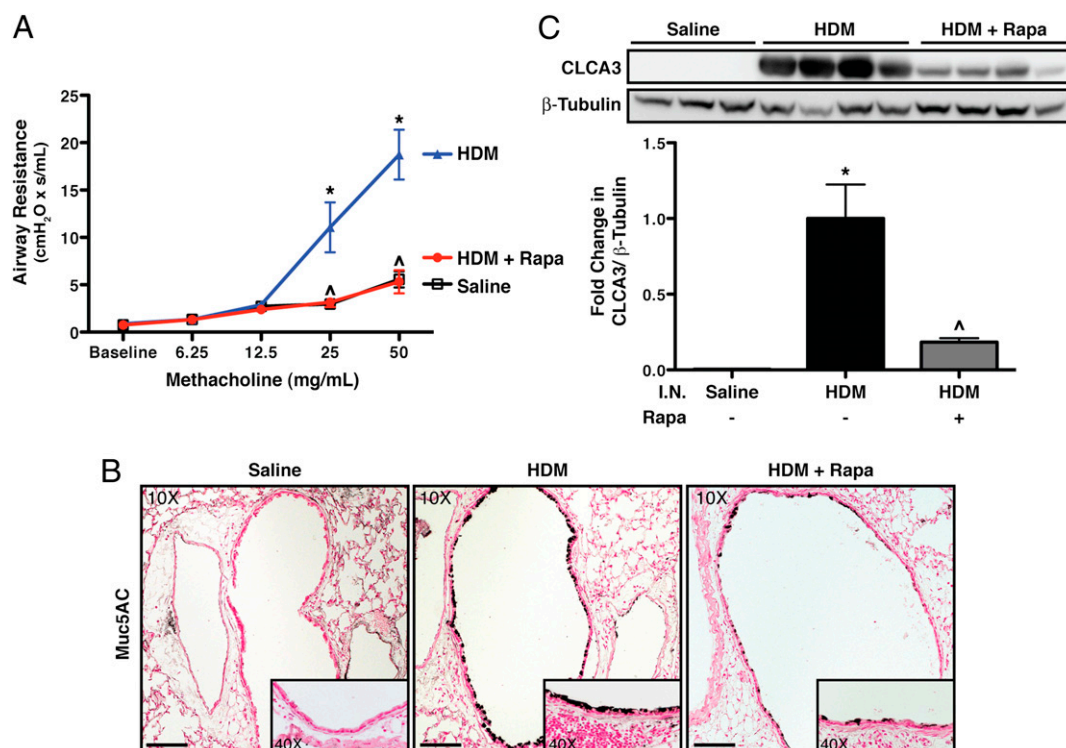


FIGURE 3. AHR and goblet cells in mice exposed to i.n. HDM and rapamycin (Rapa). **A**, Rapamycin prevented increases in airway resistance to methacholine in HDM-exposed mice ($n = 7$ or 8 mice/group in two independent experiments). **B**, Rapamycin attenuated increases in Muc5AC immunostaining in airways after HDM exposure. Scale bars, $100\ \mu\text{m}$. **C**, Rapamycin attenuated increases in the goblet cell protein, CLCA3, by Western blot in HDM-exposed mice. $n = 3$ to 4 mice per group. * $p < 0.05$, versus saline; ^ $p < 0.05$, versus HDM.

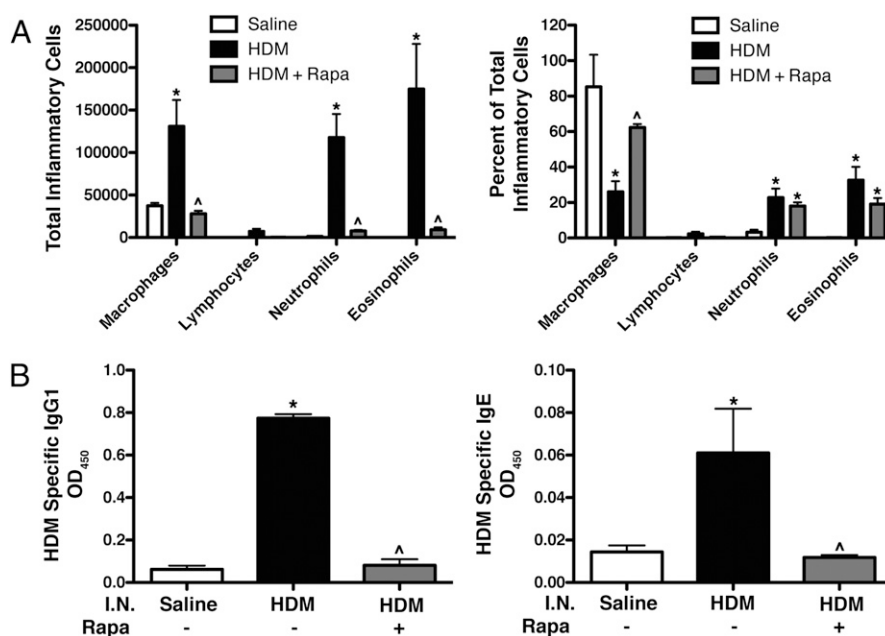
in HDM/HDM and HDM/HDM+Rapa mice compared with HDM/saline controls (Fig. 7C). IL-4 levels were undetectable in the BALF of HDM/saline controls, but they were readily detectable in HDM/HDM mice (Fig. 8A). IL-4 levels were similar in HDM/HDM+Rapa mice compared with HDM/HDM mice. IL-13 levels were also undetectable in HDM/saline control mice, but were readily detectable in HDM/HDM mice (Fig. 8A). IL-13 was not detectable in rapamycin-treated mice (HDM/HDM+Rapa). IL-5 and eotaxin 1 levels were increased in HDM/HDM mice compared with HDM/saline controls (Fig. 8B). Rapamycin treatment

decreased IL-5; however, eotaxin 1 levels remained elevated in HDM/HDM+Rapa mice. Cysteinyl leukotriene levels were increased in BALF from HDM/HDM mice compared with HDM/saline control mice (Fig. 8C). Leukotriene levels in HDM/HDM+Rapa mice were similar to HDM/saline controls.

Discussion

The goal of our study was to determine whether the mTOR inhibitor, rapamycin, would suppress key characteristics and mediators in a clinically relevant asthma model induced by the aeroallergen HDM.

FIGURE 4. Inflammatory cell counts in BALF and HDM-specific IgG1 and IgE levels in serum. **A**, HDM exposure induced increases in total inflammatory cells and the percentage of neutrophils and eosinophils compared with saline controls. Rapamycin prevented HDM-induced increases in total inflammatory cell counts in the BALF ($n = 6$ mice/group). **B**, HDM-specific IgG1 and IgE levels were increased after HDM exposure compared with saline controls and blocked by rapamycin ($n = 9$ mice/group). * $p < 0.05$, versus saline; ^ $p < 0.05$, versus HDM.



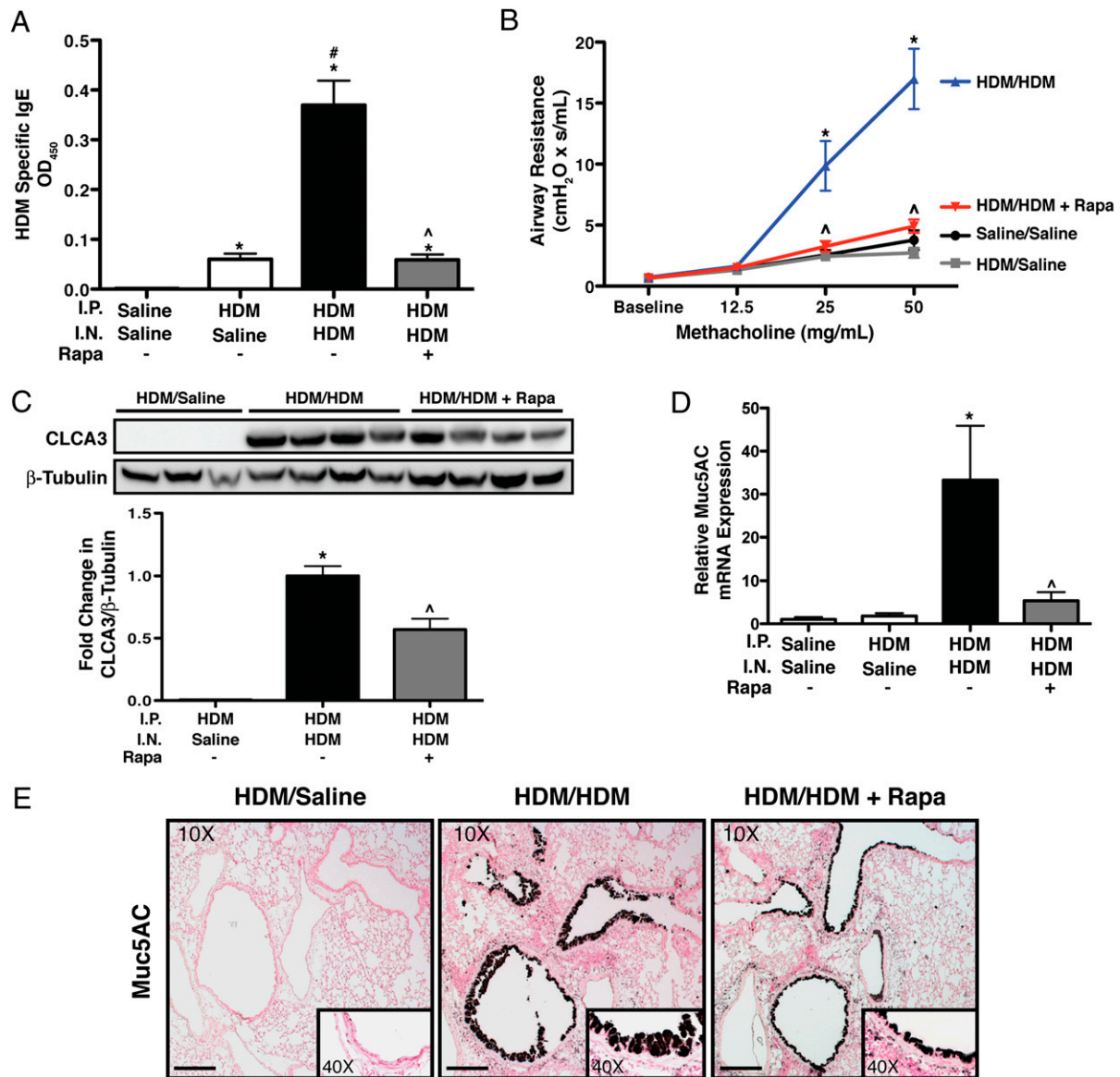


FIGURE 5. IgE, AHR, and goblet cells in sensitized mice exposed to HDM and rapamycin. **A**, Rapamycin attenuated HDM-induced IgE ($n = 4$ – 11 mice/group). * $p < 0.05$, versus saline/saline; # $p < 0.05$, versus HDM/saline; ^ $p < 0.05$, versus HDM/HDM. **B**, Rapamycin blocked HDM-induced increases in AHR after methacholine challenge ($n = 3$ – 10 mice/group in two independent experiments). * $p < 0.05$, versus saline/saline, HDM/saline; ^ $p < 0.05$, versus HDM/HDM. **C**, Western blot analysis shows that rapamycin attenuated HDM-induced increases in CLCA3 ($n = 3$ – 4 mice/group). * $p < 0.05$, versus HDM/saline; ^ $p < 0.05$, versus HDM/HDM. **D**, Quantitative real-time PCR analysis revealed decreased Muc5AC mRNA expression after HDM challenge in lungs of rapamycin-treated mice. Gene expression was normalized to β -actin ($n = 3$ – 5 mice/group). Samples were run in triplicate. * $p < 0.05$, versus HDM/saline; ^ $p < 0.05$, versus HDM/HDM. **E**, Rapamycin reduced Muc5AC immunostaining in airways of HDM/HDM mice. Scale bars, $100 \mu\text{m}$.

We used two protocols to investigate the effects of rapamycin on allergic responses to HDM, and although they cannot be directly compared, each protocol was designed to address specific questions. In the first protocol, we administered rapamycin to mice simultaneously with i.n. HDM to determine whether rapamycin could prevent HDM-induced allergic responses. Increases in AHR and inflammation were prevented by rapamycin treatment. In addition, increases in HDM-specific IgG1 and IgE levels were prevented by rapamycin, indicating that simultaneous treatment with rapamycin prevented allergic sensitization. Therefore, it was unclear whether inhibition of AHR and inflammatory cell influx into the lungs was due to direct effects on these processes or because allergic sensitization was prevented. Hence, we used a second protocol to address the question of whether rapamycin could suppress pulmonary responses to allergen exposure after allergic sensitization had been established. In this protocol, we sensitized mice to HDM (by i.p.

injections) before exposing the lung to the allergen and treating with rapamycin. In these sensitized mice, AHR, inflammatory cell influx (especially eosinophils), and IgE increased dramatically with i.n. HDM exposure. Rapamycin treatment, given after sensitization and just prior to HDM exposure, prevented these exacerbations, including the large increase in AHR and IgE. To explore potential mechanisms, Th2 cytokines and cysteinyl leukotrienes in BALF were measured. Although IL-4 and eotaxin 1 levels were unaffected by rapamycin treatment, increases in IL-13 and leukotrienes with HDM exposure were completely suppressed. Decreases in IL-13 and leukotriene levels, both important mediators of AHR and goblet cell metaplasia, provide insight into a potential mechanism for the effects of rapamycin on allergic airway disease. The suppressive effects of rapamycin on allergen-induced IL-13 and leukotriene levels, to our knowledge, have not been previously reported in studies using allergic asthma models.

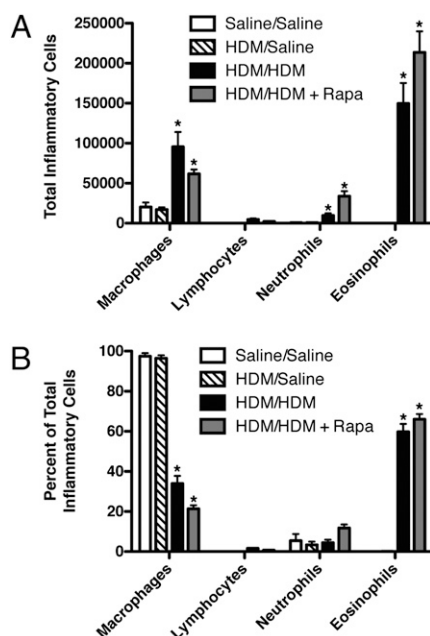


FIGURE 6. Inflammatory cells in BALF from sensitized mice exposed to HDM and rapamycin. **A**, Total numbers of macrophages, neutrophils, and eosinophils were increased with HDM and unaltered by rapamycin treatment ($n = 3-11$ mice/group in two independent experiments). **B**, The percentage of eosinophils was increased after HDM challenge and unaffected by rapamycin treatment ($n = 3-11$ mice/group in two independent experiments). * $p < 0.05$, versus saline/saline, HDM/saline.

Previous studies used SAR 943, a derivative of rapamycin, and rapamycin in the OVA-induced allergic asthma model. In Brown-Norway rats sensitized to OVA, SAR 943 given prior to OVA challenge to the lung reduced the number of CD4⁺ T cells recruited into the lung; however, AHR and eosinophil infiltration were unaffected (16). A similar study in mice showed attenuation of eosinophils, IL-4, goblet cells, and AHR responses after SAR 943 treatment (17) but no effect on serum IgE levels. In a third study, mice sensitized with OVA and treated with rapamycin showed reductions in IgE levels; however, no change in eosinophils or AHR was observed (18). Hence, these OVA studies reported mixed effects of mTOR inhibitors on allergic responses and, as mentioned earlier, did not assess IL-13 or leukotrienes.

Prior studies demonstrated an important role for inflammation in the development of AHR, with increased inflammatory cells typically correlating with increases in AHR (23). However, in our second protocol, we observed a marked disconnect between inflammatory cell influx and AHR. Despite no effect on inflammatory cell influx, rapamycin blocked the increase in AHR following HDM challenge to the lung. This dissociation between inflammatory cells and AHR has been observed previously. For example, in patients with allergic asthma, the number of inflammatory cells in the lung did not correlate with the degree of AHR (23, 24). In our second protocol, eotaxin 1 levels were increased in HDM/HDM mice but unaffected by rapamycin treatment, which is consistent with the lack of a change in eosinophils levels after rapamycin treatment, despite decreases in IL-5. This maintenance of elevated eosinophils is in contrast to the suppressive effects of rapamycin treatment on AHR, although there are conflicting reports on the role of eosinophils in AHR (25–28).

Another interesting observation in our study was that rapamycin treatment did not affect IL-4 levels in BALF, despite a major reduction in IL-13 levels. It is unclear which cell types are responsible for the differences in IL-4 and IL-13 levels that we

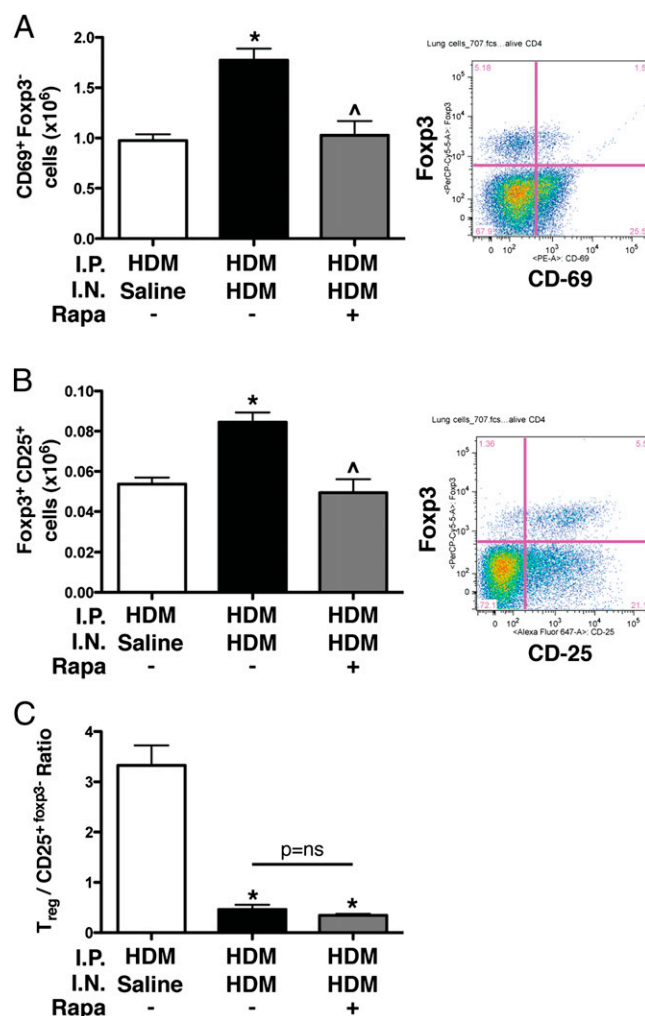


FIGURE 7. Activated T cells and regulatory T cells in lungs of sensitized mice exposed to HDM and rapamycin. **A**, Increases in the total number of CD69⁺Foxp3⁺ T cells with HDM was attenuated by rapamycin ($n = 3-11$ mice/group in two independent experiments). * $p < 0.05$, versus HDM/saline; [^] $p < 0.05$, versus HDM/HDM. **B**, The total number of Foxp3⁺CD25⁺ regulatory T cells was increased after HDM exposure and reduced to the level of HDM/saline control mice with rapamycin treatment ($n = 3-11$ mice/group in two independent experiments). * $p < 0.05$, versus HDM/saline; [^] $p < 0.05$, versus HDM/HDM. **C**, The ratio of Foxp3⁺CD25⁺ regulatory T cells/CD25⁺Foxp3⁺ T cells was lower in HDM/HDM and HDM/HDM+Rapa mice compared with HDM/saline controls ($n = 3-11$ mice/group in two independent experiments). * $p < 0.05$, versus HDM/saline.

observed, because a number of cell types are known to secrete these cytokines (29). Although mast cells can produce both IL-4 and IL-13, it was reported that rapamycin did not affect degranulation and cytokine release from mouse mast cells (30). It is unlikely that eosinophils are the primary source of IL-4 and IL-13 in our model, because only IL-13 levels are affected by rapamycin, whereas no change in IL-4 and eosinophil numbers was observed. In addition, although both mast cells and eosinophils can release Th2 cytokines, the primary source of cytokines like IL-4 and IL-13 is most likely Th2 lymphocytes (29). Interestingly, it was reported that when T cells were costimulated under Th2-skewing conditions and treated with rapamycin, IL-13 levels were more severely decreased, and only a modest decrease in IL-4 levels was observed (31). It is possible that other cellular sources are contributing to sustained levels of IL-4 in our model, and future studies will address this. Interestingly, despite no changes in IL-4, which is known to be an important mediator of the IgE response, the large

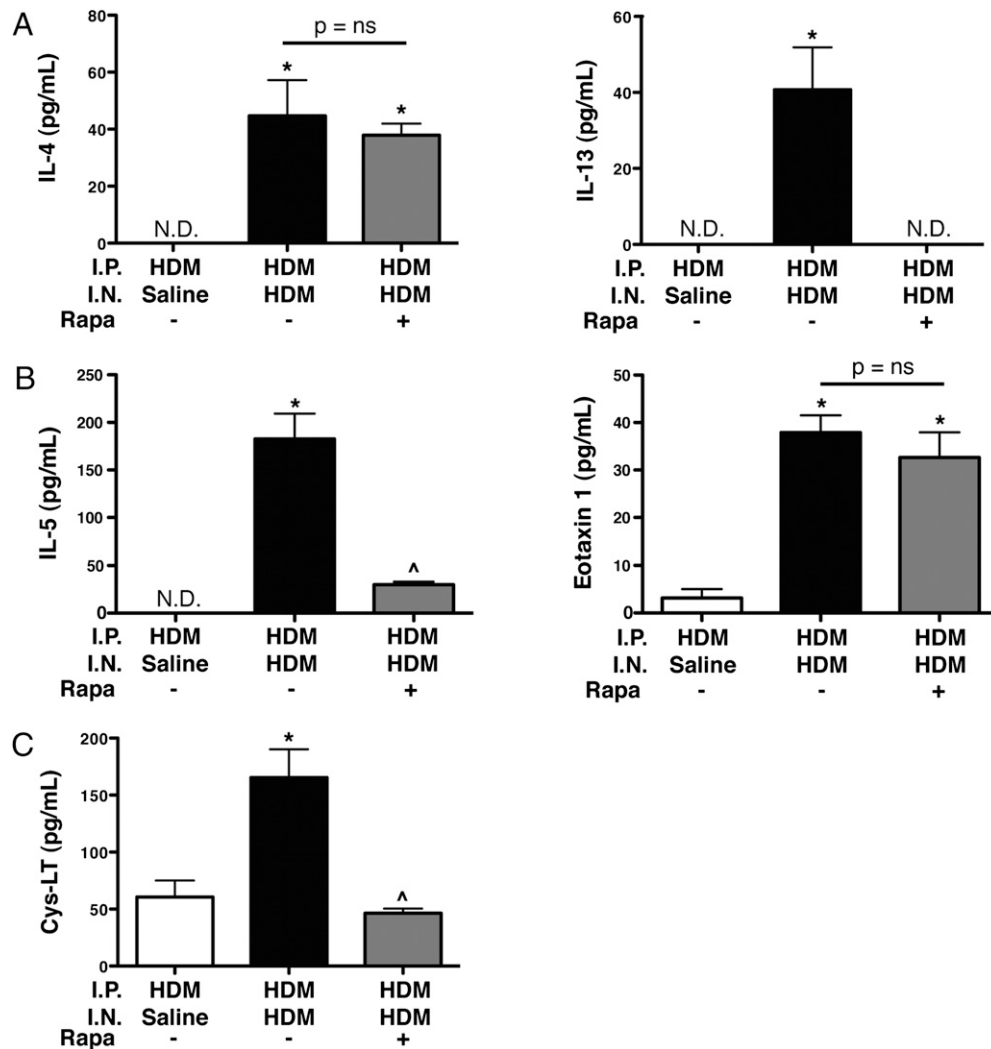


FIGURE 8. Cytokines and cysteinyl leukotrienes levels in BALF of sensitized mice exposed to HDM and rapamycin. **A**, Rapamycin blocked HDM-induced increases in IL-13, but it had no effect on IL-4 levels ($n = 4-12$ mice/group). * $p < 0.05$, versus HDM/saline; $^{\wedge}p < 0.05$, versus HDM/HDM. **B**, IL-5 and eotaxin 1 levels were increased in HDM/HDM mice. Rapamycin reduced IL-5 levels in the BALF, but it had no effect on eotaxin 1 levels ($n = 4-12$ mice/group). * $p < 0.05$, versus HDM/saline; $^{\wedge}p < 0.05$, versus HDM/HDM. **C**, Rapamycin blocked HDM-induced increases in cysteinyl leukotrienes (C_4 , D_4 , E_4) ($n = 5-10$ mice/group). * $p < 0.05$, versus HDM/saline; $^{\wedge}p < 0.05$, versus HDM/HDM.

increase in IgE detected after allergen challenge was blocked by rapamycin. In addition, AHR was still completely suppressed by rapamycin, suggesting that reductions in IL-13 and/or leukotrienes were responsible, because these are known to play important roles in allergic asthma (32–36). However, it was also reported that rapamycin can inhibit contractile proteins in airway smooth muscle cells (37). This raises the possibility that reductions in AHR in our second protocol could be due to direct effects of rapamycin on airway smooth muscle contractility.

The importance of mTOR in regulating immune responses is becoming increasingly apparent. Currently, rapamycin is used in patients after organ transplant as an immunosuppressant drug (12, 14). Early studies demonstrated a role for mTOR in regulating T cell proliferation, especially after cytokine stimulation (38). More recent studies showed that T cells deficient in mTOR fail to become Th1, Th2, or Th17 effector cells under skewing conditions and instead default to Foxp3⁺ regulatory T cells (39). These data and others highlight the importance of mTOR in T cell differentiation (39, 40). Similar to other reports (41, 42), we demonstrated reductions in activated T cells in the lung with rapamycin treatment. However, in contrast to in vitro studies (39, 43, 44), a decrease in the total

number of T regulatory cells with rapamycin treatment was seen in our in vivo study. This may be due to the fact that total T cell numbers were reduced by rapamycin treatment. Although T regulatory cell numbers were decreased with rapamycin, they did not fall below those seen in HDM/saline controls. In addition, although the total number of T regulatory cells was decreased with rapamycin, the ratio of T regulatory cells/conventional T cells was not different compared with HDM/HDM mice.

Asthma is a complex disease that manifests differently among individual patients. Currently, inhaled glucocorticosteroids are the most effective anti-inflammatory therapies for the treatment of asthma (1), although some patients are refractory to them. Omalizumab, a relatively new therapy, is being used in the treatment of asthma and targets IgE. It was shown to be an effective treatment for allergic asthma, as assessed by decreased asthma exacerbations and hospitalizations in patients receiving this therapy (45, 46). In our studies, rapamycin treatment suppressed or attenuated key characteristics of the asthmatic response, including AHR, IgE, and goblet cell metaplasia, as well as important mediators, including IL-13 and leukotrienes. Additional studies are necessary to elucidate the potential contribution of each of these mediators to the effects of

rapamycin on the allergic response and AHR. Understanding these mechanisms may provide further insight into the role of mTOR in allergic disease.

Acknowledgments

We thank Patricia Pastura for technical assistance in these studies and Dr. Satish Madala for critical review of the manuscript.

Disclosures

The authors have no financial conflicts of interest.

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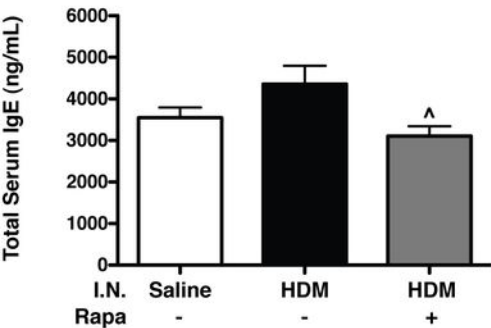
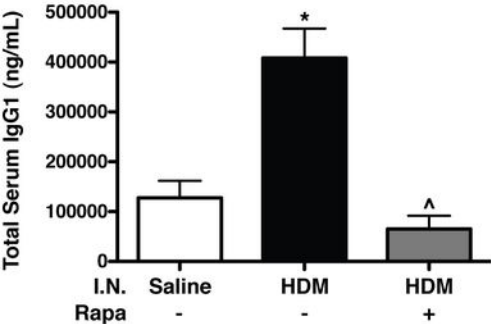
SUPPLEMENTAL MATERIALS

SUPPLEMENTAL FIGURE LEGENDS

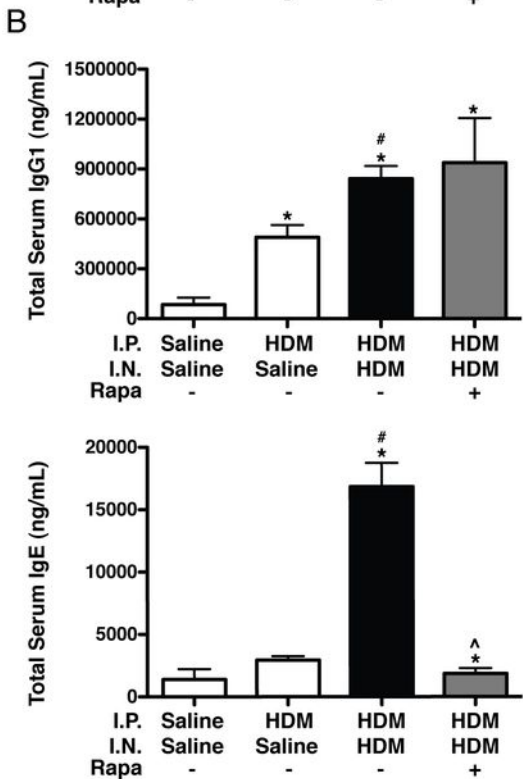
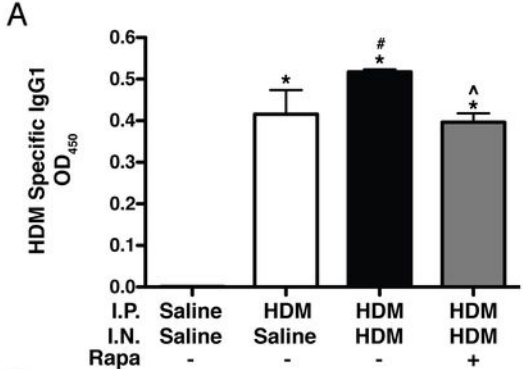
SUPPLEMENTAL FIGURE S1: Protocol 1- Total IgG1 and IgE in serum of mice exposed to HDM and rapamycin. Total levels of IgG1 were increased after HDM exposure and blocked by rapamycin with levels similar to saline controls. Total IgE levels were reduced in HDM exposed mice treated with rapamycin compared to HDM alone mice. $n = 9$ mice per group. * $p < 0.05$ vs. Saline, ^ $p < 0.05$ vs. HDM.

SUPPLEMENTAL FIGURE S2: Protocol 2- Allergic sensitization in mice sensitized with HDM. *A:* HDM-specific IgG1 levels were increased in HDM/Saline mice and further increased in HDM/HDM mice. Rapamycin blocked this response. $n = 4-11$ mice per group. *B:* Total IgG1 and IgE levels were increased in HDM/HDM mice. Rapamycin blocked increases in total IgE, but had no effect on total IgG1. $n = 4-11$ mice per group. * $p < 0.05$ vs. Saline/Saline, ^ $p < 0.05$ vs. HDM/HDM, # $p < 0.05$ vs. HDM/Saline.

SUPPLEMENTAL FIGURE S3: Protocol 2- Activated T cells and regulatory T cells in lungs of sensitized mice exposed to HDM and rapamycin. FACS analysis showing increases in CD69⁺Foxp3⁻ T cells in HDM/HDM mice. Rapamycin attenuated the response (top panels). Labeling of Foxp3⁺CD25⁺ regulatory T cells was similar between HDM/HDM and HDM/HDM+Rapa mice (bottom panels).



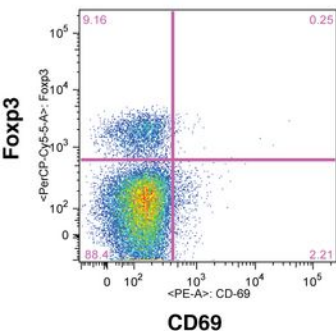
Supplemental Figure S1



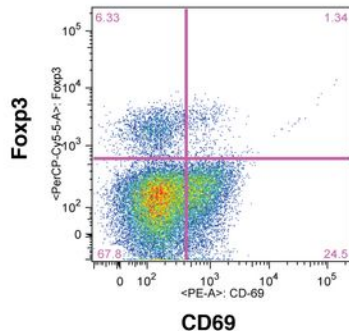
Supplemental Figure S2

HDM/Saline**HDM/HDM****HDM/HDM + Rapa**

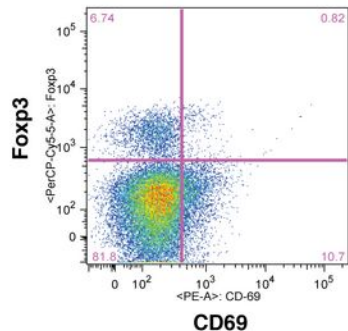
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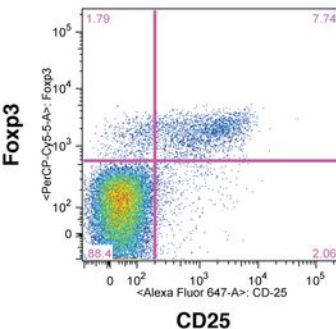
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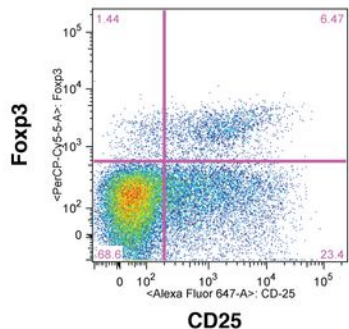
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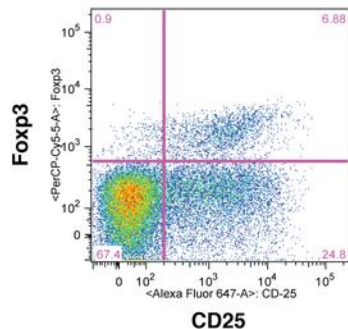
Lung cells_696.fcs...alive CD4



Lung cells_708.fcs...alive CD4



Lung cells_715.fcs...alive CD4



Supplemental Figure S3