Paradoxical Effects of Rapamycin on Experimental House Dust Mite-Induced Asthma

Karin Fredriksson1, Jill A. Fielhaber4*, Jonathan K. Lam1*, Xianglan Yao1, Katharina S. Meyer1, Karen J. Keeran2, Gayle J. Zywicke2, Xuan Qu3, Zu-Xi Yu3, Joel Moss1, Arnold S. Kristof4, Stewart J. Levine4*

1 Cardiovascular and Pulmonary Branch, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, Maryland, United States of America, 2 Laboratory of Animal Medicine and Surgery, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, Maryland, United States of America, 3 Pathology Core Facility, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, Maryland, United States of America, 4 Critical Care and Respiratory Divisions and Meakins-Christie Laboratories, Department of Medicine, McGill University Health Centre, Montreal, Quebec, Canada

Abstract

The mammalian target of rapamycin (mTOR) modulates immune responses and cellular proliferation. The objective of this study was to assess whether inhibition of mTOR with rapamycin modifies disease severity in two experimental murine models of house dust mite (HDM)-induced asthma. In an induction model, rapamycin was administered to BALB/c mice coincident with nasal HDM challenges for 3 weeks. In a treatment model, nasal HDM challenges were performed for 6 weeks and rapamycin treatment was administered during weeks 4 through 6. In the induction model, rapamycin significantly attenuated airway inflammation, airway hyperreactivity (AHR) and goblet cell hyperplasia. In contrast, treatment of established HDM-induced asthma with rapamycin exacerbated AHR and airway inflammation, whereas goblet cell hyperplasia was not modified. Phosphorylation of the S6 ribosomal protein, which is downstream of mTORC1, was increased after 3 weeks, but not 6 weeks of HDM-challenge. Rapamycin reduced S6 phosphorylation in HDM-challenged mice in both the induction and treatment models. Thus, the paradoxical effects of rapamycin on asthma severity paralleled the activation of mTOR signaling. Lastly, mediastinal lymph node re-stimulation experiments showed that treatment of rapamycin-naive T cells with ex vivo rapamycin decreased antigen-specific Th2 cytokine production, whereas prior exposure to in vivo rapamycin rendered T cells refractory to the suppressive effects of ex vivo rapamycin. We conclude that rapamycin had paradoxical effects on the pathogenesis of experimental HDM-induced asthma. Thus, consistent with the context-dependent effects of rapamycin on inflammation, the timing of mTOR inhibition may be an important determinant of efficacy and toxicity in HDM-induced asthma.

Introduction

Rapamycin (Sirolimus, Rapamune®) is a macrolide product of Streptomyces hygroscopicus that was initially discovered in a soil sample from Easter Island (Rapa Nui) in the early 1970s [1]. Rapamycin inhibits signaling by targeting the mammalian target of rapamycin (mTOR) with resultant immunosuppression and inhibition of cellular proliferation. Rapamycin is in clinical use for the prevention of kidney transplant rejection and has also been investigated as a treatment for tuberous sclerosis and lymphangioleiomyomatosis [2]. Similarly, rapamycin-eluting coronary artery stents have been developed to prevent restenosis [3,4]. Rapamycin has also been proposed as a candidate treatment for asthma. Consistent with this, the ability of rapamycin-derivatives to inhibit asthma has been investigated in experimental animal models. The rapamycin derivative, 32-deoxorapamycin (SAR 943), was shown to be as effective as corticosteroids in inhibiting eosinophilic and lymphocytic airway inflammation, Th2 cytokine production, epithelial cell proliferation, goblet cell hyperplasia, and airway hyperreactivity in a murine model of ovalbumin (OVA)-induced asthma [5]. In contrast, intratracheal administration of 32-deoxorapamycin prior to a single OVA challenge in sensitized Brown-Norway rats, did not inhibit the number of bronchoalveolar lavage fluid eosinophils, lymphocytes or neutrophils, nor did it suppress airway hyperreactivity [6]. In another Brown-Norway rat...
model, repeated oral administration of 32-deoxyrapamycin to sensitized animals that had already begun to receive multiple OVA challenges, inhibited airway smooth muscle and epithelial cell proliferation and reduced the number of CD4+ T cells, but did not inhibit airway hyperreactivity or pulmonary eosinophilia [7]. Thus, previous studies report conflicting results regarding the utility of mTOR inhibitors for the treatment of asthma.

Here, we sought to define the role of mTOR signaling on the pathogenic manifestations of asthma using a clinically relevant house dust mite (HDM)-induced model of murine disease. We selected HDM to induce airway disease because it is an important environmental allergen that has been identified as a risk factor for persistent asthma in human subjects [8,9]. HDM has a heterogeneous composition that includes multiple proteins and lipopolysaccharide, which induces airway inflammation via both allergic and non-allergic pathways [10,11,12]. These pathways include toll-like receptor 4 signaling in airway epithelial cells, which activates both innate and adaptive immune responses [13]. We show that inhibition of mTOR signaling by rapamycin has paradoxical effects on the manifestations of HDM-induced asthma. Inhibition of mTOR signaling with rapamycin prior to the induction of asthma effectively suppressed airway inflammation, goblet cell hyperplasia and airway hyperreactivity, whereas inhibition of mTOR signaling in established asthma exacerbated airway inflammation and airway hyperreactivity, but did not modify HDM-induced increases in goblet cell hyperplasia.

Methods

Murine Models of Experimental House Dust Mite-induced Asthma

Female Balb/c mice were purchased from Jackson Laboratories (Bar Harbor, Maine). Asthma was induced by daily intranasal administration of HDM (Dermatophagoides pteronyssinus) (25 µg) or saline in a volume of 12 µl for 5 days each week [8]. In the induction model, mice received rapamycin (3 mg/kg) or vehicle (Phosal 50 PG®), by gavage daily, 5 days per week for 3 weeks concurrent with administration of nasal HDM. In the treatment model, mice received daily nasal HDM for 6 weeks and were treated with rapamycin (3 mg/kg) or vehicle (Phosal 50 PG®), by gavage daily, 5 days per week, during weeks 4 through 6. HDM was purchased from Greer Laboratories (Lenoir, NC), while the rapamycin oral solution was from Wyeth (Philadelphia, PA) and Phosal 50 PG® was from the American Lecithin Company (Oxford, CT). All experimental protocols (Protocols H-0210 and H-0244) were approved by the Animal Care and Use Committee of the National Heart, Lung, and Blood Institute. Results are representative of two independent experiments for each model.

Bronchoalveolar Lavage and Lung Histopathologic Examination

Bronchoalveolar lavage (BAL) was performed utilizing three instillations of ice cold PBS (0.5 ml). Red blood cells present in BAL fluid (BALF) were lysed using ACK buffer (2 min at 4°C), followed by re-suspension of cells in 0.3 ml RPMI-1640 with 20% fetal bovine serum. The total number of BALF cells were counted using a hemocytometer. The differential cell counts were performed on Diff-Quick-stained cytospin slides (Siemens Healthcare Diagnostics, Deerfield, IL). For histopathological examination, lungs were inflated to a pressure of 25 cm of H₂O and fixed in 10% formalin for 24 h. Lungs were then dehydrated through gradient ethanol prior to embedding in paraffin. Sagittal sections were cut at a thickness of 5 µm and stained with hematoxylin and eosin or periodic acid Schiff (PAS). Representative histology images were selected by one of the authors who was blinded to the identity of the groups. Quantification of goblet cell hyperplasia was performed as previously described [14]. Briefly, all the airways present (large (conducting), medium (central), and small (distal)) within representative lung sections were analyzed and the number of airways containing PAS-positive cells were recorded. Goblet cell hyperplasia is presented as the percentage of airways containing PAS-positive cells. The number of airways inspected in each animal is also presented.

Quantitative RT-PCR

Lungs were minced prior to storage in RNAlater® (Applied Biosystems Inc., Foster City, CA) at −70°C. Total RNA was subsequently isolated using the lipid tissue kit from Qiagen (Qiagen Inc, Valencia, CA) and on-column DNase treatment was performed using RNase-Free DNase from Qiagen. Reverse transcription was performed utilizing random hexamer primers and High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). PCR was performed utilizing the TaqMan Universal PCR Master Mix and the following FAM dye-labeled Taqman® MGB probes; IL-4: Mm00445259_m1, IL-10: Mm00439614_m1, IL-13: Mm00434204_m1, IL-17a: Mm00439618_m1, Muc5Ac: Mm01267559_m1, Ccl11: Mm00449859_m1, CCL7: Mm00443113_m1, Ccl11: Mm00441239_m1, CCL17: Mm00516136_m1, CCL24: Mm00444701_m1 and 18S: Hs99999901_s1. One µg of cDNA was used as a template. and samples were amplified utilizing the 7500 Real Time PCR System running Sequence Detector version 2.1 software (ABI systems, Foster City, CA). Gene expression was quantified relative to the expression of 18S mRNA using the control sample as calibrator to calculate the difference in Ct values (ΔΔCt) and results are presented as relative mRNA expression.

HDM-specific Re-stimulation of Mediastinal Lymph Node Cultures and Measurement of Th2 Cytokine Production

Mediastinal lymph nodes were removed, disrupted by gentle pressure with a syringe plunger and passed through a 100 µm strainer to yield single cell suspensions [15]. Following lysis of red blood cells with ACK buffer, mediastinal lymph node cells were suspended in RPMI 1640 medium containing 10% fetal calf serum, penicillin (50 units/ml), streptomycin (50 µg/ml), and L-glutamine (2 mM) and 4 x 10⁵ cells per well were cultured in 96-well plates with “U”-shaped bottoms. Cells were stimulated with or without HDM (100 µg/ml) and with or without rapamycin (10 nM) (Sigma-Aldrich, St. Louis, MO). After 96 hours, medium was collected and analyzed for cytokines by sandwich ELISAs with a limit of sensitivity of 15.6 pg/ml for IL-4 and IL-17A, 31.25 pg/ml for IL-5 and 62.5 pg/ml for IL-13 (R & D Systems, Minneapolis, MN).

Measurement of Plasma IgE

Total plasma IgE was measured using an OptEIA™ kit (BD Biosciences Pharmingen, San Diego, CA). Total plasma IgG1 was measured using an ELISA Quantitation Set from Bethyl Laboratories, Inc. (Montgomery, TX), whereas total plasma IgG2a was measured using an ELISA Set from BD Biosciences Pharmingen (San Diego, CA).

Detection and Quantification of Protein Levels

Whole lungs were removed en bloc, snap frozen in liquid nitrogen, and stored at −80°C prior to protein extraction. Lungs were mechanically disrupted using a Brickman mechanical homogenizer in homogenization buffer (20 mM Tris pH 8.0,
0.5% Nonidet P-10, 1 mM phenylmethanesulphonilfluoride, 50 mM NaF, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 100 μM sodium orthovanadate). Homogenates were snap frozen on dry ice, thawed, and cleared by centrifugation at 16,000 xg for 30 min at 4°C. Supernatants were assayed for protein content by Bradford assay. Lung proteins (80 μg) were separated by SDS-PAGE and transferred to nitrocellulose membrane before immunohorserd conjugated antibodies and developed using Super-Signal West Pico chemiluminescence detection kit (Pierce). Antibodies that react with S6, phospho-S6, Akt, phospho-Akt, STAT6 and phospho-STAT6 were from Cell Signaling Technology, Inc. (Danvers, MA), whereas the antibody that reacted with β-actin was from Sigma-Aldrich (St. Louis, MO).

Measurement of Airway Hyperreactivity
Mice were anesthetized with ketamine and xylazine and a 19 gauge beveled metal catheter was inserted into the trachea. Mice were mechanically ventilated with a tidal volume of 0.2 ml at 2 Hz, while PBS or increasing doses of methacholine (0, 2.5, 5, 7.5 and 10 mg/ml) were administered by nebulization. Airway resistance was directly measured utilizing an Elan RC Fine Pointe system (Buxco Research Systems, Wilmington, N.C.). Airway resistance was recorded at 10 s intervals for 3 min and average values are presented as cm H20/ml/s.

Statistics
Results are presented as mean ± SEM. A one-way ANOVA with Bonferroni’s multiple comparison test was utilized for all analyses except for airway hyperreactivity experiments, which instead utilized a two-way ANOVA with a Bonferroni post-test test. A P value less than 0.05 was considered significant. Statistical analyses were performed with GraphPad Prism version 5.0a (Graphpad Software, Inc., La Jolla, CA).

Results
Paradoxical Effects of Rapamycin on Airway Inflammation in Murine Models of House Dust Mite-induced Asthma
To induce airway disease, Balb/c mice received daily nasal administration of HDM (25 μg) for 5 days per week. In the induction model, mice received rapamycin or vehicle coincident with the initiation of HDM administration for 3 weeks. In the treatment model, HDM challenges were performed for 6 weeks, whereas treatment with rapamycin or vehicle was administered during weeks 4 through 6 (Figure S1). In the induction model (Figure 1A), mice that had received rapamycin had a significant decrease in the total number of bronchoalveolar lavage fluid (BALF) inflammatory cells, specifically in the number of BALF eosinophils and lymphocytes. In contrast, in the treatment model (Figure 1B), rapamycin significantly increased the total number of BALF inflammatory cells, specifically in the number of eosinophils, lymphocytes, neutrophils and macrophages. As shown in Figure 2, the magnitude of peri-bronchial inflammatory cell infiltrates reflected the opposite effects of rapamycin on the number of BALF inflammatory cells in the induction and treatment models.

The pulmonary expression of Th2 and Th17 cytokines was assessed to investigate further the mechanisms mediating the differential effects of rapamycin in the induction and treatment models of HDM-induced asthma. As shown in Figure 3, administration of rapamycin prior to the induction of asthma significantly inhibited lung mRNA levels of canonical Th2 (IL-4 and IL-13) and Th17 (IL17A) cytokines. Treatment of established asthma with rapamycin, however, did not significantly alter the HDM-induced increases in lung mRNA levels of IL-4, IL-13 and IL-17A.

The effect of inhibition of mTOR on the expression of lung chemokines was also assessed. As shown in Figure 4, administration of rapamycin in the induction model significantly reduced the lung mRNA levels of key chemokines that mediate the chemotaxis of eosinophils and T cells to asthmatic lungs via binding to CCR3, including CCL11 (eotaxin-1), CCL24 (eotaxin-2) and CCL7 (MCP-3) [16,17,18]. Rapamycin treatment of established asthma, however, increased mRNA levels of CCL11 and did not affect the expression of CCL7 or CCL24. Rapamycin did not alter mRNA levels of CCL17 (TARC) in either the induction or treatment models of HDM-induced asthma.

These data demonstrate that administration of rapamycin prior to nasal HDM administration inhibits the induction of airway inflammation via a mechanism that involves the reduced expression of Th2- and Th17-type cytokines, as well as C-C chemokines. Conversely, treatment of established asthma with rapamycin increased both the number of BALF inflammatory cells, as well as lung mRNA levels of the C-C chemokine, CCL11.

Paradoxical Effects of Rapamycin on Th2 T Cell Responses
To investigate further the paradoxical effects of rapamycin on HDM-mediated airway inflammation, we used the induction model to assess the effects of ex vivo rapamycin on antigen-specific Th2 cytokine production by T cells isolated from the draining mediastinal lymph nodes of mice that had previously been sensitized to HDM in vivo with or without rapamycin administered by gavage. HDM re-stimulation of mediastinal lymph node cells recovered from mice that had been challenged with HDM for 3 weeks without in vivo rapamycin treatment showed a significant reduction in the production of the Th2 cytokines, IL-4, IL-5 and IL-13 (Figure 5). This result is consistent with a requirement for mTOR in Th2 cytokine production by HDM-sensitized T cells. Levels of the Th17 cytokine, IL-17A, were below the limit of detection of the assay. In contrast, HDM re-stimulation of mediastinal lymph node cells recovered from HDM-challenged mice that had been treated with rapamycin both in vivo and ex vivo showed no reduction in the production of IL-4, IL-5 or IL-13 as compared to cells that were only treated with rapamycin in vivo. Taken together, this shows that treatment of rapamycin-naïve T cells with rapamycin decreases the antigen-specific production of Th2 cytokines, whereas prior exposure of T cells to HDM and rapamycin for 3 weeks in vivo makes them refractory to the suppressive effects of ex vivo rapamycin treatment on HDM-mediated Th2 cytokine production.

Rapamycin has Divergent Effects on IgE Production in HDM-induced Asthma Disease
Experiments were performed to assess whether rapamycin also has paradoxical effects on plasma IgE production. As shown in Figure 6, administration of rapamycin prior to the induction of HDM-induced asthma markedly attenuated plasma IgE, IgG1 and IgG2a levels, whereas treatment of established asthma with rapamycin did not significantly modify plasma levels of IgE, IgG1 or IgG2a. These results suggest that inhibition of mTOR signaling with rapamycin inhibited sensitization to HDM allergens in the induction model, but did not reduce production of IgE, IgG1 or IgG2a once HDM sensitization had already occurred.
Effect of Rapamycin on HDM-induced Goblet Cell Hyperplasia

In the induction model, rapamycin administration was associated with small, but significant, decreases in the number of airways demonstrating goblet cell hyperplasia and Clca3 (chloride channel calcium activated 3) mRNA levels, whereas mRNA levels for Muc5AC (mucin 5, subtypes A and C) were not modified (Figure 7). In contrast, rapamycin did not modify goblet cell hyperplasia or mRNA levels of Muc5AC or Clca3 in the treatment model.

Paradoxical Effect of Rapamycin on HDM-induced Airway Hyperreactivity

The effect of rapamycin on HDM-induced airway hyperreactivity (AHR) was also assessed. As shown in Figure 8, administration of rapamycin prior to the induction of HDM-induced asthma resulted in a small, but significant decrease in AHR, whereas treatment of established HDM-induced airway disease with rapamycin was associated with a small, but significant increase in AHR.

Temporal Association between mTOR Signaling and the Paradoxical Effects of Rapamycin in Induction and Treatment Models of HDM-induced Asthma

Western blots of lung proteins were performed to identify the mechanism by which rapamycin mediates paradoxical effects on HDM-induced asthma. After 3 weeks in the induction model, phosphorylation of the S6 ribosomal protein was increased in the lungs of HDM-challenged mice as compared to saline-challenged mice (Figure 9). In contrast, after 6 weeks in the treatment model, S6 phosphorylation was no longer increased in HDM-challenged mice when compared to saline-challenged mice. In both the induction and treatment models, rapamycin attenuated S6 phosphorylation in HDM-challenged mice as compared to mice that received the vehicle control, which demonstrates inhibition of mTORC1 signaling. In contrast, Akt phosphorylation at serine 473 was neither increased by HDM-challenge nor inhibited by rapamycin, indicating that rapamycin did not inhibit TORC2 signaling. Lastly, although STAT6 phosphorylation was increased in HDM-challenged mice in both the induction and treatment models, it was only inhibited by rapamycin in the induction model.
Rapamycin is a specific and potent inhibitor of mTOR, a highly conserved and ubiquitous serine-threonine kinase that nucleates two distinct multi-protein mTOR complexes [19]. mTOR complex 1 (mTORC1) controls nutrient-sensitive protein synthesis and cell growth primarily via the initiation of translation and ribosomal biogenesis, whereas mTORC2 controls cytokinesis and cell survival via distinct effector kinases [20,21]. Novel adaptor proteins define the functions of these mTOR complexes. mTORC1 contains the protein raptor and controls cell growth; mTORC2 contains the protein rictor and regulates cytoskeletal rearrangement. Rapamycin specifically blocks signaling via mTORC1 by binding to the immunophilin FK506-binding protein 1A (FKBP12), thereby disrupting the interaction between raptor and mTOR [1]. In contrast, mTORC2, which does not contain raptor, is thought to be resistant to direct inhibition by rapamycin. Multiple signaling pathways, such as antigen receptors, co-stimulatory molecules, growth factors, cytokines, hypoxia, cellular stress, low cellular energy levels and DNA damage regulate mTOR signaling. Activated mTORC1 phosphorylates at

**Figure 2.** Paradoxical Effect of Rapamycin on Lung Histology in Induction and Treatment Models of House Dust Mite-induced Asthma. Histologic sections of lung were stained with hematoxylin and eosin (H & E) or periodic acid-Schiff (PAS) stains and images obtained at 200× or 1000×. Results are representative of 2 independent experiments. doi:10.1371/journal.pone.0033984.g002

**Discussion**

Rapamycin is a specific and potent inhibitor of mTOR, a highly conserved and ubiquitous serine-threonine kinase that nucleates two distinct multi-protein mTOR complexes [19]. mTOR complex 1 (mTORC1) controls nutrient-sensitive protein synthesis and cell growth primarily via the initiation of translation and ribosomal biogenesis, whereas mTORC2 controls cytokinesis and cell survival via distinct effector kinases [20,21]. Novel adaptor proteins define the functions of these mTOR complexes. mTORC1 contains the protein raptor and controls cell growth;
least two targets, S6 kinase 1 (S6K1) and the eukaryotic translation initiation factor-binding protein 1 (EIF4EBP1). Phosphorylation activates S6K1 and inhibits EIF4EBP1, with resultant enhancement of mRNA translation and cellular growth by augmentation of the cellular translational apparatus.

Inhibition of mTOR signaling by rapamycin has immunosuppressive effects on antigen-presenting cells and T cells, which thereby modulate adaptive immune responses. For example, rapamycin inhibits IL-4-dependent dendritic cell (DC) maturation, fms-like tyrosine 3 kinase ligand (Flt3L)-induced DC mobilization, as well as co-stimulatory molecule expression, pro-inflammatory cytokine production and T-cell allostimulation by DCs [1,22]. Rapamycin attenuates DC-mediated antigen uptake and presentation via the inhibition of macropinocytosis and mannose receptor-mediated endocytosis [1,23]. mTOR signaling also plays an important role in modulating the differentiation of effector T cells. mTOR activation is necessary for the differentiation and proliferation of Th1, Th2, and Th17 effector T cells, whereas T cells lacking mTOR differentiate into FOXP3+ regulatory T cells, which mediate immunological tolerance [24,25]. Consistent with its therapeutic use as an immunosuppressant in organ transplant patients, rapamycin potently inhibits the expansion of T-cell populations [1]. Based upon its ability to inhibit the DC and effector T cell functions, rapamycin induces immune tolerance to solid organ transplants and is utilized clinically to prevent rejection in kidney transplantation [1]. mTOR inhibition also has effects on other immune cells that participate in the pathogenesis of asthma [1]. Rapamycin suppresses B cell responses and antibody production, inhibits neutrophil chemotaxis and prevents NKT cell proliferation.

mTOR signaling also plays an important role in airway smooth muscle and epithelial cell proliferation. mTORC1-related changes in the size, proliferation, and survival of smooth muscle or epithelial cells may contribute to hypertrophy and remodeling of the airway wall in asthmatics [26,27]. Rapamycin prevents airway myocyte differentiation into a contractile phenotype via blockade of the mTORC1/p70 S6 kinase pathway, which may reduce the intrinsic contractile properties of airway smooth muscle [28]. Rapamycin also inhibits TGF-β-induced pulmonary fibrotic responses, which could contribute to sub-epithelial fibrosis and airway remodeling [29]. Similarly, mTOR signaling may modulate angiogenesis and lymphangiogenesis, both of which play important roles in asthma pathogenesis [30,31]. Inhibition of mTOR blocks the synthesis of vascular endothelial growth factor (VEGF), an angiogenesis factor that induces an asthma phenotype in mice [32,33]. mTOR physically interacts with the transcription factor HIF-1α, which regulates the expression of multiple angiogenesis genes [34]. Furthermore, mechanical strain of airway

Figure 3. Paradoxical Effect of Rapamycin on Lung Th2 and Th17 Cytokine Expression in Induction and Treatment Models of House Dust Mite-induced Asthma. Quantification of lung mRNA levels for IL-4, IL-13, and IL-17A by qRT-PCR presented as relative mRNA expression. Results for the induction experiment are shown in Panel A (n = 6–8 animals per group, * P < 0.05, HDM + Vehicle vs. HDM + Rapamycin), while results for the treatment experiment are shown in Panel B (n = 6–10 animals per group, * P < 0.001). Results are representative of 2 independent experiments. doi:10.1371/journal.pone.0033984.g003
smooth muscle induces HIF-1α-dependent VEGF expression via mTOR and ERK pathways, which may thereby contribute to angiogenesis in asthma [32].

Based upon the important role of mTOR signaling in inflammatory and remodeling responses, we investigated whether rapamycin could be utilized to modify the pathogenic manifestations of asthma as well as provide new insights into disease pathogenesis. Here, we show that rapamycin has paradoxical effects depending on whether it is administered prior to the induction of HDM-induced asthma or as a treatment during the effector phase of HDM-induced asthma. Administration of rapamycin coincident with HDM exposure significantly attenuated eosinophilic and lymphocytic airway inflammation, which was mediated by the reduced expression of Th2- and Th17-type cytokines and C-C chemokines, as well as production of IgE, IgG1 and IgG2a. This is consistent with the role of mTOR in mediating the differentiation of CD4+ T cells into Th2 and Th17 subsets [24,35]. Rapamycin also reduced the induction of AHR and goblet cell hyperplasia.

In contrast, treatment of established HDM-induced asthma with rapamycin augmented airway inflammatory responses, as indicated by significant increases in the number of BALF eosinophils, lymphocytes and neutrophils. Similarly, treatment of established HDM-induced asthma with rapamycin worsened AHR and did not reduce goblet cell hyperplasia or IgE production. Taken together, these results demonstrate that inhibition of mTOR signaling by rapamycin represents a “molecular switch” with divergent effects on asthma pathogenesis that are dependent upon the temporal relationship between rapamycin administration and allergen sensitization. When administered concurrent with HDM, rapamycin blocked allergic sensitization and the induction of the key manifestations of asthma, but when...
administered in the setting of established asthma, rapamycin exacerbated disease severity as evidenced by enhanced airway inflammation and AHR.

The mechanism underlying the paradoxical effects of rapamycin in the induction and treatment models of HDM-induced asthma may in part have reflected the temporal association between HDM challenge and activation of mTOR signaling pathways. Activation of mTORC1 signaling was assessed by phosphorylation of its downstream target, the S6 ribosomal protein. S6 phosphorylation was increased after 3 weeks of HDM challenge in the induction model, but returned to levels similar to those of saline-challenged mice at 6 weeks in the treatment model. Consistent with this finding, inhibition of mTOR signaling with rapamycin attenuated HDM-induced asthma only in the induction model when mTOR signaling was up-regulated, but not in the treatment model when mTOR

Figure 6. Paradoxical Effect of Rapamycin on Plasma Immunoglobulin Levels in House Dust Mite-induced Asthma. Plasma levels of IgE, IgG1 and IgG2a were quantified. Results for the induction experiment are shown in Panels A, C and E, while results for the treatment experiment are shown in Panels B, D and F (n = 8–20 animals per group, * P < 0.05 vs. Saline+Vehicle, ** P < 0.001). doi:10.1371/journal.pone.0033984.g006
signaling was no longer increased. Changes in STAT6 phosphorylation paralleled the effects of rapamycin on HDM-induced asthma. Furthermore, Akt phosphorylation was neither upregulated by HDM challenge nor inhibited by rapamycin, which

Figure 7. Paradoxical Effect of Rapamycin on Goblet Cell Hyperplasia in House Dust Mite-induced Asthma. Quantification of lung mRNA levels for Muc5AC and Clca3 by qRT-PCR are presented as relative mRNA expression. Results for the induction experiment are shown in Panel A (n = 6 animals per group, * P<0.01), while the results for the treatment experiment are shown in Panel B (n = 5–6 animals per group, P = NS). Results are representative of 2 independent experiments. Goblet cell hyperplasia is presented as the percentage of airways containing PAS-positive cells (n = 8–10 animals per group, * P<0.001). 35.3±0.6 airways were inspected in each mouse.

doi:10.1371/journal.pone.0033984.g007

Figure 8. Paradoxical Effect of Rapamycin on Airway Hyperreactivity in House Dust Mite-induced Asthma. Airway resistance (cm H2O/ml/s) was directly measured following administration of increasing doses of nebulized methacholine. Results for the induction experiment are shown in Panel A (n = 10 animals per group, * P<0.05), while results form the treatment experiment are shown in Panel B (n = 9–10 animals per group, * P<0.05). Results are representative of 2 independent experiments.

doi:10.1371/journal.pone.0033984.g008
is consistent with the conclusion that mTORC2 signaling did not modulate HDM-induced asthma in this model.

We also found that rapamycin had paradoxical effects on Th2 cytokine production by T cells. The ex vivo treatment of mediastinal lymph node cells from HDM-challenged, rapamycin-naïve mice with rapamycin attenuated the production of Th2 cytokines, which is consistent with the ability of rapamycin to inhibit the induction of allergen-mediated Th2-type inflammatory responses by HDM-sensitized T cells. In contrast, ex vivo rapamycin treatment of mediastinal lymph node cells from HDM-challenged mice that had already been exposed in vivo to rapamycin did not suppress Th2 cytokine production. This shows that prior rapamycin treatment renders mediastinal T cells refractory to the suppressive effects of rapamycin on Th2 cytokine production. Thus, these data are additional evidence supporting the context-dependent effects of mTOR inhibition on Th2 cytokine production.

Our findings are consistent with prior reports that showed paradoxical pro-inflammatory effects of rapamycin. For example, although rapamycin has immunosuppressive effects and is utilized clinically to prevent kidney transplant rejection, its use has been complicated by the development of a lymphocytic interstitial pneumonitis [36]. Glomerulonephritis and anemia secondary to complications by the development of a lymphocytic interstitial pneumonitis [36]. Glomerulonephritis and anemia secondary to clinically complicated by the development of a lymphocytic interstitial pneumonitis [36]. Glomerulonephritis and anemia secondary to immunosuppressive effects and is utilized clinically to prevent kidney transplant rejection, its use has been complicated by the development of a lymphocytic interstitial pneumonitis [36]. Glomerulonephritis and anemia secondary to complications by the development of a lymphocytic interstitial pneumonitis [36]. Glomerulonephritis and anemia secondary to clinically complicated by the development of a lymphocytic interstitial pneumonitis [36]. Glomerulonephritis and anemia secondary to complications by the development of a lymphocytic interstitial pneumonitis.

Acknowledgments

We are extremely appreciative of the staff of the NHLBI Laboratory of Animal Medicine and Surgery, whose commitment, professional advice and excellent technical support made this study possible. We are also very appreciative of Filipina Giacometti, B.Sc. in the Pathology Core Facility, NHLBI for her assistance with the histopathological analyses. We are most appreciative of Dr. Martha Vaughan for her helpful discussions and advice.

Author Contributions

Conceived and designed the experiments: KF JAF JKL XY JM ASK SJL. Performed the experiments: KF JAF JKL KSM KJJ GJJ XQ ZY ASK SJL. Analyzed the data: KF JAF JKL XY ZY JM ASK SJL. Contributed reagents/materials/analysis tools: KF JAF JKL XY ZY JM ASK SJL. Wrote the paper: KF JKL ASK SJL.

References

1. Thomson AW, Turnquist HR, Raimondi G (2009) Immunoregulatory functions of mTOR inhibition. Nat Rev Immunol 9: 324–337.
2. Bisler JJ, McCormack FX, Young LR, Elwing JM, Chuck G, et al. (2008) Sirolimus for angiomyolipoma in tuberous sclerosis complex or lymphangioleiomyomatosis. N Engl J Med 358: 140–151.
3. Serruys PW, Kutryk MJ, Ong AT (2006) Coronary-artery stents. N Engl J Med 354: 483–495.
4. Halloran FP (2004) Immunosuppressive drugs for kidney transplantation. N Engl J Med 351: 2715–2729.
5. Fujita T, Trifilieff A (2003) In vivo and in vitro effects of SAR 943, a rapamycin analogue, on airway inflammation and remodeling. Am J Respir Crit Care Med 167: 193–198.
6. Huang TJ, Eynott P, Salmon M, Nicklin PL, Chung FK (2002) Effect of topical immunomodulators on acute allergic inflammation and bronchial hyperresponsiveness in sensitised rats. Eur J Pharmacol 437: 187–194.
7. Eynott P, Salmon M, Huang TJ, Oates T, Nicklin PL, et al. (2003) Effects of cyclosporin A and a rapamycin derivative (SAR4968) on chronic allergic inflammation in sensitized rats. Immunology 109: 461–467.
8. Johnson JR, Wiley RE, Fatouh R, Swinski FK, Gajewskia BU, et al. (2004) Continuous exposure to house dust mite elicits chronic airway inflammation and structural remodeling. Am J Respir Crit Care Med 169: 378–383.
9. Sears MR, Greene JM, Willian AR, Wieczek EM, Taylor DR, et al. (2003) A longitudinal, population-based, cohort study of childhood asthma followed to adulthood. N Engl J Med 349: 1414–1422.
25. Hom JT, Raedmouck K, Dekkak A, Collins M, Wong S, et al. (2009) HDM induces direct airway inflammation in vivo: implications for future disease therapy? Eur Respir J 35: 1377–1387.

24. Delgoffe GM, Kole TP, Zheng Y, Zarek PE, Matthews KL, et al. (2009) The Wnt signaling pathway is a potential therapeutic target for asthma. Int Arch Allergy Immunol 129: 1–18.

23. Hackstein H, Taner T, Logar AJ, Thomson AW (2002) Rapamycin inhibits IL-4-induced dendritic cell maturation in vitro and type 17 responses. American journal of respiratory and critical care medicine 166: 1228–1238.

22. Hackstein H, Taner T, Zahorchak AF, Morelli AE, Logar AJ, et al. (2003) Rapamycin inhibits IL-4 signaling is critical for house dust mite-specific helper T cell type 2 and type 17 responses. American journal of respiratory and critical care medicine 174: 883–893.

21. Laplante M, Sabatini DM (2009) mTOR signaling at a glance. J Cell Sci 122: 3585–3594.

20. Walsh ER, Sahu N, Kearley J, Benjamin E, Kang BH, et al. (2000) Strain-specific requirement for eosinophils in the recruitment of T cells to the lung during the development of allergic asthma. J Exp Med 288: 1295–1292.

19. Ying S, Robinson DS, Meng Q, Barata LT, McEwen AR, et al. (1999) C-C chemokines in allograft-induced acute-phase cutaneous responses in atomic subjects: association of rotavirus with early t-cell responses, and of rotavirus-2 and monocye chemoattractant protein-4 with the later 24-hour tissue eosinophilia, and relationship to basophils and other C-C chemokines (monocyte chemoattractant protein-3 and RANTES). J Immunol 163: 3976–3984.

18. Barnes PJ (2008) The cytokine network in asthma and chronic obstructive pulmonary disease. J Clin Invest 118: 3546–3556.

17. Jacinto E, Hall MN (2003) Tor signalling in bugs, brain and brawn. Nat Rev Mol Cell Biol 4: 117–126.

16. Phipps S, Lam CE, Kaiko GE, Foo SY, Collison A, et al. (2009) Toll/IL-1 receptor-associated protein (TIRAP) expression promotes dendritic cell mobilization and function in vivo. Blood 101: 4457–4463.

15. Weichhart T, Costantino G, Poglitsch M, Rosner M, Zeyda M, et al. (2008) The B7-H1/p75 tumor necrosis factor receptor, a suppressor of mTOR signaling, is an essential mediator of cigarette smoke-mediated sensitization and inflammation in the lung. Nat Med 10: 1095–1103.

14. Yoshida T, Mett I, Bhunia AK, Bowman J, Perez M, et al. (2010) Rtp801, a LDL Receptor-mediated Pathway. Am J Respir Crit Care Med 182: 2035–2043.

13. Thaunat O, Beaumont C, Chatenoud L, Lechaton S, Mamzer-Bruneel MF, et al. (2006) rapamycin-associated post-transplantation glomerulonephritis and its remission. Transplantation 80: 1212–1219.

12. Schmitz F, Heit A, Dreher S, Eisenacher K, Mages J, et al. (2008) Mammalian target of rapamycin increases STAT1 nuclear content and transcriptional activity in alpha4- and protein phosphatase 2A-dependent fashion. J Biol Chem 283: 24141–24153.

11. Zhou L, Goldsmith AM, Bendley JK, Jia Y, Rodriguez ML, et al. (2005) 4E-binding protein phosphorylation and eukaryotic initiation factor-4E release are required for airway smooth muscle hypertrophy. Am J Respir Cell Mol Biol 33: 195–202.

10. De Alba J, Raedmouck K, Dekkak A, Collins M, Wong S, et al. (2009) HDM induces direct airway inflammation in vivo: implications for future disease therapy? Eur Respir J 35: 1377–1387.

9. Lambrecht BN, Hammad H (2009) Biology of lung dendritic cells at the origin of asthma. Immunity 31: 412–424.

8. Weichhart T, Costantino G, Poglitsch M, Rosner M, Zeyda M, et al. (2009) The B7-H1/p75 tumor necrosis factor receptor, a suppressor of mTOR signaling, is an essential mediator of cigarette smoke-mediated sensitization and inflammation in the lung. Nat Med 10: 1095–1103.

7. Hammad H, Chieppa M, Perros F, Willart MA, Germain RN, et al. (2009) C-C chemokines in allograft-induced acute-phase cutaneous responses in atomic subjects: association of rotavirus with early t-cell responses, and of rotavirus-2 and monocye chemoattractant protein-4 with the later 24-hour tissue eosinophilia, and relationship to basophils and other C-C chemokines (monocyte chemoattractant protein-3 and RANTES). J Immunol 163: 3976–3984.

6. Delgoffe GM, Kole TP, Zheng Y, Zarek PE, Matthews KL, et al. (2009) The Wnt signaling pathway is a potential therapeutic target for asthma. Int Arch Allergy Immunol 129: 1–18.

5. Hackstein H, Taner T, Logar AJ, Thomson AW (2002) Rapamycin inhibits IL-4-induced dendritic cell maturation in vitro and type 17 responses. American journal of respiratory and critical care medicine 166: 1228–1238.

4. Phipps S, Lam CE, Kaiko GE, Foo SY, Collison A, et al. (2009) Toll/IL-1 receptor-associated protein (TIRAP) expression promotes dendritic cell mobilization and function in vivo. Blood 101: 4457–4463.

3. Hackstein H, Taner T, Logar AJ, Thomson AW (2002) Rapamycin inhibits macrophocytosis and mannose receptor-mediated endocytosis by bone marrow-derived dendritic cells. Blood 100: 1084–1087.

2. Delgoffe GM, Kole TP, Zheng Y, Zarek PE, Matthews KL, et al. (2009) The mTOR kinase differentially regulates effector and regulatory T cell lineage commitment. Immunity 30: 832–844.

1. Hackstein H, Taner T, Zahorchak AF, Morelli AE, Logar AJ, et al. (2003) Rapamycin inhibits IL-4-induced dendritic cell maturation in vitro and dendritic cell mobilization and function in vivo. Blood 101: 4457–4463.

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27. Zhou L, Goldsmith AM, Bendley JK, Jia Y, Rodriguez ML, et al. (2005) 4E-binding protein phosphorylation and eukaryotic initiation factor-4E release are required for airway smooth muscle hypertrophy. Am J Respir Cell Mol Biol 33: 195–202.

26. Goniocarova EA, Goncharov DA, Esserhas A, Hunter DS, Glassberg MK, et al. (2002) Tuberin regulates p70 S6 kinase activation and ribosomal protein S6 phosphorylation. A role for the TSC2 tumor suppressor gene in pulmonary lymphangioleiomyomatosis (LAM). J Biol Chem 277: 30958–30967.

25. Hom JT, Raedmouck K, Dekkak A, Collins M, Wong S, et al. (2009) HDM induces direct airway inflammation in vivo: implications for future disease therapy? Eur Respir J 35: 1377–1387.

24. Delgoffe GM, Kole TP, Zheng Y, Zarek PE, Matthews KL, et al. (2009) The Wnt signaling pathway is a potential therapeutic target for asthma. Int Arch Allergy Immunol 129: 1–18.

23. Hackstein H, Taner T, Logar AJ, Thomson AW (2002) Rapamycin inhibits macrophocytosis and mannose receptor-mediated endocytosis by bone marrow-derived dendritic cells. Blood 100: 1084–1087.

22. Delgoffe GM, Kole TP, Zheng Y, Zarek PE, Matthews KL, et al. (2009) The mTOR kinase differentially regulates effector and regulatory T cell lineage commitment. Immunity 30: 832–844.