RESEARCH ARTICLE

Oral tolerance induction does not resolve gastrointestinal inflammation in a mouse model of food allergy

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Scope: Oral immunotherapy (OIT) involving continuous oral administration of allergenic foods has gained attention as a therapy for food allergies. To study the influence of oral administration of allergenic foods on gastrointestinal symptoms including inflammation, we established a mouse model of food-induced intestinal allergy.

Methods and results: BALB/c mice were fed an egg white (EW) diet containing ovalbumin (OVA, a major EW allergen) after intraperitoneal sensitisation with OVA and Alum. The mice on the EW diet for one wk presented gastrointestinal symptoms (i.e. weight loss and soft stools) and inflammation in the small intestines (i.e. duodenum, jejunum and ileum). Further continuous EW diet resolved the weight loss but not the soft stools. Splenic $CD4^+$ T-cells of EW diet-fed mice on the continuous diet showed less proliferation and cytokine production compared with those of control mice, suggesting tolerance induction by the diet. The continuous EW diet reduced levels of OVA-specific IgE antibodies, but significantly aggravated the inflammation in the jejunum.

Conclusion: Our mouse model would be useful to investigate inflammatory and regulatory mechanisms in food-induced intestinal allergies. Our results suggest potential gastrointestinal inflammation in patients undergoing OIT as continuous administration of allergenic foods, even though the therapy may induce clinical tolerance.

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1 Introduction

The prevalence of food allergies has apparently increased over the past decade. Epidemiologic studies based on food challenges indicate that 1–10.8% of the population have immune-mediated nontoxic food hypersensitivity [1]. Food allergies cause inflammation and clinical symptoms

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Abbreviations: Alum, aluminium hydroxide adjuvant; **CA,** casein; **Con A,** concanavalin A; **DFS,** direct fast scarlet; **EW,** egg white; **i.p.,** intraperitoneal or intraperitoneally; **mMCP-1,** mouse mast cell protease-1; **MLN,** mesentric lymph node; **OVA,** ovalbumin; **OIT,** oral immunotherapy; **RBL-2H3 cells,** rat basophil leukaemia (RBL)-2H3 cells

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systemically and/or locally in cutaneous, respiratory, optical and gastrointestinal tissues [2, 3]. Some childhood food allergies, for instance allergies to milk and eggs, are frequently resolved by the time the patient reaches early adolescence [2–4]. It is hypothesised that maturation of the mucosal gut barrier and/or development of oral tolerance leads to this resolution [2–5]. Oral tolerance is classically defined as the suppression of immune responses to antigens that have been administered by the oral route [3, 6, 7]. The impact of oral tolerance is highlighted by the fact that a majority of the population have lifelong immunological tolerance to food antigens.

So far, the only proven treatment for food allergies is elimination of the allergenic food from the diet. Therapeutic application of oral tolerance in allergic diseases has gained increasing attention. Similar to sublingual immunotherapy, oral immunotherapy (OIT), sometimes called specific oral tolerance induction (SOIT), involving oral administration of allergenic foods has been applied as an allergen-specific immunotherapy for persistent food allergies [2, 3, 8–10]. There are clinical reports suggesting the efficacy of OIT in allergies against foods, including milk, egg, peanut and fish [reviewed in 8–10]. Understanding cellular and molecular mechanisms of allergic responses and oral tolerance induction in food allergies is of importance to develop new strategies for preventing and treating the disease.

To investigate mechanisms underlying food allergies and oral tolerance by means of continuous oral administration with allergenic foods, we set out to establish a mouse model presenting allergic symptoms and induction of oral tolerance using BALB/c mice in combination with ovalbumin (OVA, a major allergen of egg white) as a model allergen. We previously showed that feeding of OVA23-3 transgenic mice, whose $CD4^+$ T-cells express an OVA-specific T-cell receptor, with an egg white diet (EW diet) containing OVA induced gastrointestinal symptoms and inflammation in the small intestines [11]. When the transgenic mice were continuously fed the EW diet, they resolved the symptoms and inflammation, and gained tolerance. However, it was not known whether the resolution of symptoms and induction of tolerance could be achieved in inbred mice expressing polyclonal T-cells.

In this study, BALB/c mice were fed the EW diet, after intraperitoneal (i.p.) sensitisation with OVA and aluminium hydroxide adjuvant (Alum). Mice on the EW diet for 7 days presented dominant Th2 immune responses, gastrointestinal symptoms (i.e. weight loss and soft stools) and inflammation in the small intestines (i.e. duodenum, jejunum and ileum). The pathological features are similar to those in patients with allergic enteropathy [2, 4, 12]. Importantly, further continuous EW diet induced tolerance against OVA, resolved the symptoms except soft stools, but significantly aggravated the inflammation in the jejunum, which was not observed in EW diet-fed OVA23-3 mice. Our results suggest that tolerance induction by continuous oral administration of allergenic foods could suppress allergen-specific immune responses but not local inflammation in the intestinal tissue. Furthermore, the results also suggest a risk of OIT in the treatment of food allergies if the patients present gastrointestinal inflammation.

2 Materials and methods

2.1 Animals

BALB/c mice (Female, 8–10 weeks) were purchased from Charles River Laboratories International (Kisslegg, Germany) and Japan SLC (Hamamatsu, Japan). Mice were housed under pathogen-free conditions and animal experiments were performed in compliance with local legislations.

2.2 OVA sensitisation and challenge

Mice were sensitised with 50 μg of OVA (Sigma-Aldrich) and 1 mg of Alum (Perbio, Bonn, Germany) in PBS by i.p. injection twice at a two-week interval. Two weeks after the second sensitisation, the mice were fed an EW diet containing OVA, a casein (CA) diet containing cow CA, or a conventional diet (CN diet) free from OVA. The diet was pellets formed through extrusion, and contained 20% protein, 57.47% starch, 5.0% sugar, 5.0% cellulose, 6.0% soy oil, 5.0% mineral mixture, 1.3% vitamin mixture (AIN-76 composition) and 0.23% choline chloride. The EW and CA diets contained lyophilised EW and CA as the source of protein, respectively. The EW contains around 45% of OVA. Therefore, the content of OVA in the EW diet is around 9.0%. By feeding the EW diet, it is estimated that a mouse ingests around 250 mg of OVA per day. The diets were prepared at ssniff Spezialdiäten GmbH (Soest, Germany) and Funabashi farm (Chiba, Japan). The diets were identical in both countries. Induction of oral tolerance was evaluated by immunising the mice by i.p. injection with 50 μg of OVA and 1.0 mg of Alum after receiving the EW diet for 28 days.

2.3 Measuring levels of OVA-specific antibodies in sera

Microtitre plates (MaxiSorp F96; Nunc, Langenselbold, Germany) were coated with 100 μg/mL of OVA in 50 mM sodium carbonate buffer (pH 10.5) at 4°C overnight. After blocking, serum samples were applied to the wells, and IgE binding was detected by biotin-conjugated rat anti-mouse IgE antibodies (R35-72, BD Bioscience, Heidelberg, Germany) and HRPO-conjugated streptavidin (BD Bioscience). For detection of IgG_1 and IgG_{2a} antibodies, HRPO-conjugated rabbit anti-mouse IgG_1 and HRPO-

conjugated rabbit anti-mouse IgG_{2a} antibodies were used (Invitrogen, Karlsruhe, Germany). The peroxidase substrate was 3,3′,5,5′-tetramethylbenzidine (BD Bioscience). The titres of OVA-specific antibodies were determined as described previously [11].

2.4 Measuring levels of functional IgE antibodies in sera

The level of functional IgE antibodies specific for OVA in the sera was measured by mediator release assays using rat basophil leukaemia (RBL)-2H3 cells, as described previously [13].

2.5 Measuring T-cell proliferation and cytokine production

$CD4^+$ T-cells were isolated from the spleens and mesentric lymph nodes (MLNs) of the mice using an isolation kit from Miltenyi Biotec (Bergisch Gladbach, Germany). To assess T-cell proliferation, $CD4^+$ T-cells (5.0×10^5 cells/mL) were co-cultured with mitomycin-treated syngenic splenocytes (2.0×10^6 cells/mL) as antigen-presenting cells in the presence of LPS-free OVA (100 or 1000 μg/mL; Seikagaku Cooperation, Tokyo, Japan), or concanavalin A (Con A; 2.5 μg/mL) for 72 h. After the co-culture, 1.0 μCi/mL of [^{3}H] methyl thymidine (GE Healthcare, Munich, Germany) was added to the wells. Incorporation of [^{3}H]methyl thymidine into the cells for a further 20 h was measured by a liquid scintillation counter (Perkin Elmer, Rodgau, Germany). To measure T-cell cytokine production, $CD4^+$ T-cells (1.0×10^6 cells/mL) were co-cultured with the syngenic splenocytes (2.0×10^6 cells/mL) in the presence of the LPS-free OVA (100, or 1000 μg/mL) for 72 h. The concentration of cytokines in the culture supernatant was determined by ELISA.

2.6 Measuring T-cell frequency in spleens and MLNs

Spleens and MLNs were isolated from the OVA-sensitised and/or EW diet-fed mice. $CD4^+$ T-cells in the tissue's cell suspension were stained with FITC-conjugated anti-mouse CD4 monoclonal antibody (eBioscience, Frankfurt, Germany) after treating with anti-CD16/CD32 monoclonal antibody (eBioscience), and analysed using a flow cytometer, LSR II (BD Bioscience).

2.7 ELISA

Antibody sets for cytokine ELISA were purchased from eBioscience. Mouse mast cell protease-1 (mMCP-1) in sera was measured using sheep anti-mMCP-1 IgG antibodies [14].

2.8 Histological analysis

Longitudinal sections of intestinal tissue (3 cm) were taken from the duodenum, jejunum (11 cm distal to the duodenum) and ileum (the superior part of the caecum). Tissues were fixed in 10% formalin and embedded in paraffin. Sections, 3 or 5 mm thick, were prepared and stained with haematoxylin and eosin for morphologic analysis, with Direct Fast Scarlet (DFS) staining for detection of eosinophils, and with toluidine blue for detection of mast cells.

2.9 Statistical analysis

Significant differences between mean values were assessed by student *t*-test. A *p*-value of <0.05 was considered significant.

3 Results

3.1 EW diet induced gastrointestinal symptoms and subsequent resolution in OVA-sensitised mice

To investigate the influence of continuous feeding with allergenic foods after allergen sensitisation, BALB/c mice were sensitised by i.p. injection with OVA plus Alum, and subsequently fed an EW diet (OVA/EW mice) for 28 days. The immunisation schedule for sensitisation and feeding is shown in Fig. 1A. As controls, mice were i.p. injected with Alum alone and fed the EW diet (Alum/EW mice), or injected with OVA plus Alum and fed a conventional diet (OVA/CN mice), or casein diet (OVA/CA mice).

OVA/EW mice started presenting gastrointestinal symptoms such as weight loss (Fig. 1B) and soft stools on day 3 or 4 of the EW diet. We observed increased levels of OVA-specific IgE antibodies and mMCP-1, a measure of mast cell activation, in the sera of OVA/EW mice on day 7 of the EW diet (Fig. 1C and D). OVA-specific IgE antibodies were not detectable in the sera of Alum/EW mice (Fig. 1C). The increased levels of mMCP-1 and the gastrointestinal symptoms were not observed in the control mice (Fig. 1B and D and Supporting Information Fig. S1B).

Remarkably, OVA/EW mice regained weight if the mice were continuously fed with the EW diet for more than 7 days (Fig. 1B). The levels of OVA-specific IgE antibodies and mMCP-1 in the sera of OVA/EW mice decreased after 14 days of the diet, and were slightly lower than those in the sera of OVA/CN mice on day 28 of the diet (Fig. 1C and D). The levels of OVA-specific IgG_1 and IgG_{2a} antibodies in OVA/EW mice were not significantly increased during the EW diet (Supporting Information Fig. S2B). Mediator release assay using RBL-2H3 cells [13] indicated that the levels of functional IgE antibodies to trigger the cell activation were almost comparable during the EW diet in OVA/EW mice (Supporting Information Fig. S2C). The

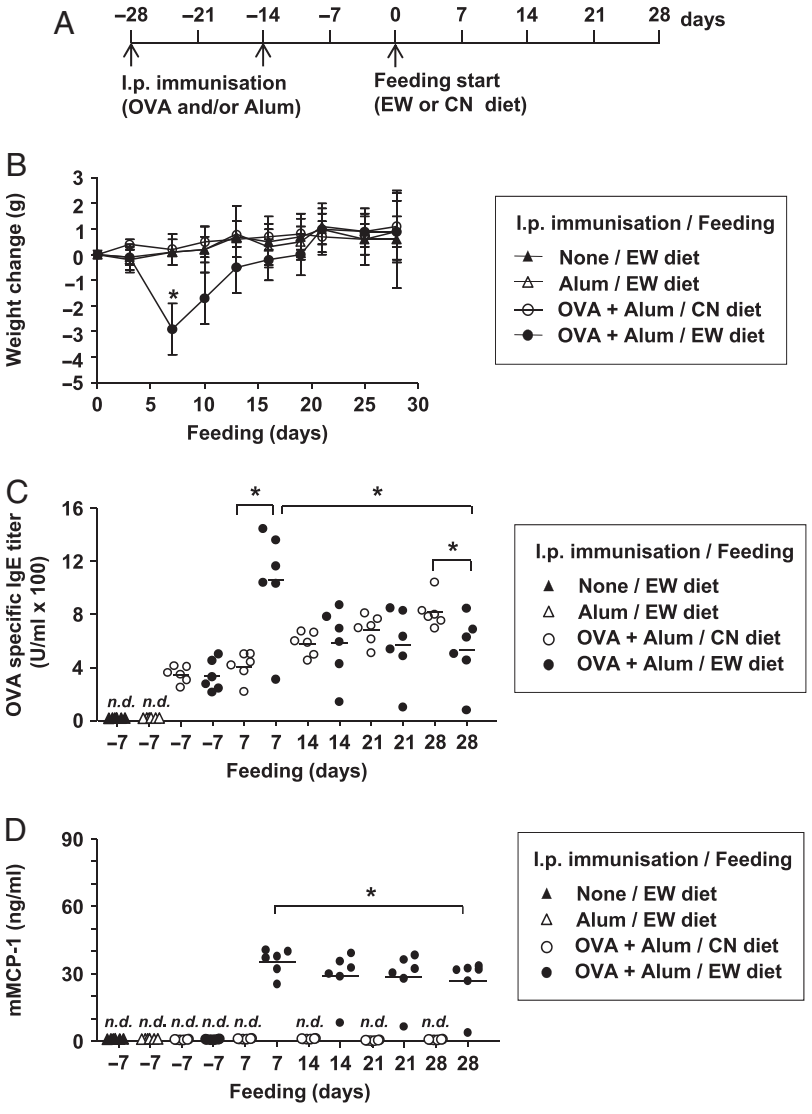

Figure 1. Induction of gastrointestinal symptoms and subsequent resolution by the EW diet in OVA-sensitised BALB/c mice. (A) Immunisation schedule for the experiment. (B) The weight of the mice was measured from the first day of the EW diet. (C) Levels of OVA-specific IgE antibodies in the sera of OVA-sensitised mice on days −7, 7, 14, and/or 28 of the EW or CN diet. *n.d.*, not detectable < 1.95 U/mL. $^*p < 0.001$. (D) Levels of mMCP-1 in sera of OVA-sensitised mice on days 7, 14, and/or 28 of the EW diet. *n.d.*, not detectable < 0.3 ng/mL. $^*p < 0.05$. The data are representative for three independent experiments with six mice per group.

result suggested that the recovery of weight was not due to induction of blocking IgG antibodies, which antagonise the recognition of OVA by IgE antibodies captured on the surface of mast cells and basophils. Soft stools were still observed in OVA/EW mice on the continuous EW diet.

3.2 Short-term EW diet induced IL-10 production in MLN CD4$^+$ T-cells of OVA-sensitised mice

To examine the influence of short-term EW diet on T-cell responses in the OVA-sensitised mice, CD4$^+$ T-cells were isolated from MLNs and spleens of OVA/EW mice on day 7 of the diet (Fig. 2A), when the animals started regaining weight. MLN CD4$^+$ T-cells from OVA/EW mice showed higher proliferation and IL-4 production in response to OVA compared with OVA/CN mice (Fig. 2B). In contrast, splenic CD4$^+$ T-cells from OVA/EW mice showed lower proliferation and cytokine production than the cells from OVA/CN mice (Fig. 2B). MLN or splenic CD4$^+$ T-cells from OVA/EW and OVA/CN mice proliferated in response to Con A, a positive stimuli for polyconal T-cell activation, at comparable levels (Supporting Information Fig. S3A). The results suggested that short-term EW diet could induce systemic but not local tolerance against OVA. OVA-specific IFN-γ production by MLN CD4$^+$ T-cells from OVA/EW mice was not detected (data not shown), suggesting the induction of predominant Th2 type immune responses in the OVA-sensitised mice on the EW diet. Notably, IL-10 was also detectable in the culture supernatant of MLN CD4$^+$ T-cells from OVA/EW mice (Fig. 2B). High levels of IL-10 production by the splenic CD4$^+$ T-cells in response to OVA were also observed (Fig. 2B). Production of a pro-inflammatory cytokine TNF-α and an anti-inflammatory cytokine TGF-β was not detected in either MLN or splenic CD4$^+$ T-cells (data not shown). The frequency of CD4$^+$ T-cells in

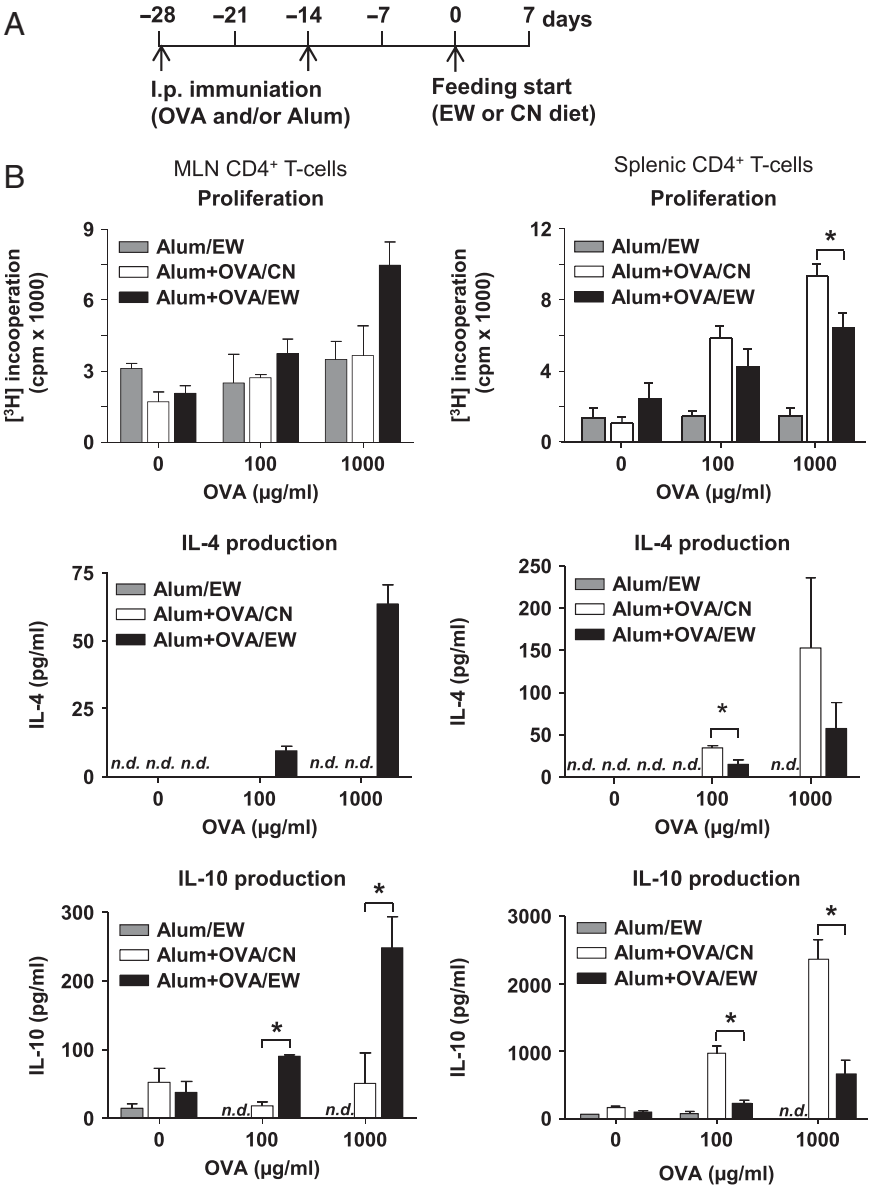

Figure 2. Influence of short-term EW diet on the proliferation and cytokine production of MLN and splenic $CD4^+$ T-cells in OVA-sensitised BALB/c mice. (A) Immunisation schedule for the experiment. (B) $CD4^+$ T-cells were isolated from MLNs (left) and spleens (right) of the mice on day 7 of the EW diet and co-cultured with mitomycin-treated syngenic splenocyte in the presence of OVA. Proliferation of $CD4^+$ T-cells was measured by incorporation of $[^3H]$ thymidine into the cells (top panels). Concentrations of cytokines in the cell culture supernatants (lower panels) were measured by ELISA. The data are representative for three independent experiments with three mice per group. *n.d.*, not detectable; IL-4 < 2.5 pg/mL, IL-10 < 10.0 pg/mL. $^*p < 0.05$.

the MLNs or spleens was comparable among OVA/EW and control mice (Table 1).

3.3 Long-term EW diet induced T-cell deletion in OVA-sensitised mice

As long-term analysis, MLN and splenic $CD4^+$ T-cells were isolated from the mice after 28 days of the EW diet. These T-cells showed only marginal levels proliferation in response to OVA (data not shown). To amplify T-cell responses, the OVA-sensitised mice received another i.p. immunisation with OVA plus Alum after 28 days of the EW diet (Fig. 3A). Seven days after the i.p. immunisation, MLN

Table 1. Influence of short-term EW diet on T-cell frequency in the MLN and spleen of OVA-sensitised mice

I.p. sensitisation	Feeding	Frequency of $CD4^+$ T-cells (%)	
		MLN	Spleen
None	EW diet	46.7±1.5	25.9±2.6
Alum	EW diet	50.1±7.5	26.2±3.1
OVA+Alum	CN diet	51.9±3.9	24.9±0.9
OVA+Alum	EW diet	49.8±6.9	27.6±3.6

BALB/c mice were i.p. sensitised with or without OVA and/or Alum, and subsequently fed the EW or CN diet for 7 days. The frequency of $CD4^+$ T-cells in the MLN and spleen of the mice was analysed by flow cytometry. The data are ±SEM of three independent experiments.

and splenic $CD4^+$ T-cells were isolated from the mice and co-cultured with the antigen-presenting cells in the presence of OVA.

OVA-specific proliferation of MLN $CD4^+$ T-cells from OVA/EW mice and OVA/CN mice were low and comparable (Fig. 3B). Both IL-4 and IL-10 production by MLN $CD4^+$ T-cells from OVA/EW mice was lower than that of the control mice, suggesting local tolerance could be induced by the long-term EW diet (Fig. 3B). Levels of proliferation and IL-4 production of splenic $CD4^+$ T-cells from OVA/EW mice were lower than these $CD4^+$ T-cells from OVA/CN mice (Fig. 3B). MLN or splenic $CD4^+$ T-cells from OVA/EW and OVA/ON mice proliferated in response to Con A at comparable levels (Supporting Information Fig. S3B). The results suggested that long-term EW diet induced and maintained systemic tolerance against OVA. The frequency of MLN and splenic $CD4^+$ T-cells in OVA/EW mice was also lower than

Table 2. Influence of long-term EW diet on T-cell frequency in the MLN and spleen of OVA-sensitised mice

I.p. sensitisation	Feeding	Frequency of $CD4^+$ T cells (%)	
		MLN	Spleen
OVA+Alum	CN diet	54.1±1.6	30.1±1.8
OVA+Alum	EW diet	41.7±2.8*	23.9±0.9*

BALB/c mice were i.p. sensitised with or without OVA and Alum twice, and subsequently fed the EW or CN diet for 28 days. The mice then received the third i.p. immunisation with OVA and Alum. Seven days after the i.p. immunisation, the frequency of $CD4^+$ T-cells in their MLN and spleen was analysed by flow cytometry. The data are±SEM of three independent experiments. *$p<0.05$ versus *CN diet-fed mice*.

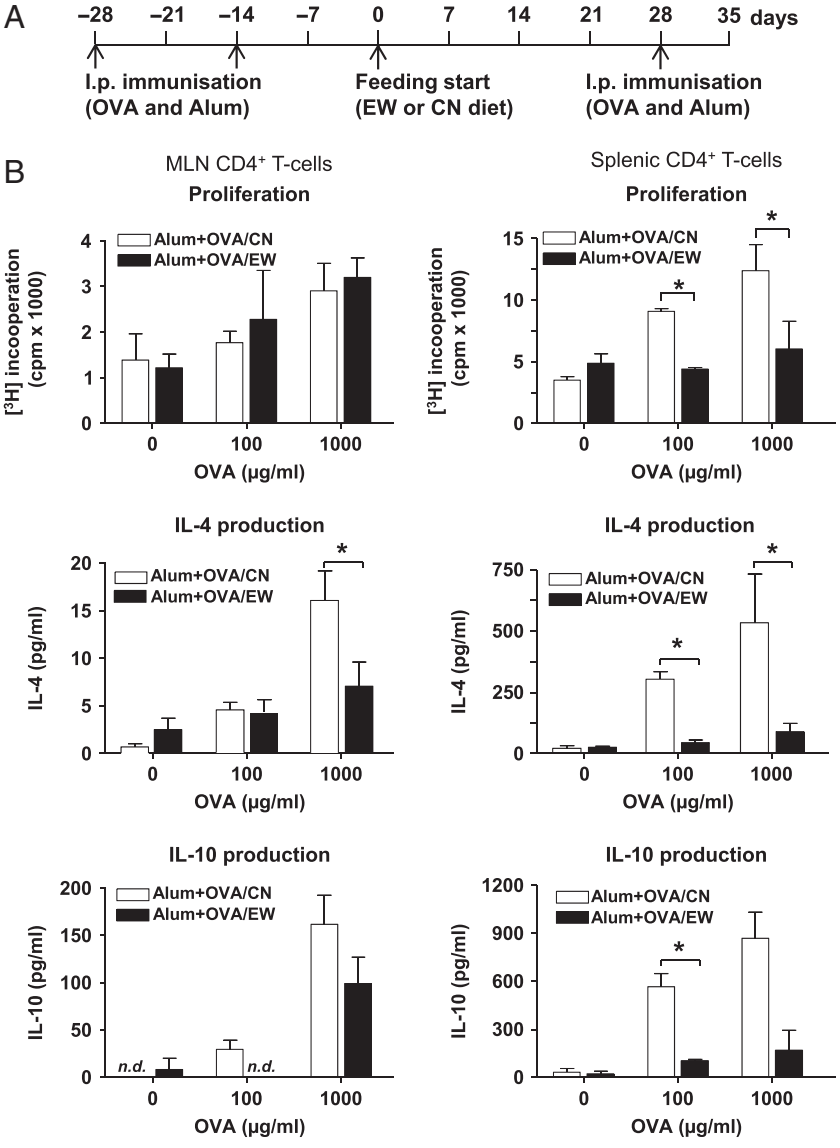

Figure 3. Influence of long-term EW diet on proliferation and cytokine production of MLN and splenic $CD4^+$ T-cells in OVA-sensitised BALB/c mice. (A) Immunisation schedule for the experiment. (B) Seven days after the third i.p. immunisation, $CD4^+$ T-cells were isolated from MLNs (left) and spleens (right) of the mice and co-cultured with mitomycin-treated syngenic splenocyte in the presence of OVA. Proliferation of $CD4^+$ T-cells was measured by incorporation of $[^3H]$ thymidine into the cells (lower panels). Concentrations of cytokines in the cell culture supernatants (lower panels) were measured by ELISA. The data are representative for four independent experiments with three mice per group. *n.d.*, not detectable; IL-4 < 2.5 pg/mL, IL-10 < 10.0 pg/ml. *$p<0.05$.

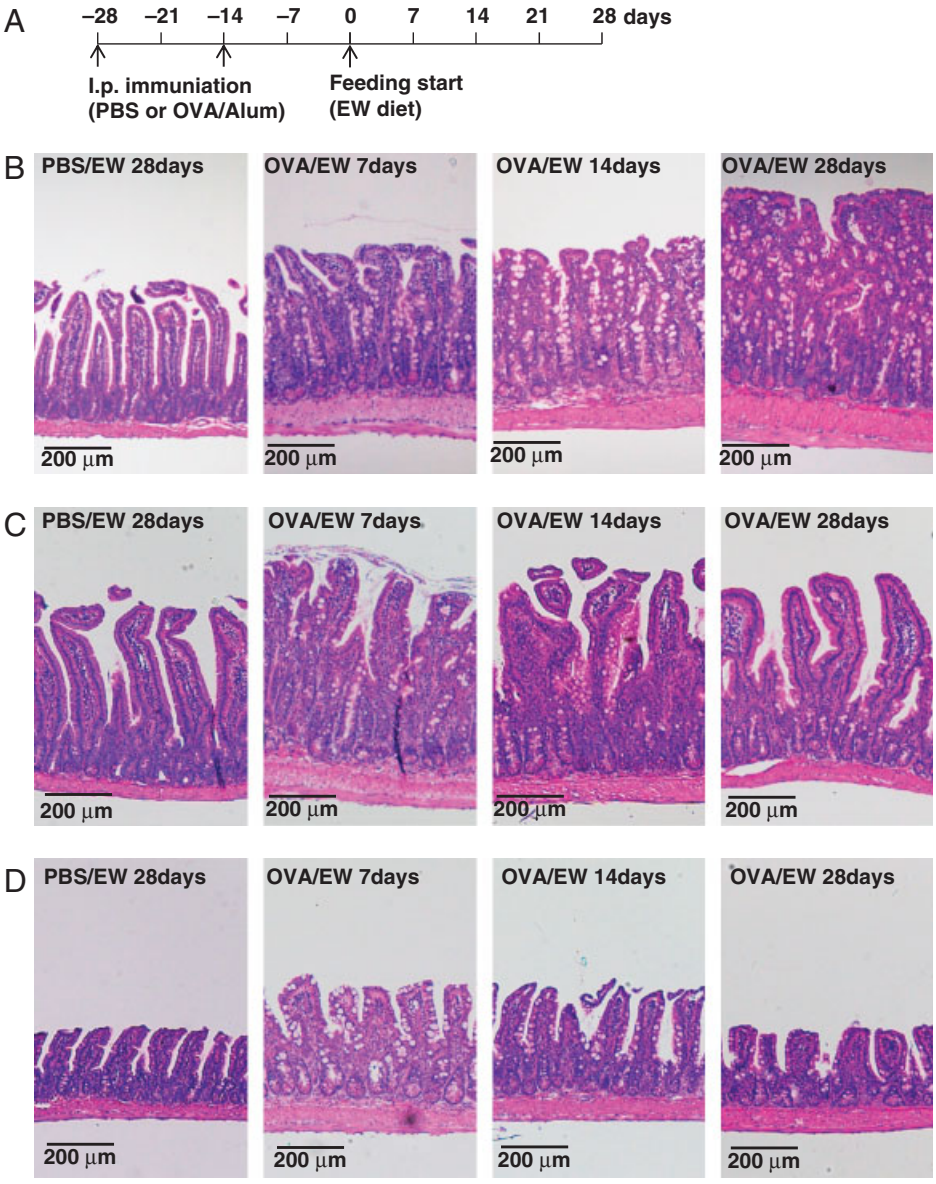

Figure 4. Influence of the EW diet on intestinal inflammation in OVA-sensitised BALB/c mice. (A) Immunisation schedule for the experiment. (B) Jejunum, (C) duodenum and (D) ileum tissues were harvested from the mice for histological analysis. The tissues were stained with haematoxylin–eosin. All images were taken at the same magnification. The data are representative for the two independent experiments.

that in OVA/CN mice (Table 2). About 22.9 and 20.6% reduction was observed in the T-cell frequency in MLNs and spleens of OVA/EW mice ($p<0.05$ versus OVA/CN mice), respectively. The results suggested that the long-term EW diet could induce T-cell deletion in the OVA/EW mice.

3.4 EW diet induced inflammation in the small intestines of OVA-sensitised mice

Histological analysis showed inflammation in the duodenum, jejunum and ileum of OVA/EW mice on day 7 of the EW diet (Fig. 4B–D). Inflammatory features, such as goblet cell hyperplasia, thicken muscular layer, crypt elongation and villous atrophy, were remarkable in these tissues. Importantly, repair of inflammation was observed in the duodenum and ileum of the mice on days 14 and 28 of the EW diet, although moderate inflammation was still observed in the duodenum after the long-term diet (Fig. 4C and D). In contrast, in the jejunum of OVA/EW mice, the inflammation was significantly aggravated by the continuous EW diet (Fig. 4B). On day 28 of the EW diet, the inflammation was severe and chronic in the greater part of the jejunal area (Fig. 4B). The continuous EW diet increased the numbers of mast cells in the duodenum and jejunum of OVA/EW mice (Supporting Information Fig. S4). Aggregated eosinophils were observed in the inflammatory region of the duodenum and jejunum on day 7 of the diet (Supporting Information Fig. S5). The inflammatory features were not observed in the intestinal tissues of non-sensitised mice on the EW diet

(PBS/EW) and OVA-sensitised mice on CA diet (OVA/CA), as shown in Supporting Information Figs. 4, S4–S7.

4 Discussion

Here, we successfully established a mouse model for intestinal food allergy developing subsequent tolerance using an inbred mouse strain. Although several mouse models for food allergy have been established [15–18], there are a few to develop tolerance and resolving allergic symptoms and/or intestinal inflammation [11, 19–21]. These models have used transgenic mice expressing monoclonal TCR against OVA artificially and/or adaptive transfer of regulatory T-cells to develop tolerance. Our mouse model using inbred BALB/c mice would be more versatile and useful for investigating inflammatory and regulatory mechanisms underlying food allergens.

Surprisingly, continuous feeding of the EW diet to the OVA-sensitised BALB/c mice induced chronic inflammation in the jejunum, although the animals resolved the allergic symptoms (i.e. weight loss) and gained tolerance against the allergen. The results suggest that the resolution of clinical symptoms and/or induction of clinical tolerance might not be a reliable indicator of recovery from food-related gastrointestinal inflammation. Continuous oral administration of allergenic foods has been performed recently in OIT to induce desensitisation or tolerance against the allergens in patients with persistent food allergy [2, 3, 8–10]. Although the study protocols and processes cannot be directly transferred from mice into humans, continuous feeding of allergen-sensitised mice with an allergenic food could mirror OIT to some extent. Our results suggest that the application of OIT in food-allergic patients with gastrointestinal inflammation may need to be reconsidered, since continuous administration with allergic foods may aggravate inflammation in local tissues.

We found that inflammation in the duodenum and the ileum was resolved in OVA/EW mice on the long-term diet, but chronic inflammation was induced in the jejunum. The results suggest that inflammatory and regulatory mechanisms may be different in these intestinal tissues. Our previous study showed that continuous feeding of the EW diet to OVA23-3 mice induced inflammation and subsequent resolution not only in the duodenum and the ileum, but also in the jejunum [11]. In the transgenic mice, T-cell-mediated mechanisms appear to be critical for the intestinal inflammation, since RAG-2 deficiency, namely deficiency of mature B cells did not attenuate induction of the inflammation [11]. It has been shown that migration of T-cells to intestinal tissues is critical for the induction of allergic reaction in the local tissues [22]. In the OVA-sensitised mice on the EW diet, T-cells would also be involved in the induction of the allergic inflammation in the intestinal tissues. Importantly, a predominant difference between the OVA23-3 mice and OVA-sensitised BALB/c mice is levels of allergen-specific IgE antibodies in our experimental setting. Levels of the IgE antibodies were low in the transgenic mice, since the animals were fed the EW diet without sensitisation using OVA and Alum. Notably, the number of mast cells, which express a high-affinity IgE receptor FcεRI, is higher in the jejunum compared with the ileum (Supporting Information Fig. S2). These observations suggest that IgE-associated mechanisms are responsible for the chronic inflammation in the jejunum of OVA/EW mice.

The long-term EW diet for 28 days appears to induce both systemic and local tolerance against OVA. However, severe inflammation was observed in the jejunum of OVA/EW mice on the long-term feeding. We hypothesise that it may be difficult to resolve inflammation in the jejunum by means of oral tolerance induction with allergenic foods: if high levels of allergen-specific IgE antibodies are present, the allergens would constantly activate IgE-bearing inflammatory cells in the local tissues.

Several studies have shown that IL-10 could suppress activation of mast cells and eosinophils [23, 24]. IL-10 appears to have a suppressive effect on local allergic inflammation in both humans and rodents, although the effect was mainly observed in allergic asthma and rhinitis [25–28]. In this study, MLN $CD4^+$ T-cells produced not only IL-4, but also IL-10 in response to OVA if the cells were isolated from the OVA/EW mice on day 7 of the EW diet, when the animals started resolving the weight loss (Fig. 2B). We further observed that long-term EW diet induced T-cell deletion in both spleen and MLN of OVA/EW mice. The MLN $CD4^+$ T-cells produced less IL-10 in response to OVA after the long-term diet (Fig. 3B). Notably, antigen dose is a primary factor determining mechanisms of oral tolerance; high dose of antigens induce T-cell deletion, whereas low dose of antigens induce active suppression mediated by regulatory T-cells [6, 7]. Taken together, our observations suggest that mechanisms of tolerance could shift from active suppression to T-cell deletion during the continuous EW diet. Loss of regulatory T-cells producing IL-10 by the long-term EW diet may also lead to the severe inflammation in the jejunum. It is necessary in future work to examine whether low doses of OVA in the diet could maintain IL-10 production by $CD4^+$ T-cells and resolve the jejunal inflammation in the OVA/EW mice.

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B. M. performed the majority of cellular assays and animal work in Germany. H. A. performed the animal work and the majority of histological analyses, and reviewed the manuscript in Japan. A. I. contributed to set up the animal works in Germany. A. P. supported to establish mMCP-1 measurement and reviewed the manuscript. S. H., H. K. and S. V. reviewed the manuscript.

M. T. wrote the manuscript and supervised the study. The results of cellular assay and histology were reproduced and confirmed both in Germany and Japan.

The authors have declared no conflict of interest.

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