

Preventive and therapeutic effects of rapamycin, a mammalian target of rapamycin inhibitor, on food allergy in mice

K. Yamaki & S. Yoshino

Department of Pharmacology, Kobe Pharmaceutical University, Kobe, Hyogo, Japan

To cite this article: Yamaki K, Yoshino S. Preventive and therapeutic effects of rapamycin, a mammalian target of rapamycin inhibitor, on food allergy in mice. *Allergy* 2012; DOI: 10.1111/all.12000.

Keywords

anaphylaxis; food allergy; mammalian target of rapamycin; mast cells; rapamycin.

Correspondence

Kouya Yamaki, Department of Pharmacology, Kobe Pharmaceutical University, 4-19-1 Motoyamakita-machi, Kobe, Hyogo 658-8558, Japan.
Tel.: +81 78 441 7577
Fax: +81 78 441 7577
E-mail: yam@kobepharm-u.ac.jp

Accepted for publication 11 July 2012

DOI:10.1111/all.12000

Edited by: Hans-Uwe Simon

Abstract

Background: Because few curative treatments are available for food allergy, we investigated the therapeutic potential of rapamycin, a mammalian target of rapamycin (mTOR) inhibitor, on mouse food allergy.

Methods: The preventive and therapeutic effects of oral rapamycin on anaphylactic symptoms induced by oral ovalbumin (OVA) challenge in food allergy mice were investigated. Mast cell functions in response to rapamycin were also measured in the passive systemic anaphylaxis model and bone marrow-derived mast cells (BMMCs).

Results: Daily rapamycin from the first challenge (preventive protocol) attenuated food allergy symptoms including diarrhea, anaphylactic reactions, and hypothermia in mice. The treatment decreased the challenge-induced increases in mouse mast cell protease-1 in serum and mast cell numbers in the intestine. Notably, the mice that already showed food allergy symptoms by previous challenges recovered from the disease with daily administration of rapamycin (therapeutic protocol). Anti-OVA IgG1 and IgE levels in serum, as well as IFN- γ , IL-4, IL-13, IL-9, IL-10, and IL-17 secretion from splenocytes, were decreased by the treatments. In contrast, a single dose of rapamycin failed to affect passive systemic anaphylaxis. Spontaneous and IL-9-dependent survival and IgE-induced IL-13 secretion, but not degranulation, of BMMCs were reduced by rapamycin.

Conclusion: Our data show that mouse food allergy was attenuated by rapamycin through an immunosuppressive effect and inhibition of intestinal mast cell hyperplasia. Inhibition of the IL-9 production–mast cell survival axis is one of the mechanisms of the therapeutic effect of rapamycin. Rapamycin and other mTOR inhibitors might be good candidates for therapeutic drugs for food allergy.

Five percent or more of infants worldwide are estimated to suffer from food allergy (FA) (1, 2). In FA patients, ingestion of food allergens causes gastrointestinal (diarrhea, vomiting), cutaneous (urticaria), bronchopulmonary (dyspnea), cardiovascular (hypotension) symptoms and, in severe case, lethal

anaphylactic shock (3, 4). Increases in the prevalence of FA among UK children from 1989 to 2002 (5) remind us that FA is a serious public health concern. Although quick treatment of the acute symptoms, such as the injection of epinephrine, saves many lives (6), casual treatment and prophylactic treatment should be established to suppress the anaphylactic symptoms and to improve the life of the allergic patients.

Animal and clinical studies have revealed that sensitization to the allergens and subsequent mast cell activation by cross-linking of Fc ϵ RI with IgE and food antigen are critical for the induction of FA (7–9). Many intracellular signaling molecules are considered to contribute to the

Abbreviations

BMMC, bone marrow-derived mast cell; CTMC, connective tissue mast cell; FA, food allergy; IMC, intestinal mast cell; MMC, mucosal mast cell; MMCP, mouse mast cell protease; mTOR, mammalian target of rapamycin; OVA, ovalbumin; PSA, passive systemic anaphylaxis; Tr, regulatory T-cell type.

sensitization stage and mast cell-dependent anaphylaxis in response to stimulation. Thus, molecular-targeted drugs affecting the activities of these molecules involved in immune responses and mast cell activation might be effective for FA. However, although various studies have investigated the efficacy of molecular-targeted drugs on FA, little is known about which intracellular signaling molecules are involved in FA (10, 11).

Among the signaling pathways, the c-kit-PI3K-Akt-mammalian target of rapamycin (mTOR) signaling cascade is important for proliferation/survival and function of mast cells *in vitro* (12) as well as for immune responses (13). In accordance with these reports, we reported that the c-kit inhibitor sunitinib inhibits FA in mice via attenuation of intestinal mast cell (IMC) activation and hyperplasia as well as via the suppression of immune responses against food allergen (14). To expand information in this field, we investigated the therapeutic effect of rapamycin (Rapamune®; Pfizer Inc., New York, NY, USA), a mTOR inhibitor used as an immunosuppressant to prevent transplant rejection in clinic (15), on FA in mice and the role of mTOR in the development of FA. In addition, the effects of rapamycin on IgE-dependent passive systemic anaphylaxis (PSA) *in vivo* were examined, together with degranulation, cytokine production, and survival by IL-9, an indispensable cytokine for IMC hyperplasia in the mouse FA model (16), of bone marrow-derived mast cells (BMMCs).

Methods

Animals

Male Balb/c mice (Japan SLC, Inc., Shizuoka, Japan) were maintained in a temperature-controlled environment with standard chow and water. All animal procedures were approved by the Animal Ethics Committee of Kobe Pharmaceutical University.

Reagents

Anti-ovalbumin (OVA) IgE (OE-3)-producing hybridoma was newly established by using Polyethylene Glycol 1500 (Roche Diagnostics GmbH, Mannheim, Germany). Rapamycin (LC Laboratories, Woburn, MA, USA) was dissolved in 0.3 ml of ethanol, and then the solution was dispersed in 2.7 ml of saline containing 0.1% TWEEN20 (Sigma-Aldrich, Co., St. Louis, MO, USA) and administered intragastrically (200 µl/mouse).

Experimental oral antigen-induced anaphylaxis

Mice were sensitized by the injection of 50 µg OVA i.p. (Sigma-Aldrich, Co.) in 100 µl of phosphate-buffered saline with 100 µl of Inject alum (Thermo Scientific, Rockford, IL, USA) at days 0 and 14 (Fig. 1A). To investigate the preventive effect, rapamycin (0.25 or 0.75 mg) was administered every day from days 27 to 45 (preventive protocol). The drug administration on the day before challenge was

carried out at 12 h or more before the challenge on the next day. On the challenge day, the drug was administered 2 h or more after the challenge. From day 28, mice were orally challenged with 50 mg OVA in saline every 3 days, a total of six times. Before each challenge, mice fasted for 3 h. Diarrhea and anaphylactic symptoms were scored by visually monitoring mice for 60 min after challenge as follows (14): diarrhea – 0, normal stools; 1, a few wet and unformed stools; 2, a number of wet and unformed stools with moderate perianal staining of the coat; 3, severe, watery stool with severe perianal staining of the coat – and anaphylactic symptoms – 0, no symptoms; 1, reduced activity, trembling of limbs; 2, loss of consciousness, no activity upon prodding; 3, convulsions, death. Rectal temperature and mouse mast cell protease (MMCP)-1 concentration in serum were determined as described previously (14). To investigate the therapeutic effect (Fig. 3A), rapamycin (0.75 mg) was administered every day from days 45 to 54 to mice already immunized and challenged six times (therapeutic protocol). On days 46, 49, and 52, the mice were orally challenged with 50 mg OVA.

PSA model

Passive systemic anaphylaxis in mice was induced as described previously (14) except for using 200 µg OE-3 for i.v. sensitization.

Measurement of cytokines from cultured spleen cells and anti-OVA antibodies in serum

On day 46 in the preventive protocol or on day 55 in the therapeutic protocol, serum and spleens were obtained. Splenocyte suspensions were cultured with 100 µg/ml OVA for 3 days (14). The cytokine concentration in the supernatants was quantified by ELISA kits for IFN-γ, IL-4, IL-13 (R&D Systems, Inc., Minneapolis, MN, USA), IL-9, IL-10, and IL-17A (eBioscience, Inc., San Diego, CA, USA). The levels of anti-OVA IgE, IgG1, and IgG2a in serum were measured as described previously (14).

Quantification of IMCs

Mast cell numbers in the jejunum were counted with chloroacetate esterase staining (14, 17).

Assay of degranulation, cytokine production, survival, and sub-G1 population of BMMCs

Bone marrow-derived mast cells were generated as described previously (14) and were sensitized with 200 ng/ml OE-3 for 24 h. After pretreatment with the indicated concentrations of rapamycin for 20 min, the cells were stimulated for 20 min with 0.5 µg/ml OVA with rapamycin to measure the release of β-hexosaminidase (14). OE-3-sensitized BMMCs were also cultured with medium containing OVA and rapamycin for 8 h. The IL-13 concentration in the cultured supernatant was determined by IL-13 ELISA

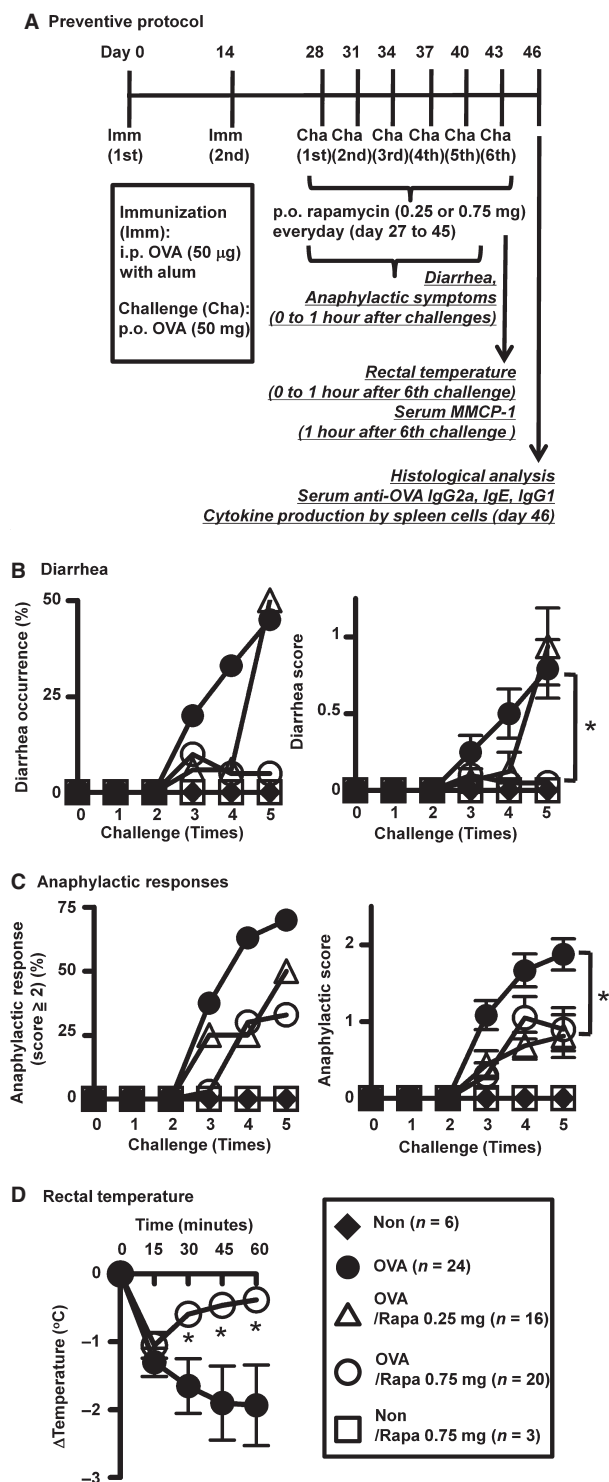


Figure 1 Preventive administration of rapamycin attenuated the symptoms of oral antigen-induced food allergy including allergic diarrhea, anaphylactic symptoms, and hypothermia. With the protocol (A), diarrhea occurrence, diarrhea score (B), anaphylactic response, and anaphylactic score (C) were monitored within 1 h from oral ovalbumin (OVA) administration to immunized (OVA) or

nonimmunized (Non) mice treated with rapamycin (Rapa). The numbers of mice in each group were as follows: control 'Non' group ($n = 6$), 'OVA' group ($n = 24$), 'OVA/Rapa 0.25 mg' group ($n = 16$), 'OVA/Rapa 0.75 mg' group ($n = 20$), and 'Non/Rapa 0.75 mg' group ($n = 3$). Rectal temperature was assessed for 1 h after the sixth challenge (D). * $P < 0.05$ vs OVA group.

kit (eBioscience, Inc.). The viability of BMMCs assayed using MTT (18) was not affected by the treatment with rapamycin at the concentration used for 8 h or less (data not shown).

Survival of BMMCs cultured with IL-9 (50 ng/ml) and rapamycin for 48 h was measured by MTT assay (18). To analyze the sub-G1 population, BMMCs cultured with or without IL-9 (50 ng/ml) and rapamycin for 24 and 48 h were collected and fixed in ethanol (70%) for 24 h. Then, the cells were stained by 50 µg/ml of propidium iodide (Wako Pure Chemical Industries, Ltd., Osaka, Japan) containing 10 µg/ml of RNase (Wako Pure Chemical Industries, Ltd.) and analyzed for fluorescence (FL2) by FACScalibur (BD Bioscience, San Jose, CA, USA).

Statistical analysis

To analyze data statistically, the Mann–Whitney *U*-test and one-way ANOVA followed by Tukey's *post hoc* test were used.

Results

Preventive and therapeutic effects of rapamycin on FA in mice

Administration of rapamycin with the preventive protocol (Fig. 1A) obviously inhibited oral OVA challenge-induced FA, namely diarrhea occurrence and score (Fig. 1B), anaphylactic response and score (Fig. 1C), and hypothermia (Fig. 1D), suggesting a preventive effect of rapamycin on FA in mice. The treatment decreased the concentration of MMCP-1 released from mucosal mast cells (MMC) in serum at 1 h after the sixth oral OVA challenge (Fig. 2A). Increase in mast cells in the intestinal villi, but not crypts of FA mice at the last day of the experiment, was diminished by rapamycin (Fig. 2B).

To investigate the therapeutic effect of rapamycin, we administered rapamycin with the therapeutic protocol to mice that had already received oral OVA challenge six times without the drug treatment (Fig. 3A). Their FA gradually decreased

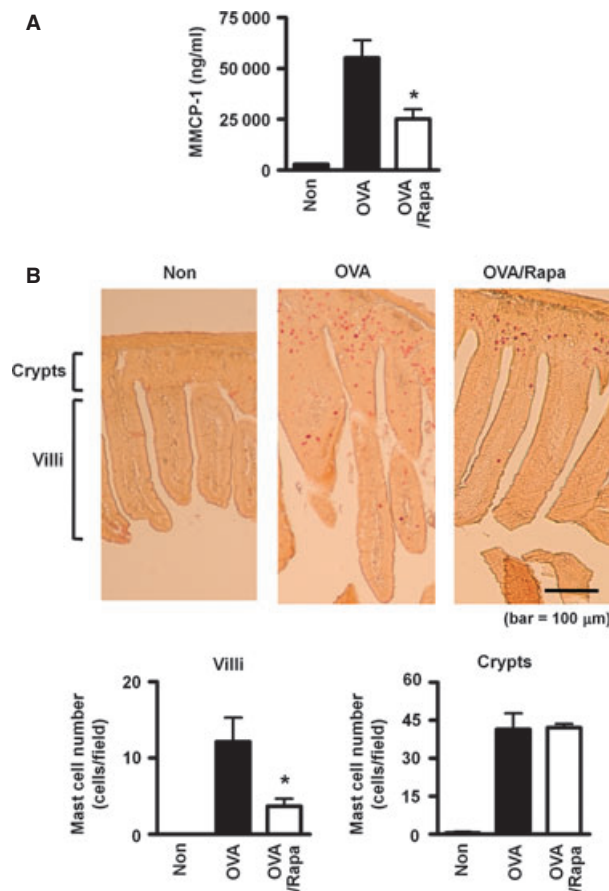


Figure 2 Preventive administration of rapamycin reduced increases in mouse mast cell protease (MMCP)-1 release and mast cell number in the intestine upon intake of ovalbumin (OVA) in food allergy mice. Serum MMCP-1 concentrations [A: control 'Non' group ($n = 6$), 'OVA' group ($n = 24$), and 'OVA/Rapa' group

($n = 20$)], mast cells per high-power field in the small intestine [B: control 'Non' group ($n = 3$), 'OVA' group ($n = 12$), and 'OVA/Rapa' group ($n = 10$)] in immunized (OVA) or nonimmunized (Non) mice treated with 0.75 mg of rapamycin (Rapa) according to the protocol shown in Fig. 1A. * $P < 0.05$ vs OVA group.

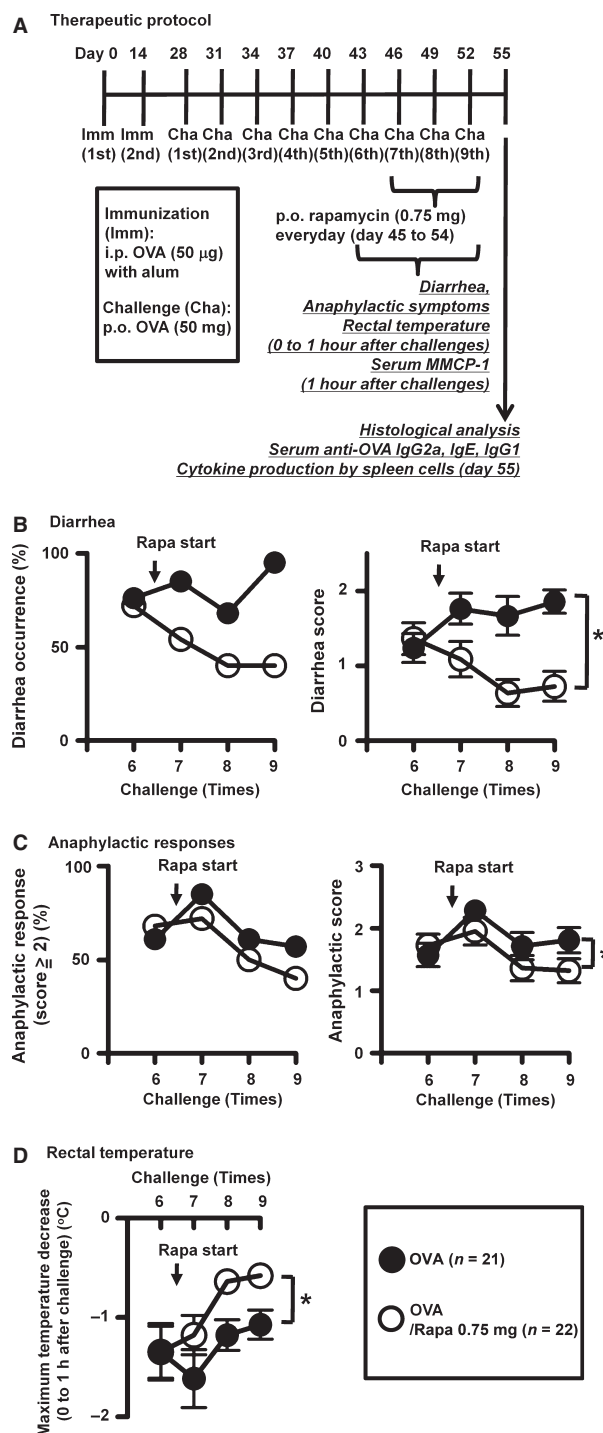


Figure 3 Relief by therapeutic administration of rapamycin from allergic diarrhea, anaphylactic symptoms, and hypothermia upon subsequent antigen challenge in mice that had already developed food allergy. With the protocol (A), diarrhea occurrence, diarrhea score (B), anaphylactic response, anaphylactic score (C), and rectal

temperature (D) in the sixth to ninth challenge were monitored in immunized (ovalbumin; OVA) mice treated with 0.75 mg of rapamycin (Rapa). The numbers of mice in each group were as follows: 'OVA' group (n = 21) and 'OVA/Rapa 0.75 mg' group (n = 22). * $P < 0.05$ vs OVA group.

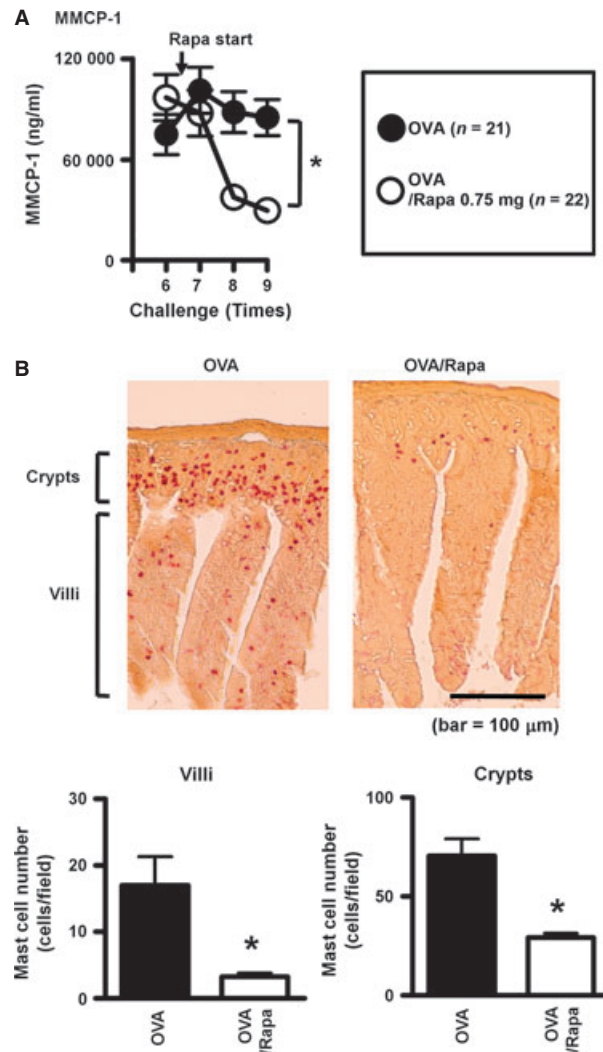


Figure 4 Therapeutic administration of rapamycin recovered the mice that had already developed food allergy from morbid oral antigen-induced mouse mast cell protease (MMCP)-1 release and mast cell hyperplasia in the intestine. Time course changes in serum MMCP-1 concentrations [A: 'ovalbumin (OVA)' group ($n = 21$) and

'OVA/Rapa' group ($n = 22$)] from sixth to ninth challenge, mast cells per high-power field in the small intestine after ninth challenge [B: 'OVA' group ($n = 7$) and 'OVA/Rapa' group ($n = 8$)] in immunized (OVA) mice treated with 0.75 mg of rapamycin (Rapa) according to the protocol shown in Fig. 3A. * $P < 0.05$ vs OVA group.

according to the number of rapamycin administrations (Fig. 3A–C). By repeated rapamycin treatment, the MMCP-1 concentration in serum steadily decreased up to the ninth challenge in parallel with the symptoms of FA (Fig. 4A). Therapeutic administration of rapamycin lowered the IMC number not only in villi but also in crypts of FA mice after the ninth challenge compared to that in vehicle-treated mice (Fig. 4B).

Rapamycin could not inhibit PSA *in vivo* and degranulation responses *in vitro*

Next, we tried to address the inhibitory mechanism of rapamycin on FA by analyzing various mast cell functions with PSA model and BMDC culture. Single rapamycin

administration at 24 h before challenge (Fig. 5A) could not inhibit hypothermia (Fig. 5B) or the increase in MMCP-1 concentration in serum (Fig. 5C) with PSA. Consistent with the absence of an effect of rapamycin on the IgE-dependent acute mast cell responses in the *in vivo* model, rapamycin pretreatment failed to affect IgE and antigen-induced degranulation of BMDCs (Fig. 6A) (12).

Rapamycin blunted IgE-induced Th2 cytokine IL-13 production and spontaneous and IL-9-enhanced cell survival of BMDCs

Th2 cytokine IL-13 release was increased dramatically within hours and obviously decreased at 8 h after stimulation by ra-

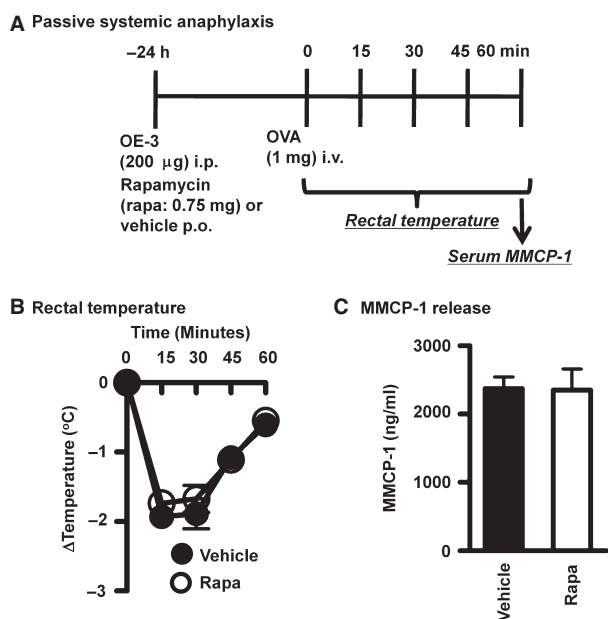


Figure 5 No inhibition of parenteral antigen-induced passive systemic anaphylaxis by rapamycin administration. Mice were sensitized by intravenous injection of 200 µg OE-3 and administered vehicle or 0.75 mg of rapamycin (Rapa) (A). Passive systemic anaphylaxis was induced by intravenous injection of 1 mg ovalbumin (OVA) and assessed by hypothermia [B: 'Vehicle' group ($n = 14$) and 'Rapa' group ($n = 14$)] and mouse mast cell protease (MMCP)-1 release [C: 'Vehicle' group ($n = 14$) and 'Rapa' group ($n = 14$)]. Similar results were obtained from two independent experiments.

pamycin pretreatment (Fig. 6B). Rapamycin induced the reduction in the BMMC numbers *in vitro* (Fig. 6C) after 2 days' culture. Rapamycin also suppressed the enhancement of BMMC survival by exogenous IL-9 (Fig. 6C), which is an essential cytokine for the increase in mast cells in the intestines of FA mice (16). In addition, percentages of BMDCs with sub-G1 DNA content were increased by rapamycin treatment in both the presence and absence of IL-9 (Fig. 6D).

Immunoregulation by rapamycin in FA mice

Preventive treatment with rapamycin significantly decreased Th2-dependent anti-OVA IgE, IgG1 levels, but did not significantly reduce Th1-dependent anti-IgG2a levels in FA mice (Fig. 7A). On the other hand, therapeutic administration significantly but only moderately decreased the anti-OVA IgG1 level, while the levels of anti-IgE and anti-IgG2a were almost unaffected by the treatment (Fig. 7C). All of Th1 cytokine IFN- γ , Th2 cytokine IL-4, and IL-13, Th9 cytokine IL-9, regulatory T-cell type (Tr) 1 cytokine IL-10, and Th17 cytokine IL-17 productions from OVA-restimulated spleen cells were lowered by both preventive and therapeutic administration (Fig. 7B,D). Similar to the antibody production, a greater effect of the drug on cytokine production was obtained by preventive administration among the two protocols. Interestingly, the inhibitory effect of rapamycin on regulatory cytokine IL-10 production was much weaker than that

on other cytokine productions in both protocols, especially the preventive protocol (Fig. 7B,D).

Discussion

Both preventive and therapeutic administration of rapamycin attenuated the symptoms of FA (Figs 1 and 3), promising that mTOR inhibitors could be used for therapeutic treatment as well as for prophylactic treatment. The parallel decrease in oral antigen-induced MMCP-1 release from mast cells by the treatments (Figs 2A and 4A) indicated that one of the primary target cells of rapamycin is mast cells. Because rapamycin could not inhibit PSA (Fig. 5B) and the degranulation response of BMMC induced by IgE and antigen (Fig. 6A), the reduction in MMCP-1 release by rapamycin in FA mice should be mediated by a mechanism other than the direct inhibition of acute mast cell responses including degranulation machinery. Rather, the inhibition of MMCP-1 release by rapamycin was thought to be dependent on the decrease in IMC numbers in FA mice (Figs 2B and 4B). Decreased number (Fig. 6C) (12) and increased sub-G1 population (Fig. 6D) of BMMC *in vitro* by rapamycin treatment strongly supported the notion that direct promotion of cell death by rapamycin occurred in the intestines of the mice. Surprisingly, although the importance of mTOR on mast cells was clearly shown in *in vitro* studies (12), only a few reports have indicated a role of mTOR in allergic reactions *in vivo*, such as asthma (19). In the present study, we suggested the contribution of mTOR in FA and the therapeutic potential of mTOR inhibitors against this disease for the first time. In addition, BMMC survival by IL-9, which predisposes toward IMC hyperplasia in mouse FA (16), was also inhibited by the drug. Because IL-9 enhances the mast cell survival (20) and activates the PI3K-Akt axis located upstream of mTOR (21), blunting of IL-9-dependent BMMC survival by rapamycin might be mediated by the inhibition of mTOR. These results indicated that rapamycin relieved FA via inhibition of IMC hyperplasia directly through the reduction in mTOR activity. It should also be noted that rapamycin inhibited IL-13 production of BMMC at 8 h after IgE-dependent stimulation (Fig. 6B) without affecting acute responses including degranulation at 20 min (Fig. 6A), suggesting an inhibitory activity of the drug on IgE-dependent later mast cell responses. Because IgE and antigen-induced Fc ϵ RI aggregation induces mast cell survival (22), the direct inhibition of IgE-dependent later mast cell responses by rapamycin might also contribute to the reduction in mast cell survival. In addition, rapamycin strongly inhibited Th2 and Th9 cytokine production from antigen-stimulated splenocytes (Fig. 7B,D) in FA mice, while IL-4 (23) and IL-9 (Fig. 6C) (20) enhance mast cell survival. These results suggested that the immunosuppressive effect of rapamycin also seemed to contribute to the inhibition of IMC hyperplasia in FA mice. Taken together, the inhibition of IL-9-mast cell survival axis by rapamycin via inhibiting mTOR is quite important for the attenuation of FA.

Bone marrow-derived mast cells share some characteristics such as the expressions of Fc ϵ RI and c-kit with mature mast cells. Mouse mast cell protease-1 and MMCP-2 in both BMDCs (24) and MMCs but not in connective tissue mast

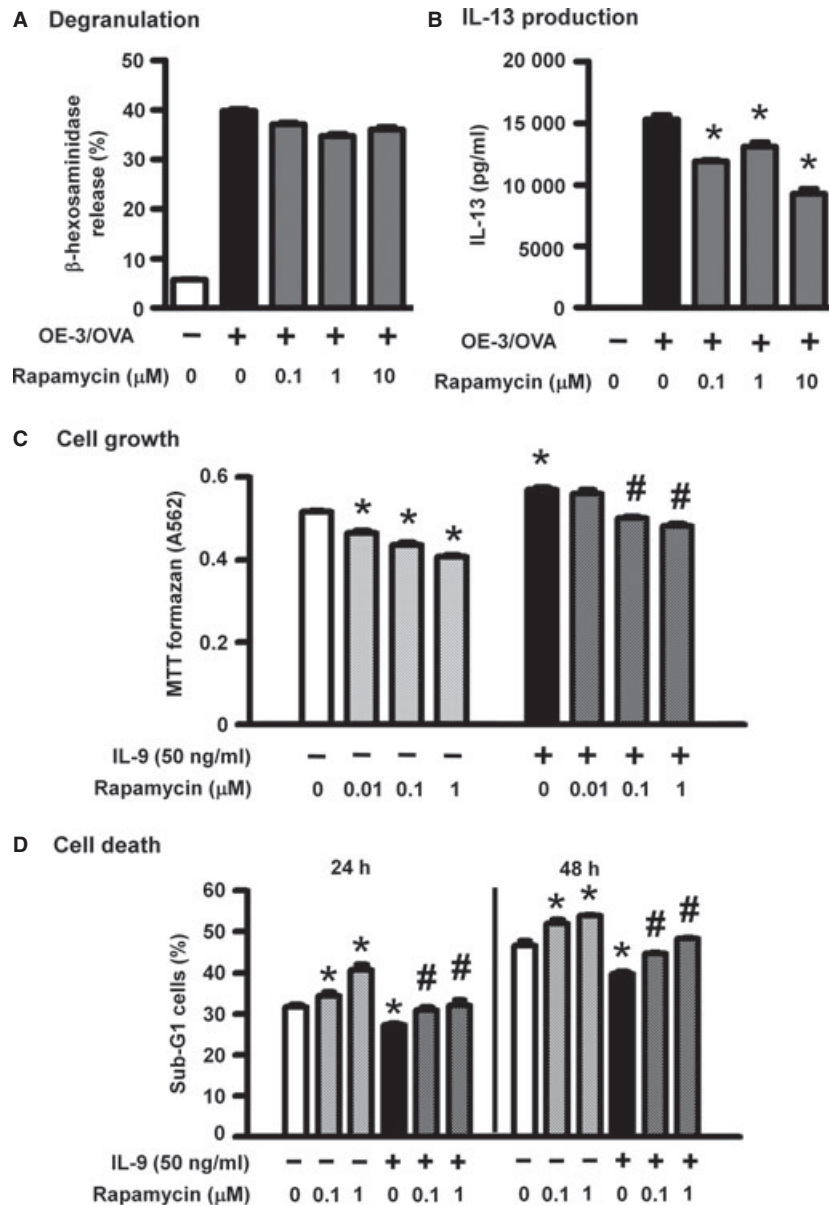


Figure 6 IgE and antigen-induced degranulation and IL-13 production, and IL-9-dependent survival of bone marrow-derived mast cells (BMMCs) in response to rapamycin. FcεRI-mediated β-hexosaminidase release of BMMCs in response to rapamycin (A). Inhibition of FcεRI-mediated IL-13 release of BMMCs by rapamycin (B). * $P < 0.05$ vs OE-3/ovalbumin (OVA) stimulation. Inhibition of spontaneous and IL-9-enhanced cell survival of BMMCs by rapamycin

(C). * $P < 0.05$ vs control. # $P < 0.05$ vs IL-9 alone. Bars show mean + SEM of six cultures. Increase in sub-G1 population of BMMCs in the presence or absence of IL-9 by rapamycin (D). * $P < 0.05$ vs control. # $P < 0.05$ vs IL-9 alone. Bars show mean + SEM of four to six cultures. Similar results were obtained from 2 or more independent experiments.

cells (CTMCs) (25, 26) indicated that BMMCs are biased to differentiate into MMCs. In contrast, from histochemical analysis using alcian blue and safranin, BMMCs were classified as immature MMCs (27). Thus, IL-3-generated BMMCs are useful for the analysis of intestinal and other mast cell functions in response to the various agents considering the difficulty of isolation of mouse intestinal MMCs. However, it

should also be noted that the results obtained from *in vitro* BMMC experiments may not necessarily accord with those from *in vivo* MMCs and CTMCs. For example, the cell survival or proliferation of BMMCs in our and other's experiments (12) and mast cell lines LAD2, HMC-1.1, HMC-1.2 (12) were decreased by rapamycin. In contrast, the cell numbers of differentiated human mast cells from peripheral blood

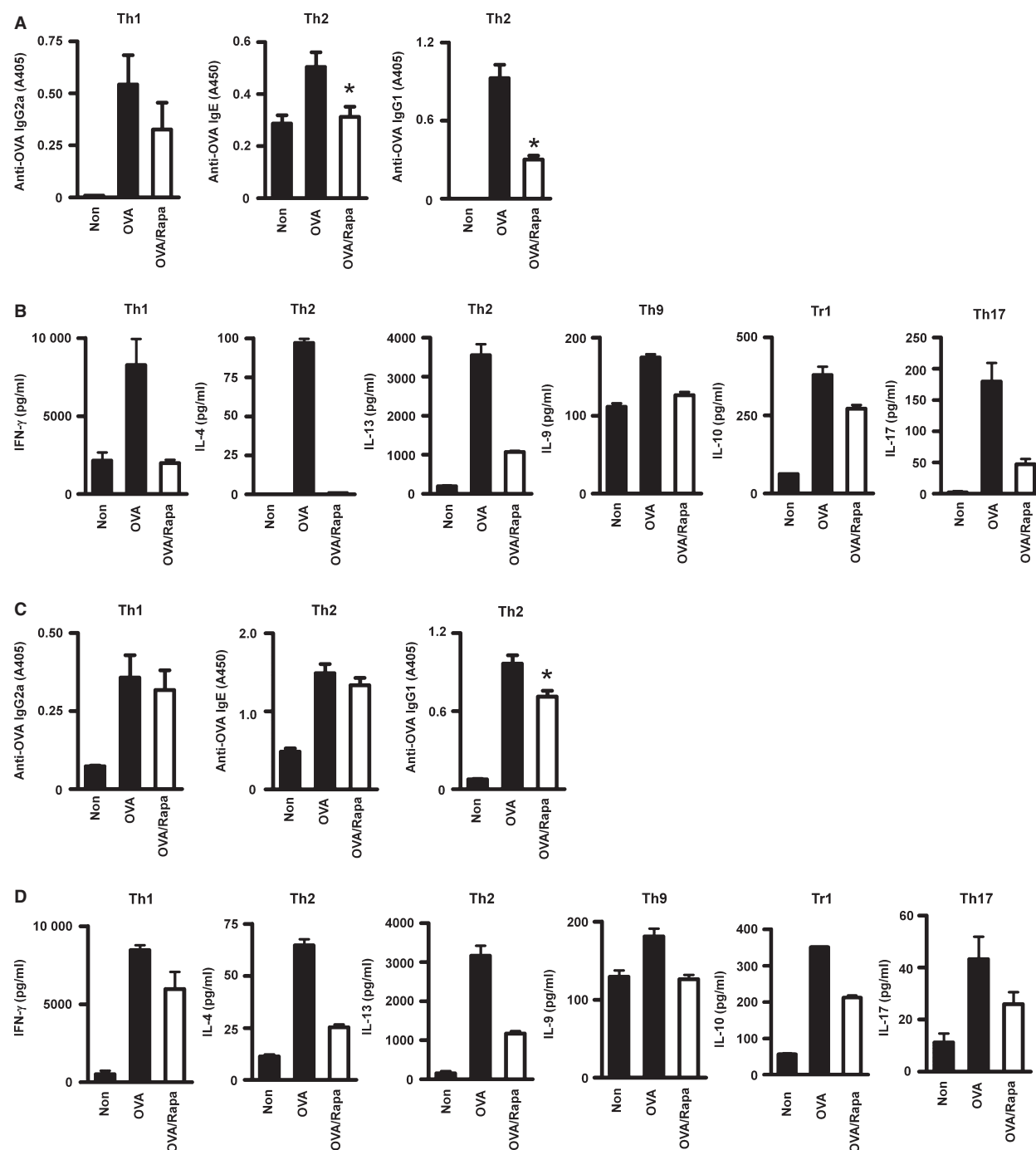


Figure 7 Increases in anti-ovalbumin (OVA) antibody level in serum and cytokine production of splenocytes were attenuated by preventive and therapeutic treatment of rapamycin in food allergy mice. Serum OVA-specific IgG2a, IgE, IgG1 levels [preventive protocol (A): control 'Non' group ($n = 3$), 'OVA' group ($n = 10$), and 'OVA/Rapa' group ($n = 10$); therapeutic protocol (C): control 'Non' group ($n = 3$), 'OVA' group ($n = 21$), and 'OVA/Rapa' group ($n = 22$)],

IFN- γ , IL-4, IL-13, IL-9, IL-10, and IL-17 secretion of splenocytes (preventive protocol (B): control 'Non' group ($n = 3$), 'OVA' group ($n = 12$), and 'OVA/Rapa' group ($n = 10$); therapeutic protocol (D): control 'Non' group ($n = 3$), 'OVA' group ($n = 7$), and 'OVA/Rapa' group ($n = 8$)] in immunized (OVA) or nonimmunized (Non) mice treated with rapamycin (Rapa) according to the protocol shown in Figs 1A and 3A. * $P < 0.05$ vs OVA group.

progenitors were not affected by the drug treatment (28). These facts indicated that the effects of rapamycin on cell survival and proliferation might vary among the mast cells at various stages of differentiation *in vivo*. In FA mice, rapamycin may reduce the mast cell number in the intestine through decreasing the cell survival and proliferation of mast cells at some, but not all, stages of differentiation. Distinctions of the cell functions are also observed among BMMCs, CTMCs, and MMCs (29); especially, rapamycin should affect the activation of BMMCs, CTMCs, and MMCs differently because of the different roles of SHIP in those cells, which modulates PI3K–Akt–mTOR cascade by hydrolyzing phosphatidylinositol-3,4,5-triphosphate (29).

While both mTOR1 and mTOR2 are inhibited by rapamycin (30), mTOR2 is indispensable for Th2 differentiation (30). Thus, the immunosuppressive effect on Th2 by rapamycin should be mediated by mTOR2 inhibition and attenuate FA (Figs 1 and 3) through the reduction in anti-OVA IgE and IgG1 levels (Fig. 7A,C). Weaker inhibition of rapamycin on Th1 responses than Th2 responses (Fig. 7A,C, IgG2a vs IgG1; Fig. 7D, IFN- γ vs IL-4) might indicate that the drug biases the Th1/Th2 immune balance toward Th1 dominance, as observed in humans receiving solid organ transplantation with rapamycin treatment (31), and also contributes to the attenuation of allergic responses. The inhibition of Th2 cytokine production of mast cells by rapamycin described above (Fig. 6B) also seemed to contribute to the immunosuppressive effect. In addition to Th1, Th2, and Th9, immunosuppressive effects of rapamycin on Tr1 and Th17 were observed (Fig. 7B,D). Among them, Th17, of which differentiation is dependent on mTOR1 (30), is considered to contribute to the development of FA. Upregulations of IL-17 mRNA in intestinal tissues (32) and IL-17 protein release from antigen-restimulated lymphocytes (33) are observed in FA mice. Fc ϵ RI gene knockout causes both attenuation of allergic symptoms and IL-17 expression in the intestines of FA mice (32). Although the detailed role of IL-17 in FA remains to be elucidated, suppression of IL-17 production by rapamycin might be involved in the reduction in FA in our model mice. It should be pointed out that the suppressive effect of rapamycin on Tr1 cytokine IL-10 production seemed to be weaker than that on other cytokines (Fig. 7B,D). Consistent with our results, rapamycin promotes the emergence of IL-10-secreting donor T cells in mice with lymphohematopoietic graft-versus-host response after donor-lymphocyte injection (34). These results indicated that one possible mechanism of the immunosuppressive effect of rapamycin is the induction of a higher ratio of IL-10 to other cytokine productions. In fact, IL-10-generating *Lactococcus lactis* strain administered into the gut prevents FA in mice (35).

In clinic, the immunosuppressant FK506-dependent FA is a major concern after transplantation (36). Although the calcineurin inhibitors cyclosporine and FK506 (37) and mTOR inhibitor rapamycin have been used for the suppression of rejection reaction after transplantation, FK506 relatively frequently induces sensitization to food allergens and causes FA

(38) by the modification of intestinal functions (39). In such cases, substitution of another calcineurin inhibitor, cyclosporine, for FK506 is almost the only way (40). Our present results suggested that rapamycin might be a good substituent for FK506 for transplant patients with FK506-induced FA, because the drug targets mTOR other than calcineurin and is considered to reduce rejection reactions and to relieve FK506-induced FA. At least, for cyclosporine-intolerant patients with FK506-induced FA, rapamycin could be a new choice. Unfortunately, the supposed concentration in the serum of rapamycin-administered mice in our present basic animal study (1–5 μ g/ml) (41) is about 50–250 times higher than the therapeutic concentration used clinically for the prevention of rejection in humans (about 10–15 ng/ml) (42), although rapamycin at concentrations of 8–24 mg/kg is often used for the analysis of the effects of the drug on various disease models of mice (43, 44). However, the clinical concentration of rapamycin for the prevention of transplant rejection may potentially be effective for the suppression of FA in humans (45). Although establishment of the appropriate application method of rapamycin or other mTOR inhibitors to prevent side effects will be needed before its use in clinical treatment for FA, our present results indicating the essential role of mTOR in FA progression and the therapeutic potential of rapamycin and other mTOR inhibitors on FA offer important information for the development of therapies for FA.

In conclusion, mTOR inhibitor rapamycin could inhibit FA in mice via suppression of mast cell activation and survival and immune responses against the allergen. These results also indicate the essential role of the mTOR pathway in FA induction through the activation of mast cells and immune functions. Rapamycin and other mTOR inhibitors are potential preventive, therapeutic, and definitive treatments for FA.

Acknowledgements

The authors thank Medical English Service (Kyoto) for English editing.

Financial support

All authors declare that they did not receive financial support from any company for this study.

Authors contribution

Kouya Yamaki performed all the experiments described herein and drafted the manuscript. Shin Yoshino acted as a laboratory supervisor to Kouya Yamaki and assisted in the preparation and proofing of this manuscript.

Conflict of interest

The authors have no financial conflicts of interest.

References

1. Sicherer SH. Epidemiology of food allergy. *J Allergy Clin Immunol* 2011;**127**:594–602.
2. Clark AT, Skypala I, Leech SC, Ewan PW, Dugue P, Brathwaite N et al. British Society for Allergy and Clinical Immunology guidelines for the management of egg allergy. *Clin Exp Allergy* 2010;**40**:1116–1129.
3. Ben-Shoshan M, Clarke AE. Anaphylaxis: past, present and future. *Allergy* 2011;**66**:1–14.
4. Benhamou AH, Caubet JC, Eigenmann PA, Nowak-Węgrzyn A, Marcos CP, Reche M et al. State of the art and new horizons in the diagnosis and management of egg allergy. *Allergy* 2010;**65**:283–289.
5. Venter C, Hasan Arshad S, Grundy J, Pereira B, Bernie Clayton C, Voigt K et al. Time trends in the prevalence of peanut allergy: three cohorts of children from the same geographical location in the UK. *Allergy* 2010;**65**:103–108.
6. Frew AJ. What are the 'ideal' features of an adrenaline (epinephrine) auto-injector in the treatment of anaphylaxis? *Allergy* 2011;**66**:15–24.
7. Brandt EB, Strait RT, Hershko D, Wang Q, Muntel EE, Scribner TA et al. Mast cells are required for experimental oral allergen-induced diarrhea. *J Clin Invest* 2003;**112**:1666–1677.
8. Lin XP, Magnusson J, Ahlstedt S, Dahlgren-Hoglund A, Hanson LL, Magnusson O et al. Local allergic reaction in food-hypersensitive adults despite a lack of systemic food-specific IgE. *J Allergy Clin Immunol* 2002;**109**:879–887.
9. Schwartz LB, Metcalfe DD, Miller JS, Earl H, Sullivan T. Tryptase levels as an indicator of mast-cell activation in systemic anaphylaxis and mastocytosis. *N Engl J Med* 1987;**316**:1622–1626.
10. Kulis M, Wan CK, Gorenz BK, Burks AW, Zhong XP. Diacylglycerol kinase zeta deficiency in a non-CD4(+) T-cell compartment leads to increased peanut hypersensitivity. *J Allergy Clin Immunol* 2011;**128**:212–214.
11. Osterlund P, von Willebrand M, Andersson LC, Suomalainen H. T-cell signal transduction in children with cow's milk allergy – increased MAP kinase activation in patients with acute symptoms of cow's milk allergy. *Pediatr Allergy Immunol* 2003;**14**:163–168.
12. Kim MS, Kuehn HS, Metcalfe DD, Gilfillan AM. Activation and function of the mTORC1 pathway in mast cells. *J Immunol* 2008;**180**:4586–4595.
13. Weichhart T, Saemann MD. The PI3K/Akt/mTOR pathway in innate immune cells: emerging therapeutic applications. *Ann Rheum Dis* 2008;**67** (Suppl 3):iii70–iii74.
14. Yamaki K, Yoshino S. Tyrosine kinase inhibitor sunitinib relieves systemic and oral antigen-induced anaphylaxes in mice. *Allergy* 2012;**67**:114–122.
15. Morath C, Arns W, Schwenger V, Mehrabi A, Fonouni H, Schmidt J et al. Sirolimus in renal transplantation. *Nephrol Dial Transplant* 2007;**22**(Suppl 8):viii61–viii65.
16. Forbes EE, Groschwitz K, Abonia JP, Brandt EB, Cohen E, Blanchard C et al. IL-9- and mast cell-mediated intestinal permeability predisposes to oral antigen hypersensitivity. *J Exp Med* 2008;**205**:897–913.
17. Brandt EB, Munitz A, Orekov T, Mingler MK, McBride M, Finkelman FD et al. Targeting IL-4/IL-13 signaling to alleviate oral allergen-induced diarrhea. *J Allergy Clin Immunol* 2009;**123**:53–58.
18. Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods* 1983;**65**:55–63.
19. Mushaben EM, Kramer EL, Brandt EB, Khurana Hershey GK, Le Cras TD. Rapamycin attenuates airway hyperreactivity, goblet cells, and IgE in experimental allergic asthma. *J Immunol* 2011;**187**:5756–5763.
20. Hultner L, Druez C, Moeller J, Uytendove C, Schmitt E, Rude E et al. Mast cell growth-enhancing activity (MEA) is structurally related and functionally identical to the novel mouse T cell growth factor P40/TCGFIII (interleukin 9). *Eur J Immunol* 1990;**20**:1413–1416.
21. Demoulin JB, Grasso L, Atkins JM, Stevens M, Louahed J, Levitt RC et al. Role of insulin receptor substrate-2 in interleukin-9-dependent proliferation. *FEBS Lett* 2000;**482**:200–204.
22. Kitaura J, Xiao W, Maeda-Yamamoto M, Kawakami Y, Lowell CA, Kawakami T. Early divergence of Fc epsilon receptor I signals for receptor up-regulation and internalization from degranulation, cytokine production, and survival. *J Immunol* 2004;**173**:4317–4323.
23. Lorentz A, Bischoff SC. Regulation of human intestinal mast cells by stem cell factor and IL-4. *Immunol Rev* 2001;**179**:57–60.
24. Pemberton AD, Brown JK, Wright SH, Knight PA, McPhee ML, McEuen AR et al. Purification and characterization of mouse mast cell proteinase-2 and the differential expression and release of mouse mast cell proteinase-1 and -2 in vivo. *Clin Exp Allergy* 2003;**33**:1005–1012.
25. Scudamore CL, McMillan L, Thornton EM, Wright SH, Newlands GF, Miller HR. Mast cell heterogeneity in the gastrointestinal tract: variable expression of mouse mast cell protease-1 (mMCP-1) in intraepithelial mucosal mast cells in nematode-infected and normal BALB/c mice. *Am J Pathol* 1997;**150**:1661–1672.
26. Friend DS, Ghildyal N, Gurish MF, Hunt J, Hu X, Austen KF et al. Reversible expression of tryptases and chymases in the jejunal mast cells of mice infected with *Trichinella spiralis*. *J Immunol* 1998;**160**:5537–5545.
27. Razin E, Stevens RL, Akiyama F, Schmid K, Austen KF. Culture from mouse bone marrow of a subclass of mast cells possessing a distinct chondroitin sulfate proteoglycan with glycosaminoglycans rich in N-acetylgalactosamine-4,6-disulfate. *J Biol Chem* 1982;**257**:7229–7236.
28. Smrz D, Kim MS, Zhang S, Mock BA, Smrzova S, DuBois W et al. mTORC1 and mTORC2 differentially regulate homeostasis of neoplastic and non-neoplastic human mast cells. *Blood* 2011;**118**:6803–6813.
29. Ruschmann J, Antignano F, Lam V, Snyder K, Kim C, Essak M et al. The role of SHIP in the development and activation of mouse mucosal and connective tissue mast cells. *J Immunol* 2012;**188**:3839–3850.
30. Delgoffe GM, Pollizzi KN, Waickman AT, Heikamp E, Meyers DJ, Horton MR et al. The kinase mTOR regulates the differentiation of helper T cells through the selective activation of signaling by mTORC1 and mTORC2. *Nat Immunol* 2011;**12**:295–303.
31. Eiwegger T, Gruber S, Geiger C, Mayer E, Dehlink E, Bannert C et al. Impact of systemic immuno-suppression after solid organ transplantation on allergen-specific responses. *Allergy* 2011;**66**:271–278.
32. Wang M, Takeda K, Shiraishi Y, Okamoto M, Dakhama A, Joetham A et al. Peanut-induced intestinal allergy is mediated through a mast cell-IgE-Fc epsilonRI-IL-13 pathway. *J Allergy Clin Immunol* 2010;**126**:306–316.
33. Perrier C, Thierry AC, Mercenier A, Corthesy B. Allergen-specific antibody and cytokine responses, mast cell reactivity and intestinal permeability upon oral challenge of sensitized and tolerized mice. *Clin Exp Allergy* 2010;**40**:153–162.
34. Durakovic N, Radojic V, Powell J, Luznik L. Rapamycin promotes emergence of IL-10-secreting donor lymphocyte infusion-derived T cells without compromising their graft-versus-leukemia reactivity. *Transplantation* 2007;**83**:631–640.
35. Frossard CP, Eigenmann PA. The role of IL-10 in preventing food-induced anaphylaxis. *Expert Opin Biol Ther* 2008;**8**:1309–1317.
36. Saeed SA, Integlia MJ, Pleskow RG, Calenda KA, Rohrer RJ, Dayal Y et al. Tacrolimus-associated eosinophilic

- gastroenterocolitis in pediatric liver transplant recipients: role of potential food allergies in pathogenesis. *Pediatr Transplant* 2006;**10**:730–735.
37. van Rossum HH, de Fijter JW, van Pelt J. Pharmacodynamic monitoring of calcineurin inhibition therapy: principles, performance, and perspectives. *Ther Drug Monit* 2010;**32**:3–10.
38. Maarof G, Krzysiek R, Decline JL, Cohen J, Habes D, Jacquemin E. Management of post-liver transplant-associated IgE-mediated food allergy in children. *J Allergy Clin Immunol* 2011;**127**:1296–1298.
39. Blanchard SS, Gerrek M, Czinn S, Chelimsky G, Seaman D, Siegel C et al. Food protein sensitivity with partial villous atrophy after pediatric liver transplantation with tacrolimus immunosuppression. *Pediatr Transplant* 2006;**10**:529–532.
40. Lykavieris P, Frauger E, Habes D, Bernard O, Debray D. Angioedema in pediatric liver transplant recipients under tacrolimus immunosuppression. *Transplantation* 2003;**75**:152–155.
41. Baker H, Sidorowicz A, Sehgal SN, Vezina C. Rapamycin (AY-22,989), a new antifungal antibiotic. III. In vitro and in vivo evaluation. *J Antibiot (Tokyo)* 1978;**31**:539–545.
42. Meier-Kriesche HU, Kaplan B. Toxicity and efficacy of sirolimus: relationship to whole-blood concentrations. *Clin Ther* 2000;**22** (Suppl B):B93–B100.
43. Warner LM, Adams LM, Sehgal SN. Rapamycin prolongs survival and arrests pathophysiologic changes in murine systemic lupus erythematosus. *Arthritis Rheum* 1994;**37**:289–297.
44. Carlson RP, Hartman DA, Tomchek LA, Walter TL, Lugay JR, Calhoun W et al. Rapamycin, a potential disease-modifying antiarthritic drug. *J Pharmacol Exp Ther* 1993;**266**:1125–1138.
45. Roque EJ, Rios MG, Vignolo AP, Pinochet VC, Schultz M, Humeres AR et al. Use of Sirolimus in five pediatric patients undergoing solid organ transplantation. *Rev Med Chil* 2008;**136**:631–636.