



Remission of food allergy by the Janus kinase inhibitor ruxolitinib in mice ☆☆☆



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ABSTRACT

To clarify the role of Janus kinase (JAK) in and the efficacy of JAK inhibitors on food allergy, we investigated the effect of the clinically available JAK inhibitor ruxolitinib on mouse food allergy and the functions of cultured mast cells *in vitro*. Anaphylactic symptoms including diarrhea and decreases in body temperature pursuant to oral ovalbumin (OVA) challenges in food allergy mice were attenuated by the daily oral administration of ruxolitinib. This drug inhibited increases in mouse mast cell protease-1 concentrations in the serum and mast cell numbers in the intestines of these mice as well as degranulation, IL-13 production, and the spontaneous and IL-9-dependent survival of mouse bone marrow-derived mast cells in spite of the absence of an effect of ruxolitinib on passive systemic anaphylaxis. Anti-OVA IgG2a, IgE, and IgG1 serum levels and the release of IFN- γ , IL-4, IL-9, and IL-10 from the OVA-restimulated splenocytes of food allergy mice were also decreased by the treatment. Moreover, ruxolitinib administration to mice that had already exhibited anaphylactic responses to previous challenges reduced anaphylactic responses to further oral OVA challenges, which suggested that ruxolitinib has a therapeutic potential on food allergy. Our results showed that ruxolitinib remitted food allergy in mice mainly through immunosuppression and the prevention of mast cell hyperplasia, and partially through the inhibition of mast cell activation. We consider JAK inhibition to be a promising strategy for the prevention of food allergy, and ruxolitinib along with its derivatives inhibiting JAK as good candidates for therapeutic drugs to treat food allergy.

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1. Introduction

Food allergy (FA) is a potentially life-threatening allergic disease triggered by the ingestion of a food allergen [1,2]. Many mild to severe symptoms including urticaria, vomiting, diarrhea, dyspnea, tachycardia, and anaphylactic shock are typically induced within minutes to hours after the intake of a food allergen. The prevalence of patients with FA has been increasing [3], and currently affects 8% of children [4] and 2% of adults [5]. FA causes 150 to 200 fatalities per year in the United States [6], even though established effective symptomatic treatments including an epinephrine injection are available [2]. A causal treatment

to overcome this disease has not yet been established; therefore, the removal of allergens from the diet is the only reliable method to prevent FA.

Intestinal mast cell (MC) hyperplasia [7,8], Th2 sensitization against allergens [7,9], and IgE-dependent MC activation in acute allergic responses [7,10] were found to be important for the development of FA in both humans and a mouse model. Since MCs have been implicated in both allergic diathesis and the induction of acute allergic responses upon allergen exposure, compounds that modulate MC functions in and improve the pathological conditions of FA may represent potential candidates for therapeutic drugs used as alternatives to definitive treatments for FA.

Janus kinase (JAK) is a tyrosine kinase that participates in intracellular signal transduction pathways from the receptors of cytokines and growth factors to the transcriptional factor signal transducer and activator of transcription (STAT) to produce cellular responses [11]. Although many studies have demonstrated that JAK controls the cell viability, growth, and functions of MCs [11], the individual roles of each JAK in various conditions have not yet been determined. Two clinical JAK inhibitors, ruxolitinib (Jakafi, Incyte Co.) [12] and tofacitinib (Xeljanz, Pfizer Inc.) [13], were recently developed and have been used to treat myelofibrosis [14] and rheumatoid arthritis [15], respectively. Since the survival and activation of MCs are controlled by JAK, we investigated the effect of the JAK inhibitor ruxolitinib on a mouse FA model to examine the

Abbreviations: BMMCs, bone marrow-derived mast cells; FA, food allergy; JAK, Janus kinase; MCs, mast cells; MMCP, mouse mast cell protease; OVA, ovalbumin; PSA, passive systemic anaphylaxis; STAT, signal transducer and activator of transcription; Tr, regulatory T cell type.

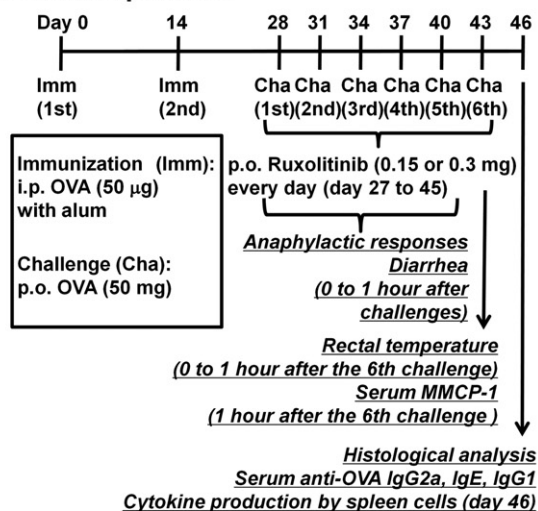
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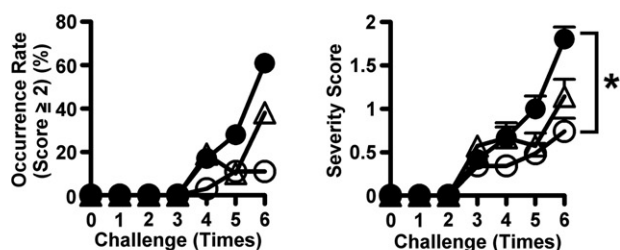
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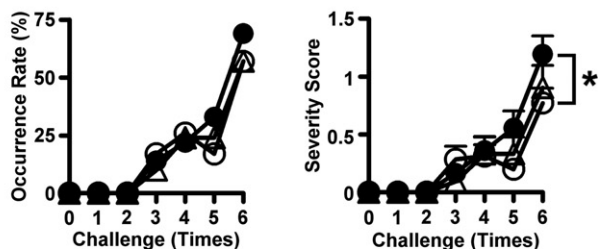
A) Prevention protocol



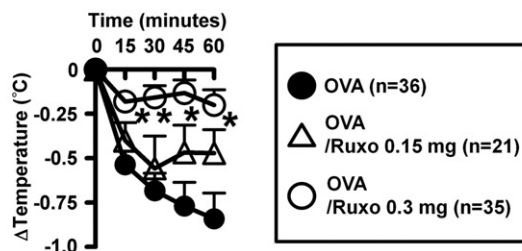
B) Anaphylactic responses



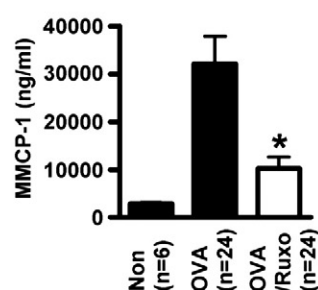
C) Diarrhea



D) Rectal temperature



A)



B)

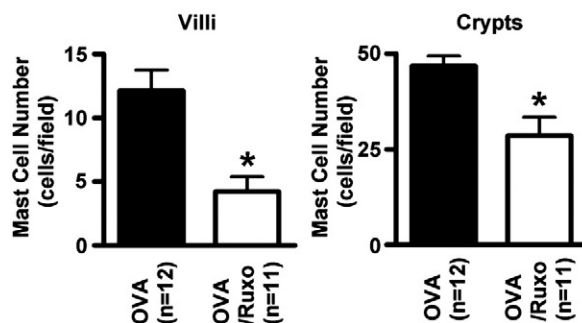
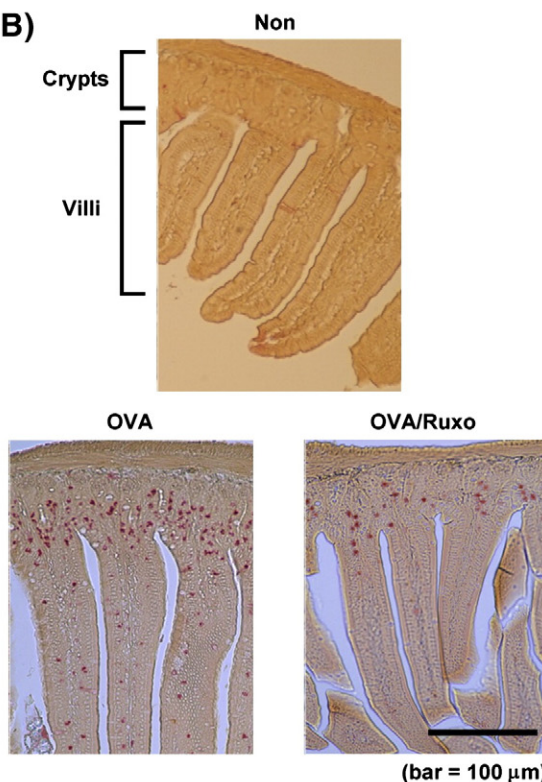


Fig. 2. Mouse mast cell protease (MMCP)-1 release at the 6th challenge and intestinal mast cell number increases in food allergy mice were reduced by the ruxolitinib treatment in the prevention protocol. (A) MMCP-1 serum concentrations in vehicle- or ruxolitinib (Ruxo, 0.3 mg)-treated food allergy mice were measured 1 h after the 6th challenge. Bars show means + SEM. Data were accumulated from two independent experiments. * $p < 0.05$, the ovalbumin (OVA) group ($n = 24$) versus the OVA/Ruxo ($n = 24$) (Tukey's post hoc test after one-way ANOVA among non ($n = 6$), OVA, and OVA/Ruxo groups). (B) Intestinal mast cells in the villi and crypts stained in red were counted to evaluate intestinal mast cell hyperplasia, an indicator of a predisposition for food allergy. Bars show means + SEM. Data were accumulated from two independent experiments. * $p < 0.05$, the OVA group ($n = 12$) versus the OVA/Ruxo ($n = 11$) (Mann-Whitney U-test).

Fig. 1. Ruxolitinib in the prevention protocol reduced the induction of the symptoms of food allergy in mice due to ovalbumin (OVA) challenges. To examine the preventive effect of ruxolitinib (Ruxo), it was orally administered to immunized mice from the day before the 1st challenge to the day before sacrifice (A). The occurrence rates and severity scores of anaphylactic responses (B) and diarrhea (C) were calculated from monitoring within 1 h of the OVA challenges. Rectal temperatures were measured for 1 h after the 6th challenge (D). Symbols show values (occurrence rates in B and C) or means + SEM (severity scores in B and C, and D). Data were accumulated from three independent experiments. * $p < 0.05$, the OVA group ($n = 36$) versus the OVA/Ruxo 0.3 mg ($n = 35$) at the 6th challenge (Mann-Whitney U-test).

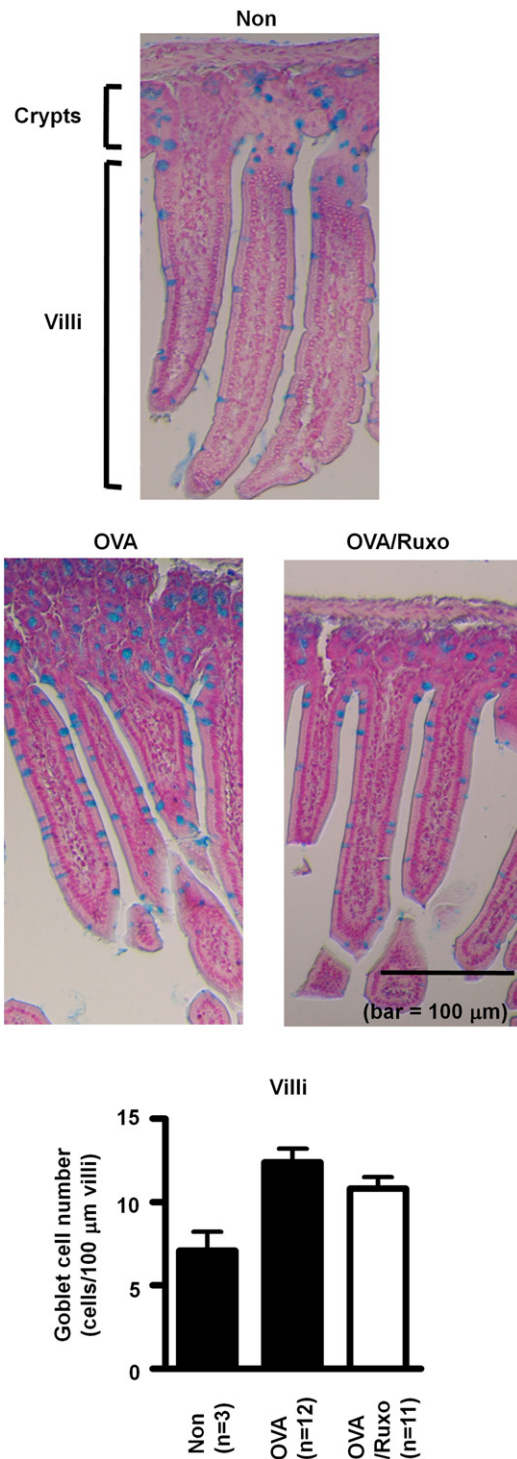


Fig. 3. Increases in intestinal goblet cell numbers in food allergy mice were not significantly inhibited by the ruxolitinib treatment in the prevention protocol. Intestinal goblet cells in the villi stained in blue were counted to evaluate intestinal goblet cell metaplasia, an indicator of a predisposition for food allergy. Bars show means + SEM. Data were accumulated from two independent experiments.

potential of the drug to treat FA and the importance of JAK in FA, with a focus on MC functions and immune responses. A passive systemic anaphylaxis (PSA) model and *in vitro* MC cultures were also used to clarify in detail the effect of ruxolitinib on MC functions.

2. Materials and methods

2.1. Animals

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

2.2. Mouse food allergy

FA was induced in mice and monitored as described previously [16]. Briefly, mice were immunized with 50 μg of ovalbumin (OVA, Sigma-Aldrich Co., St. Louis, MO, USA) in phosphate-buffered saline emulsified with Imject Alum (Thermo Fisher Scientific Inc., Waltham, MA, USA). Mice were challenged from day 28 by the oral administration of 50 mg OVA every 3 days. Anaphylactic symptoms and diarrhea were scored for 1 h after each challenge [17]. At the 6th challenge in the prevention protocol and 6 to 9th challenges in the therapy protocol, rectal temperatures were also measured for 1 h after the challenges and sera were then collected. Ruxolitinib (LC Laboratories, Woburn, MA, USA) was dissolved in 0.3 ml of ethanol and the solution was then mixed with 2.7 ml of saline containing 0.1% TWEEN20. In the prevention protocol, 200 μl of the suspension containing ruxolitinib (0.15 or 0.3 mg) or the vehicle was administered intragastrically every day from day 27 to day 45. In the therapy protocol, ruxolitinib (0.3 mg) was gavaged every day from day 45 to day 54 to mice already immunized and challenged 6 times.

2.3. Passive systemic anaphylaxis

PSA was induced and monitored in mice as described previously [17]. In brief, mice were sensitized by an intravenous injection of 200 μg of anti-OVA monoclonal IgE OE-1 [18], and subsequently orally administered with ruxolitinib (0.3 mg) or vehicle. After 24 h, mice were challenged by an intravenous injection of 1 mg OVA.

2.4. Measurements of the serum concentrations of mouse mast cell protease (MMCP)-1, serum levels of anti-OVA antibodies, and concentrations of cytokines secreted from cultured spleen cells

MMCP-1 concentrations in serum obtained 1 h after the challenges were determined using commercially available ELISA kits following the manufacturer's instructions (eBioscience, Inc., Minneapolis, MN, USA). Splenocyte suspensions were obtained on day 46 in the prevention protocol or on day 55 in the therapy protocol, and were cultured with OVA for 3 days to measure the release of cytokines using ELISA kits as described previously [16]. Serum levels of anti-OVA IgG2a, IgE, and IgG1 were measured using colorimetric ELISA [17,19].

2.5. Histological analysis for the diagnosis of intestinal mast cell hyperplasia and goblet cell metaplasia

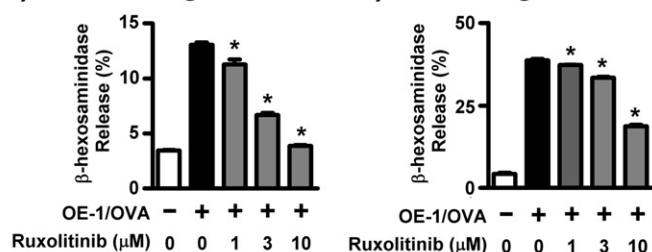
MCs in 4 μm sections of a paraffin-embedded jejunum were counted by the chloroacetate esterase staining method [7]. To determine the number of goblet cells in the intestine, the sections were deparaffinized and stained with Alcian blue (pH 2.5) for 30 min (MUTO PURE CHEMICALS CO., LTD., Tokyo, Japan) followed by nuclear counterstaining for 5 min with Kernchrot solution (Neutral Red, MUTO PURE CHEMICALS CO., LTD.).

2.6. Evaluation of degranulation, IL-13 production, and cell viability of RBL2H3 cells and bone marrow-derived mast cells

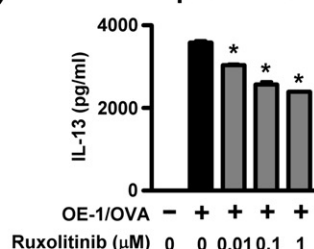
RBL2H3 cells were obtained from the Cell Resource Center for Biomedical Research, Institute of Development, Aging and Cancer, Tohoku University. The effects of ruxolitinib on the degranulation of RBL2H3 cells and bone marrow-derived mast cells (BMMCs), IL-13 production, and viability of BMMCs were analyzed as described previously [16]. BMMCs were differentiated from mouse bone marrow cells by

incubation with recombinant mouse IL-3 (30 ng/ml; Peprotech Inc., Rocky Hill, NJ, USA) for 6–8 weeks. RBL2H3 cells and BMMCs were sensitized by overnight incubation with OE-1 (200 ng/ml) and then pretreated with the indicated concentrations of ruxolitinib for 20 min. These cells were stimulated with OVA (5 µg/ml for RBL2H3 cells; 0.5 µg/ml for BMMCs) with or without ruxolitinib for 20 min and 8 h to measure the degranulation rate and IL-13, respectively. BMMCs were cultured in the presence or absence of ruxolitinib and IL-9 for 48 h and their cell viabilities were then measured with the MTT assay [20].

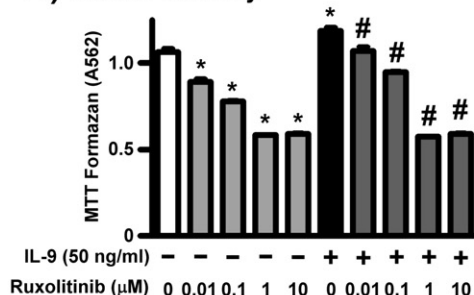
A) RBL2H3 degranulation B) BMMC degranulation



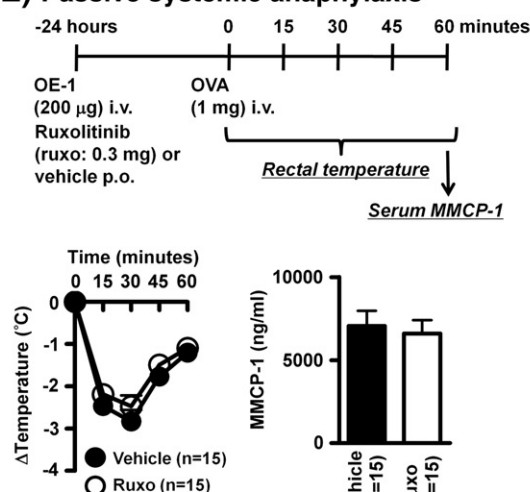
C) BMMC IL-13 production



D) BMMC viability



E) Passive systemic anaphylaxis



2.7. Statistical analysis

The Mann-Whitney U-test and one-way ANOVA followed by Tukey's post-hoc test were used to analyze the significance of the data obtained. A P value less than 0.05 was considered significant.

3. Results

3.1. Daily oral ruxolitinib prevented allergic diathesis and acute allergic symptoms of food allergy induced by OVA intake

Daily doses of ruxolitinib in the preventive protocol (Fig. 1A) strongly and moderately decreased the occurrence rates and severity scores of the anaphylactic response (Fig. 1B) and diarrhea (Fig. 1C) in a dose-dependent manner, respectively. Hypothermia induced at the 6th challenge was also strongly suppressed by the administration of ruxolitinib (Fig. 1D).

In accordance with the suppression of hypothermia, ruxolitinib diminished the increase in MMCP-1 serum concentrations after the 6th challenge (Fig. 2A). Increases in MCs in both the villi and crypts of the intestine by the challenges were diminished by ruxolitinib, which indicated that ruxolitinib could prevent allergic diathesis, namely intestinal MC hyperplasia (Fig. 2B), and thus reduce the increase in MMCP-1 serum concentrations after the 6th challenge (Fig. 2A). In contrast, goblet cell metaplasia induced in the villi of the intestine of FA mice was slightly suppressed by the treatment with ruxolitinib (Fig. 3).

Fig. 4. Reductions in IgE-induced degranulation and IL-13 secretion and cell survival with or without IL-9 in cultured mast cells, in contrast to no inhibition of passive systemic anaphylaxis (PSA), by ruxolitinib. To measure the degranulation rate, RBL2H3 cells (A) and bone marrow-derived mast cells (BMMCs) (B) sensitized with OE-1 (200 ng/ml) were stimulated with ovalbumin (OVA) for 20 min in the presence or absence of the indicated concentrations of ruxolitinib. IL-13 concentrations were also measured in the cultured supernatants of OE-1-sensitized BMMCs with OVA and ruxolitinib for 8 h (C). The viability of BMMCs cultured for 48 h with or without IL-9 and ruxolitinib was determined by the MTT assay (D). Ruxolitinib (Ruxo) was administered to mice passively sensitized by an intravenous injection of OE-1 (E). OVA was injected intravenously after 24 h to induce PSA and rectal temperatures were monitored for 1 h. Mouse mast cell protease (MMCP)-1 serum concentrations were also measured 1 h after the challenge. Symbols and bars show means ± SEM of six cultures (A, B, C, and D) and the indicated number of mice (E). Data were representative of two or three independent experiments (A, B, C, and D) and were accumulated from two independent experiments (E). * $p < 0.05$, the OVA group ($n = 6$) versus the OVA/Ruxolitinib 1 µM group ($n = 6$), 3 µM group ($n = 6$), or 10 µM group ($n = 6$) (Tukey's post hoc test after one-way ANOVA among the non-stimulated control ($n = 6$), OVA, and OVA/ruxolitinib 1 µM, 3 µM, and 10 µM groups) in (A) and (B). * $p < 0.05$, the OVA group ($n = 6$) versus the OVA/ruxolitinib 0.01 µM group ($n = 6$), 0.1 µM group ($n = 6$), or 1 µM group ($n = 6$) (Tukey's post hoc test after one-way ANOVA among the non-stimulated control ($n = 6$), OVA, and OVA/ruxolitinib 0.01 µM, 0.1 µM, and 1 µM groups) in (C). * $p < 0.05$, the non-treated control group ($n = 6$) versus the ruxolitinib 0.01 µM group ($n = 6$), Ruxolitinib 0.1 µM group ($n = 6$), Ruxolitinib 1 µM group ($n = 6$), Ruxolitinib 10 µM group ($n = 6$) or IL-9 alone group ($n = 6$); # $p < 0.05$, the IL-9 alone group versus the IL-9/ruxolitinib 0.01 µM group ($n = 6$), 0.1 µM group ($n = 6$), 1 µM group ($n = 6$), or 10 µM group ($n = 6$) (Tukey's post hoc test after one-way ANOVA among all 10 groups) in (D).

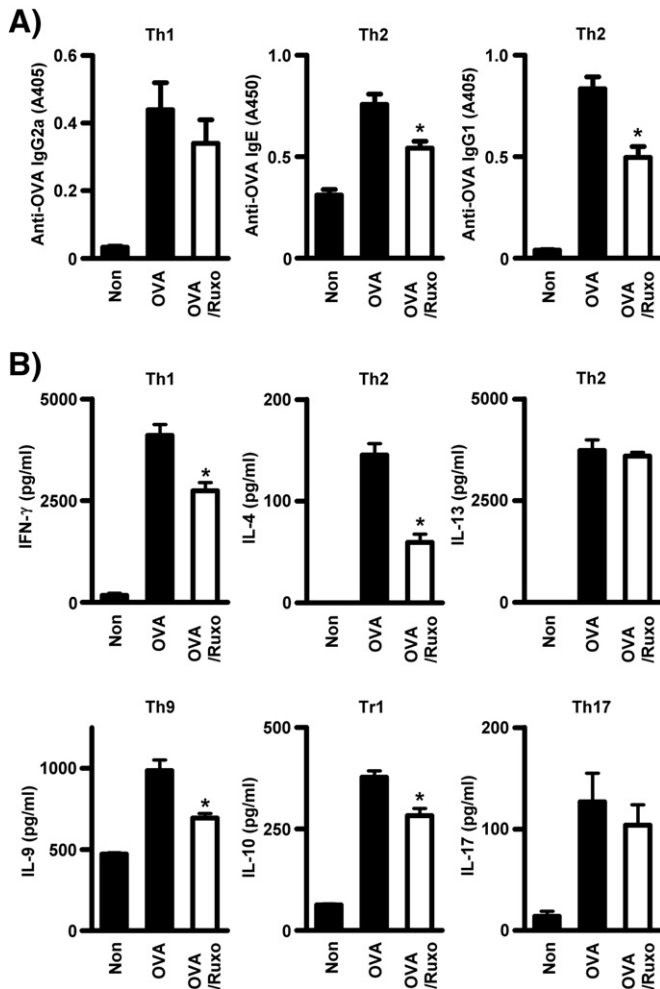
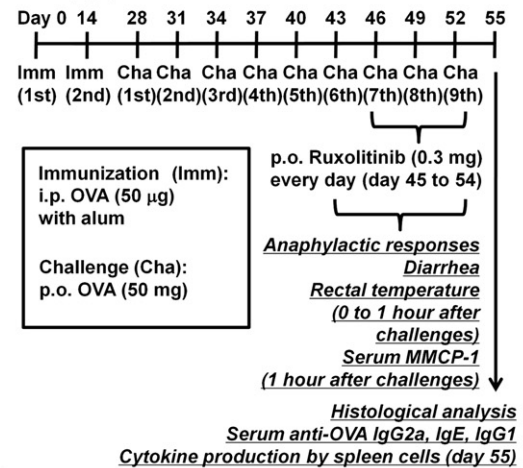


Fig. 5. Preferable inhibition by ruxolitinib of Th2/Th9 responses over Th1/Tr1/Th17 responses observed during the determination of anti-ovalbumin (OVA) IgG2a, IgE, and IgG1 levels in the serum and secretion of IFN- γ , IL-4, IL-13, IL-9, IL-10, and IL-17 from the OVA-restimulated cultured splenocytes of food allergy mice. Sera were recovered from naive (non, $n = 5$) and food allergy mice (OVA ($n = 23$), OVA/ruxolitinib (Ruxo, 0.3 mg) ($n = 23$)) 3 days after the last challenge. The Th1 antibody anti-OVA IgG2a and Th2 antibodies anti-OVA IgE and IgG1 were measured in the serum (A). Splenic cells collected from mice (non, $n = 3$; OVA, $n = 12$; OVA/Ruxo, $n = 11$) were cultured for 3 days with OVA to determine cytokine concentrations in supernatants (B). Bars show means $\pm$ SEM. Data of (A) were accumulated from two independent experiments. * $p < 0.05$, the OVA group ($n = 23$) versus the OVA/Ruxo ($n = 23$) (Tukey's post hoc test after one-way ANOVA among non ($n = 5$), OVA, and OVA/Ruxo groups). Data of (B) were representative of two independent experiments. * $p < 0.05$, the OVA group ($n = 3$) versus the OVA/Ruxo ($n = 3$) (Tukey's post hoc test after one-way ANOVA among non ($n = 3$), OVA, and OVA/Ruxo groups).

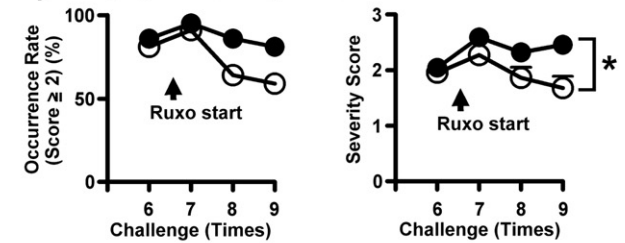
3.2. Different concentrations of ruxolitinib were required for the inhibition of IgE-dependent degranulation, IgE-dependent IL-13 production, survival, and IL-9-dependent proliferation of cultured mast cells

The ruxolitinib pretreatment blunted the degranulation of anti-OVA monoclonal IgE (OE-1)-sensitized RBL2H3 cells (Fig. 4A) and BMMCs (Fig. 4B) *in vitro* MC cultures, upon OVA stimulation for 20 min at relatively higher concentrations between 1 to 10 μ M without affecting cell viability (data not shown). In contrast, the later activation of MCs, namely IL-13 production at 8 h, induced by OE-1 and OVA was significantly inhibited by lower concentrations of ruxolitinib between 0.01 and 1 μ M (Fig. 4C). These ruxolitinib concentrations directly decreased BMMC viability as well as IL-9-induced survival after the 48-h culture (Fig. 4D); IL-9 is an important cytokine for intestinal MC hyperplasia and the development of FA [21]. These results suggest that ruxolitinib directly prevented intestinal MC hyperplasia by blunting IL-9-dependent MC survival in FA mice.

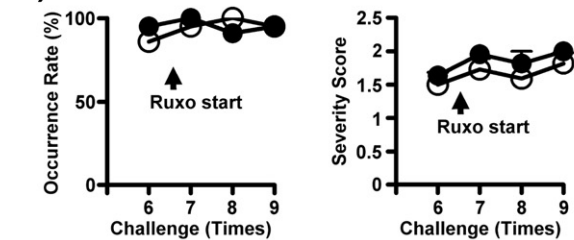
A) Therapy protocol



B) Anaphylactic responses



C) Diarrhea



D) Rectal temperature

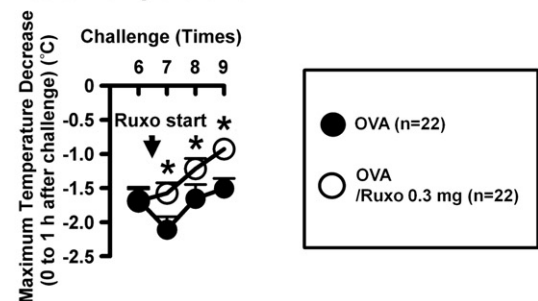
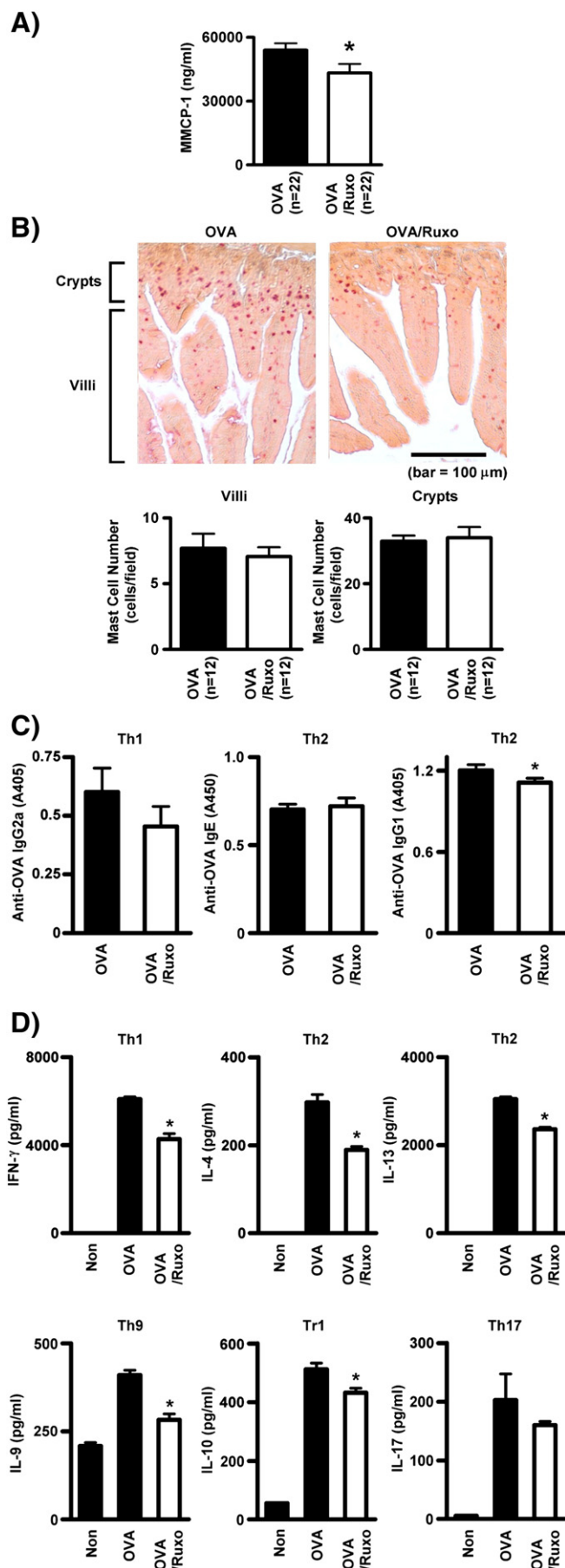


Fig. 6. Ruxolitinib in the therapy protocol attenuated anaphylactic responses and hypothermia, but not diarrhea induced by further oral ovalbumin (OVA) challenges in food allergy mice already challenged with oral OVA 6 times. Ruxolitinib (Ruxo) was administered every day to FA mice already challenged with oral OVA 6 times from the day before the 7th challenge to evaluate the therapeutic effect of ruxolitinib on food allergy (A). The anaphylactic response occurrence rate, anaphylactic score (B), diarrhea occurrence rate, and diarrhea score (C) were monitored for 1 h after the 6 to 9th challenges. Decreases in rectal temperatures induced by each challenge were measured and the data were shown as the maximum rectal temperature decrease in mice within 1 h (D). Symbols show means $\pm$ SEM. Data were accumulated from two independent experiments. * $p < 0.05$, the OVA group ($n = 22$) versus the OVA/Ruxo 0.3 mg ($n = 22$) at the 6th challenge (Mann–Whitney U-test), at the 9th challenge in (B) and (C), and at the 7 to 9th challenges in (D).



3.3. The single oral administration of ruxolitinib failed to inhibit passive systemic anaphylaxis

The single administration of ruxolitinib barely attenuated hypothermia and MMCP-1 release in the PSA model (Fig. 4E), which indicated that the attenuation of allergic symptoms as well as MMCP-1 release in FA mice by repeated ruxolitinib did not appear to be mainly mediated through the inhibition of acute MC activation, but through the prevention of MC hyperplasia.

3.4. Immunosuppressive effect of ruxolitinib in food allergy mice

The daily administration of ruxolitinib significantly reduced Th2-dependent anti-OVA IgE and IgG1 serum levels, but did not Th1-dependent anti-OVA IgG2a levels (Fig. 5A). The secretion of the Th2 cytokine IL-4 and Th9 cytokine IL-9 from OVA-restimulated cultured spleen cells was markedly lower in ruxolitinib-administered FA mice than in vehicle-administered FA mice (Fig. 5B). In contrast, oral ruxolitinib only moderately diminished the secretion of the Th1 cytokine IFN-γ and regulatory T cell type 1 (Tr1) cytokine IL-10, and did not reduced the secretion of another Th2 cytokine IL-13 and the Th17 cytokine IL-17 from splenocytes (Fig. 5B).

3.5. Therapeutic effect of ruxolitinib on food allergy in mice

We evaluated the beneficial effect of ruxolitinib on mice that had already received an oral OVA challenge 6 times without drug treatment (Fig. 6A). The daily oral administration of ruxolitinib from the day before the 7th challenge reduced the anaphylactic response (Fig. 6B), but not diarrhea (Fig. 6C). In parallel to the anaphylactic response, hypothermia induced by the 7th to 9th challenges was diminished by ruxolitinib (Fig. 6D). These results indicate that ruxolitinib could be an effective drug for the treatment of established FA.

Increases in MMCP-1 concentrations due to the 9th challenge in ruxolitinib-treated mice were lower than those in vehicle-treated mice (Fig. 7A), whereas the ruxolitinib treatment could not diminish the increased numbers of MCs in the intestines of FA mice (Fig. 7B). Although anti-OVA IgG1 serum levels were significantly lower in mice treated with ruxolitinib than in those treated with vehicle (Fig. 7C), anti-OVA IgG2a and IgE levels were not significantly affected by ruxolitinib (Fig. 7C). In contrast, the production of IFN-γ, IL-4, and IL-9 by OVA-restimulated cultured splenocytes was markedly decreased by ruxolitinib, that of IL-13 and IL-10 was moderately decreased, and IL-17 production was not affected at all (Fig. 7D). Taken together, ruxolitinib in the therapy protocol attenuated FA by suppressing immune responses including cytokine production potentiating MC functions, instead of by affecting anti-OVA immunoglobulin production and MC numbers in the intestine.

4. Discussion

In the present study, we revealed for the first time using experiments with a mouse model that the JAK inhibitor ruxolitinib, used for myelofibrosis in a clinical setting, has the potential to be a therapeutic drug for

Fig. 7. Mouse mast cell protease (MMCP)-1 release and immune responses were inhibited, whereas intestinal mast cell hyperplasia was not inhibited by ruxolitinib in food allergy mice challenged 9 times in the therapy protocol. MMCP-1 serum concentrations at 1 h (A) and the number of intestinal mast cells at 3 days (B) were determined after the 9th challenge of food allergy mice treated with or without ruxolitinib (Ruxo, 0.3 mg). Serum levels of anti-ovalbumin (OVA) IgG2a, IgE, and IgG1 (C) and the secretion of IFN-γ, IL-4, IL-13, IL-9, IL-10, and IL-17 from splenocytes stimulated with OVA *in vitro* (D) were measured in food allergy mice treated with or without ruxolitinib (Ruxo, 0.3 mg). Bars show means + SEM. Data (A, B, and C) were accumulated from two independent experiments. Data (D) were representative of two independent experiments. * $p < 0.05$, the OVA group ($n = 22$) versus the OVA/Ruxo ($n = 22$) (Mann-Whitney U-test) in (A) and (C). * $p < 0.05$, the OVA group ($n = 3$) versus the OVA/Ruxo ($n = 3$) (Tukey's post hoc test after one-way ANOVA among non ($n = 3$), OVA, and OVA/Ruxo groups) in (D).

allergic diseases including FA, and also that JAK plays an important role in the establishment of these diseases. The repeated administration of ruxolitinib within the development period of the pathological condition of FA, namely from the 1st to the 6th challenges, attenuated both the acute anaphylactic symptoms of FA upon allergen challenges (Fig. 1) and allergic diathesis such as intestinal MC hyperplasia and sensitization to the allergen (Figs. 2B and 5). Although the doses of 4.5 to 9 mg/kg/day (150 to 300 µg/mouse) used for mice in this study were 5 to 10 times higher than those of 0.4 to 1 mg/kg/day (20 to 50 mg/person) used to treat patients with myelofibrosis, similar maximum concentrations (C_{max}) were observed in the sera of mice after similar doses to those used in our study (2.09 µM after 15 mg/kg dosing) [22] and humans during the treatment of myelofibrosis (1.48 µM after 1 mg/kg (50 mg/person) dosing) [22]. Therefore, the dose of ruxolitinib used to treat myelofibrosis may exert beneficial effects on the symptoms of FA in humans, as observed in mice in the present study.

Three mechanisms by which FA in mice was significantly attenuated by ruxolitinib were identified by further analyzing MCs and immune functions. The first is the suppression of MC activation. In accordance with the reduction in MMCP-1 release observed in FA mice (Fig. 2A), the degranulation of cultured MCs was directly inhibited by ruxolitinib (Fig. 4A and B). The concentration of ruxolitinib needs to be 100 times higher in a cellular assay (IC₅₀ = 282, 281 nM) than in an enzyme assay (IC₅₀ = 3.3, 2.8 nM) to inhibit JAK1 and JAK2 [12]; therefore, ruxolitinib, which inhibits JAK3 at IC₅₀ = 428 nM in the enzyme assay [12], should inhibit JAK3 in the cellular assay at an IC₅₀ of approximately 40 µM. Only high concentrations of ruxolitinib at 1 to 10 µM partially attenuated the degranulation of RBL2H3 cells and BMMCs (Fig. 4A and B), which suggests that suppressing JAK3 inhibition, but not that of JAK1 or JAK2, was responsible for the preventive effect of the drug on acute MC activation. However, the concentration of ruxolitinib administered orally every day to FA mice should only be able to reach approximately 1 µM in the serum, as discussed above. At this concentration, ruxolitinib was considered to weakly inhibit JAK3, causing the marginal inhibition of MC degranulation in FA mice. Moreover, a single oral dose of ruxolitinib to mice could not prevent PSA by a challenge on the next day (Fig. 4E). Thus, the direct inhibition of acute MC activation by ruxolitinib only partially participated in the attenuation of FA by the drug. In contrast, ruxolitinib effectively inhibited the later activation of MCs, namely IL-13 production at 8 h, through JAK1 and JAK2 inhibition at low concentrations between 0.01 and 1 µM (Fig. 4C), which should be able to be observed in the sera of FA mice. The suppression of later MC activation by ruxolitinib should influence MC growth and immune responses, and, thus, importantly participate in the inhibitory effect on FA.

The second mechanism is the inhibition of intestinal MC hyperplasia (Fig. 2B). The requirement for lower concentrations (0.01 to 1 µM) of ruxolitinib to reduce the cell viability of cultured BMMCs (Fig. 4D) suggested a direct decreasing effect of the drug on the increase in the number of MCs in the intestine (Fig. 2B). This decreasing effect was considered to be mediated through inhibition of the JAK2–STAT5 pathway, because IL-3 and stem cell factor activate JAK2 [23,24] and STAT5 [25,26], which is indispensable for MC survival induced by the cytokine and growth factor [27].

The third mechanism is antigen-specific immunosuppression (Fig. 5). The repeated administration of ruxolitinib strongly decreased anti-OVA IgE and IgG1 levels (Fig. 5A), which are Th2-dependent antibodies capable of inducing MC degranulation [28] and anaphylactic responses in mice [29]. In parallel with the decrease in anti-OVA antibody production, the secretion of cytokines from the OVA-restimulated cultured splenocytes of FA mice was diminished by the administration of ruxolitinib (Fig. 5B). Among the cytokines, a decrease in IL-4 production by ruxolitinib was the major cause of the suppression of many events in FA. In the acute phase, IL-4 has been shown to upregulate MC functions [30] and potentiate sensitivity to anaphylactic shock [31]. IL-4 was also reported to induce Th2 differentiation [32], a class switch of B cells to produce IgE [33], and the survival/proliferation of MCs [30,34] leading

to allergic diathesis. As well as the direct inhibition of MC survival as discussed above, a decrease in the production of IL-9, which is an essential cytokine for intestinal MC hyperplasia in FA mice [21] as well as BMMC survival *in vitro* [35], by immune cells due to ruxolitinib was shown to contribute to the suppression of intestinal MC hyperplasia in FA mice.

Goblet cell metaplasia in the airways in asthma is one of the mechanisms that causes airway obstruction and is induced in asthma model mice by Th2 cytokines [36,37] through the JAK–STAT-dependent pathway [38,39]. A larger amount of mucus in the stools was observed in patients with food-related gastrointestinal symptoms than in individuals without the symptoms [40]. Previous studies reported an increase in the number of goblet cells in the intestine, and which was inhibited by treatments suppressing the symptoms of food allergy in a mouse food allergy model [41,42]. These results proposed an association between mucus secretion and the severity of food allergy. However, ruxolitinib did not effectively diminish intestinal goblet cell numbers in the present study (Fig. 3) in spite of its suppressive effect on the symptoms of food allergy (Figs. 1 and 2). The absence of the ruxolitinib-induced inhibition of IL-13 production in the prevention protocol (Fig. 5) may be one of the reasons why the drug could not inhibit intestinal goblet cell metaplasia in food allergy mice, because IL-13 is a predominant factor that induces metaplasia from epithelial cells to goblet cells in the airways of asthma model mice [37].

Ruxolitinib had beneficial effects on FA in mice in the therapeutic protocol (Fig. 6) through immunosuppression as evidenced by the lower secretion of cytokines from the splenocytes of mice (Fig. 7D), followed by the inhibition of MC activation (Fig. 7A). The reason why anti-OVA antibody production was weakly inhibited (Fig. 7C) in spite of the strong inhibition of cytokine production (Fig. 7D) was considered to be due to the large amount of antibodies that had already been produced; therefore, more time may have been needed for changes in their levels in the serum, as well as the number of MCs in the intestine, to occur (Fig. 7B). Consistent with our findings, the therapeutically administered JAK1 and JAK2 inhibitor INCB028050, a compound structurally similar to ruxolitinib, was shown to inhibit Th1 and Th17 cytokine mRNA expression in draining lymph nodes, but did not decrease anti-collagen antibodies in the sera of mice with collagen-induced arthritis [43].

In conclusion, we clarified that the JAK inhibitor ruxolitinib prevented the development of FA and improved the predisposition for FA by decreasing MC activation, intestinal MC hyperplasia, and antigen-specific immune responses. These results also indicated for the first time the importance of JAK in the development of diathesis and allergic responses of FA. JAK is primarily important for the immune and inflammatory responses closely associated with allergic diseases; thus, JAK inhibitors may be candidate drugs for the treatment of allergic diseases that only cause mild side effects. Such a novel approach to treat allergies will improve the quality of life of patients with allergies who have not obtained good therapeutic results from treatments with existing drugs.

Disclosures

The authors have no financial conflicts of interest.

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