

ORIGINAL ARTICLE

Allergen-specific antibody and cytokine responses, mast cell reactivity and intestinal permeability upon oral challenge of sensitized and tolerized mice

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Experimental
Allergy

Summary

Background Food allergy has reached an epidemic level in westernized countries and although central mechanisms have been described, the variability associated with genetic diversity underscores the still unresolved complexity of these disorders.

Objective To develop models of food allergy and oral tolerance, both strictly induced by the intestinal route, and to compare antigen-specific responses.

Methods BALB/c mice were mucosally sensitized to ovalbumin (OVA) in the presence of the mucosal adjuvant cholera toxin, or tolerized by intra-gastric administrations of OVA alone. Antibody titres and cytokines were determined by ELISA, and allergic status was determined through several physiologic parameters including decline in temperature, diarrhoea, mast cell degranulation and intestinal permeability.

Results OVA-specific antibodies (IgE, IgGs and IgA in serum and feces) were produced in sensitized mice exclusively. Upon intra-gastric challenge with OVA, sensitized mice developed anaphylactic reactions associated with a decline of temperature, diarrhoea, degranulation of mast cells, which were only moderately recruited in the small intestine, and increased intestinal permeability. Cytokines produced by immune cells from sensitized mice included T-helper type 2 cytokines (IL-5, IL-13), but also IL-10, IFN- γ and IL-17. In contrast, all markers of allergy were totally absent in tolerized animals, and yet the latter were protected from subsequent sensitization, demonstrating that oral tolerance took place efficiently.

Conclusion This work allows for the first time an appropriate comparison between sensitized and tolerized BALB/c mice towards OVA. It highlights important differences from other models of allergy, and thus questions some of the generally accepted notions of allergic reactions, such as the protective role of IFN- γ , the importance of antigen-specific secretory IgA and the role of mucosal mast cells in intestinal anaphylaxis. In addition, it suggests that IL-17 might be an effector cytokine in food allergy. Finally, it demonstrates that intestinal permeability towards the allergen is increased during challenge.

Keywords food allergy, IL-17, intestinal permeability, mast cells, murine models, oral tolerance, secretory IgA

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Introduction

Food allergy affects 2–4% of adults and 6% of children [1–3], incidences that are even currently increasing [4]. This disorder arises from a failure to induce oral tolerance towards innocuous food proteins, which generally leads to the production of allergen-specific IgE and, upon second exposure to the allergen, to various allergic manifestations [5]. Oral tolerance describes a specific suppression of

cellular and humoral immune responses towards an antigen after oral administration [6]. Although the general mechanisms of both events are known [1, 5, 6], several unknown mechanisms remain, as highlighted by the important differences of allergic manifestations associated with genetic diversity [7]. A better understanding of the mechanisms leading to allergy and oral tolerance will clarify prevention recommendations [8] and help to design adapted treatments, which are currently restricted to strict

avoidance of the allergen. This should allow the rational design of clinical studies aiming to manage food allergy.

During the last decades, murine models of food allergy and oral tolerance have been developed. In the case of allergy, a sensitization procedure with the antigen is required to bypass natural oral tolerance occurring in mice. A first sensitization procedure consists of a systemic sensitization (mostly BALB/c background) with the allergen and an adjuvant (alum or complete Freund's adjuvant), followed by repetitive oral challenges, which finally induce *intestinal* anaphylaxis [9, 10]. A second alternative consists of repetitive mucosal administrations of the allergen together with cholera toxin (CT) performed preferentially in the C3H/HeJ background. Subsequently, *systemic* anaphylaxis is triggered by a single intra-gastric challenge [11, 12]. These models allowed the identification of mechanisms involved in, or associated with, food-mediated allergic reactions. However, marked differences were observed in the intensities and types of symptoms when the combinations of mouse genetic backgrounds and allergen were shuffled [13]. In parallel, although oral tolerance has been extensively studied in various experimental models, very limited information exists as to a strict comparison between mucosally sensitized and tolerized animals. To date, such a comparison has been performed in two studies with one combination of allergen and genetic background [14, 15].

Here, we developed a model of food allergy using CT-induced sensitization and a model of oral tolerance using the allergen ovalbumin (OVA) in the BALB/c background. In both models, we used the same administration mode, time frame and allergen doses in order to ensure a strict comparison of immune and physiologic responses at the systemic and mucosal level. Analysis of antibodies, mast cells and cytokines underscored marked differences with previously published models. Furthermore, the model highlights the contribution of intestinal permeability in allergic reactions, and suggests that IL-17 might be an important cytokine in food allergies. Our results confirm the important diversity among rodent models of allergy and reveal the multi-factorial complexity of the mechanisms leading to allergy or tolerance.

Methods

Animals

Three- to 4-week-old female BALB/cOlaHsd mice (Harlan Netherlands, Horst, the Netherlands) were housed under standard conditions according to the institutional guidelines. Water and a commercial OVA-free diet were provided *ad libitum*.

Experimental protocols

The optimal sensitization protocol was established as 7 weekly intra-gastric administrations of 20 mg of OVA

(Crude stock, Fluka, Buchs, Switzerland) combined with 10 µg of the adjuvant CT (Calbiochem, Darmstadt, Germany) in a final volume of 200 µL of phosphate-buffered saline (PBS). Tolerization and CT control protocols followed the same time frame and mice were given 20 mg of OVA alone or 10 µg of CT alone, respectively. Allergic reactions were induced 1 week after the end of the experimental procedure in all groups by a single intra-gastric challenge with 100 mg OVA (Fig. 1a). In two experiments, the intra-gastric challenge was replaced by an intravenous (i.v.) challenge with 100 µg OVA (Worthington, Lakewood, NJ, USA). Each experimental group comprised 10 mice. All experimental procedures were approved by the State Veterinary Office and tested in mouse hepatitis virus containing and mouse hepatitis virus-free animal facilities.

Evaluation of allergic reactions

Rectal temperature was recorded before and 30 min after the challenge. Signs of allergic reactions (scratching, bristled fur, diarrhoea, labourious wheezing and abnormal immobility) were monitored in a blinded fashion by two independent knowledgeable individuals between 30 and 60 min post-challenge. Allergic scores were subsequently graded following an adapted pre-established scale [16]. More precisely: 0, no signs; 1, mice are scratching between four and 10 times for 15 min; 2, mice are scratching more than 10 times for 15 min, or display reduced activity or bristled fur; 3, mice have a strongly reduced activity, display liquid diarrhoea, have difficulty in walking normally, bristled fur and sometimes laboured respiration; 4, manifestations of degree 3 are stronger, mice display cyanosis around the mouth and tail; and 5, death.

Mice were killed 4 h post-challenge to recover blood, mesenteric lymph nodes (MLN), spleen (SPL) and jejunum samples for histological sections.

Processing of feces

Feces were freshly collected, solubilized in PBS containing protease inhibitors (CompleteTM; Roche Applied Science, Rotkreuz, Switzerland) and 0.001% sodium azide and adjusted to a 100 mg (wet pellet)/mL concentration. Feces were homogenized, incubated for 1 h at room temperature, centrifuged for 10 min at 10 000 g at 4 °C to remove solid material and finally frozen at -20 °C until use.

Quantification of ovalbumin-specific antibodies and seric mouse mast cell protease-1

ELISA was used to quantify OVA-specific antibodies in sera and feces as described previously [17], except that plates were coated with 500 ng OVA/well. Optimal sample dilutions avoiding assay saturation were determined as

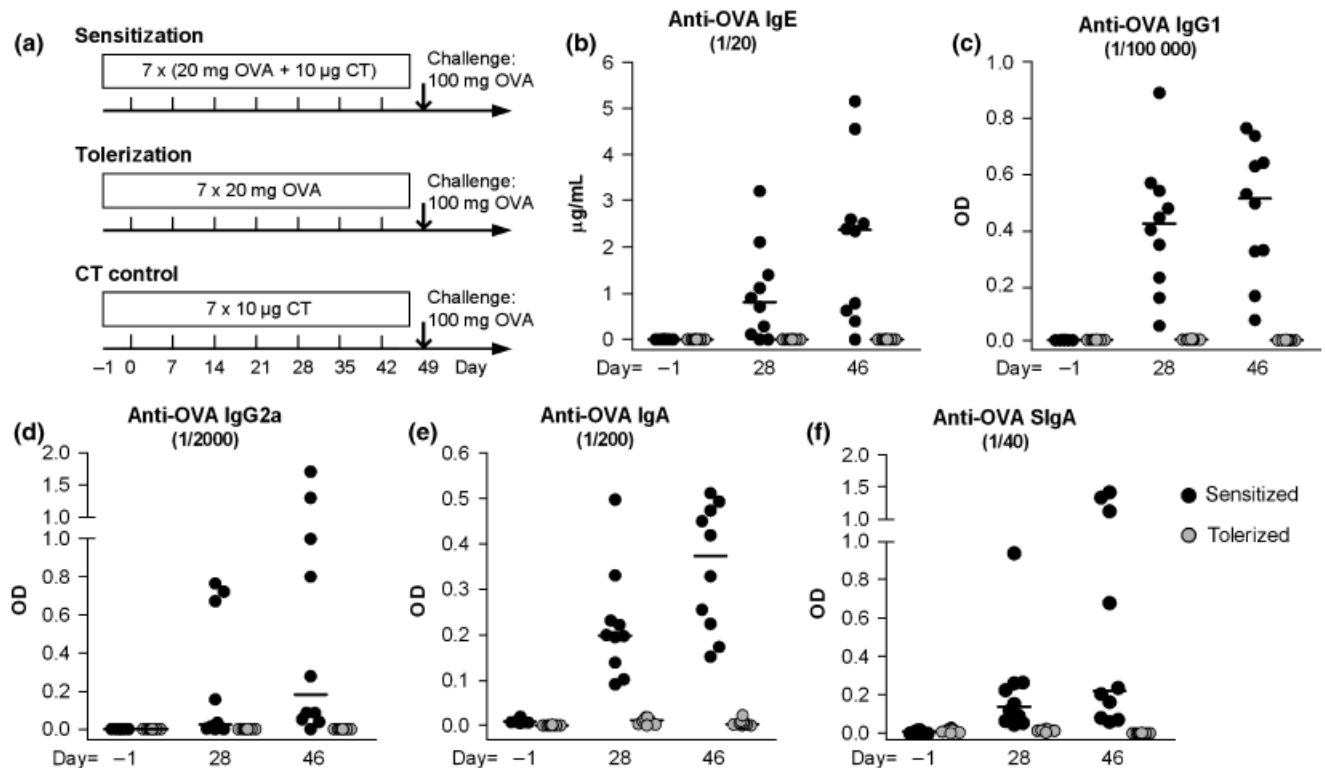


Fig. 1. Protocols and antibody responses. (a) Schematic drawing representing the experimental protocols and doses used in this work. Sera and feces were collected before (day = -1), during (day = 28) and at the end (day = 46) of the protocols; (b) serum ovalbumin (OVA)-specific IgE; (c) Serum OVA-specific IgG1; (d) serum OVA-specific IgG2a; (e) serum OVA-specific IgA; and (f) OVA-specific secretory IgA (SIgA) measured in feces. Data obtained from sensitized mice (black) and tolerized mice (grey) are presented and are expressed as optical densities (OD). OVA-specific antibodies were undetectable in the cholera toxin (CT) control groups at all dilutions (data not shown).

1/40 for secretory IgA (SIgA), 1/200 for IgA, 1/2000 for IgG2a, and 1/100 000 for IgG1. Owing to the very large amount of OVA-specific IgG, OVA-specific IgE were detected using an alternative procedure [10]. Briefly, plates were coated with anti-mouse IgE (BD Pharmingen, Basel, Switzerland), blocked and incubated with mouse sera diluted 1 : 20. Detection was achieved by a first incubation of biotinylated OVA and then of streptavidin coupled to horse radish peroxidase (HRP). Mouse mast cell protease-1 (MMCP-1) concentrations were quantified in the sera collected 1 day before and 3 h after the challenge following the manufacturer's instructions (Moredun Animal Health, Penicuik, UK).

Intestinal permeability measurement by quantification of serum ovalbumin

Intestinal permeability was assessed *in vivo* by quantifying OVA that translocated from the intestinal lumen to the blood. OVA or antibody complexed OVA were quantified by ELISA in the sera collected 1 day before and 1 h post-challenge. Plates were coated with rabbit anti-OVA (Abcam, Cambridge, UK) diluted at 10 µg/mL in 50 mM carbonate-bicarbonate buffer (pH 9.6). After 1 h of blocking with 5% non-fat dry milk, sera diluted 1/3 were incubated for 2 h at 37 °C to allow the binding of free OVA and

antibody complexed OVA. Then, detection was achieved in two steps: a first incubation with a labmade serum of sensitized but unchallenged mice was performed for 1 h at RT, and second, HRP-conjugated anti-mouse IgG (Sigma-Aldrich, Buchs, Switzerland) was added. To ensure optimal comparison between groups exhibiting or not OVA-specific antibodies, calibration curves were performed with OVA diluted in the serum of sensitized or naïve mice, respectively.

Mast cell staining

Jejunum samples were fixed in 4% paraformaldehyde, embedded in paraffin and cut into 4 µm cross-sections. Connective tissue mast cells (CTMC) were observed after toluidine blue staining and mast cells in the lamina propria (LPMC) were detected with fuchsine staining, as described previously [18, 19]. Mast cells from four independent cross-sections per mouse were counted under a microscope (Leica DM 1000, Heerbrugg, Switzerland, magnification: ×400).

Cell culture and cytokine quantification

MLN and SPL cells were isolated and processed as described [17] and a total of 5×10^5 cells/well were

stimulated for 5 days with or without 250 µg/mL of the OVA antigen. Supernatants were collected to determine cytokine concentrations by ELISA using commercial kits; IL-5 and IFN- γ (BD Pharmingen); and IL-13, IL-10 and IL-17A (R&D System, Abingdon, UK).

Statistical analyses

The Mann–Whitney test was applied for data of allergic scores and mast cell counts; otherwise, the unpaired Student's *t*-test was used. The limit of significance was set at $P = 0.05$; * $P < 0.05$, ** $P < 0.001$ and *** $P < 0.0001$. NS, not significant.

Results

The production of ovalbumin-specific antibodies is restricted to sensitized mice

Preliminary experiments allowed us to determine that optimal sensitization, assessed by OVA-specific IgE production, was achieved after seven intra-gastric administrations of 20 mg OVA and 10 µg CT. Following the same doses and time frames, we included two experimental control groups. First, mice were given OVA alone intra-gastrically and this group was named tolerized. Second, mice were treated with CT only in order to rule out potential bystander effects of the mucosal adjuvant, and referred to as CT control (Fig. 1a).

Serum and secreted allergen-specific antibodies were quantified in all experimental groups. OVA-specific antibodies were largely produced in sensitized mice (Figs 1b–f) and, as expected, were totally absent in the CT control group. Tolerized mice were also devoid of OVA-specific IgE and SIgA. OVA-specific IgA, IgG1 and IgG2a were undetectable in about 50% of tolerized mice, or only slightly produced [higher titres to obtain an optical density (OD) of 0.2 were 1/50 for IgA, 1/200 for IgG1 and 1/50 for IgG2a].

The presence of OVA-specific IgE in sensitized animals suggests that allergic reactions might occur upon challenge. Moreover, OVA-specific IgA, IgG1 and IgG2a were also markedly produced, as well as OVA-specific SIgA in feces (Fig. 1f). As abundant IgGs and SIgA might neutralize the allergen and thus prevent the onset of allergic reactions, we next examined whether sensitized mice developed allergic reactions upon an intra-gastric challenge.

Intra-gastric challenge triggers allergic manifestations in sensitized mice exclusively

Mice of all groups were challenged intra-gastrically with 100 mg OVA, and allergic reactions were evaluated according to three major read-outs: (i) the variation of body

temperature 30 min post-challenge, (ii) the allergic score, which includes the occurrence of diarrhoea (score $\geq 3/5$) and (iii) the degranulation of mucosal mast cells monitored by the presence of MMCP-1 in blood. Sensitized mice developed strong allergic reactions following OVA challenge as depicted by the pronounced decrease of body temperature, the elevated allergic scores and the high concentration of serum MMCP-1 (Figs 2a–c). In contrast, tolerized and CT control groups remained unaffected by the challenge, demonstrating the specificity of the sensitization protocol. However, the concentration of MMCP-1 before the challenge was higher in mice treated with CT as compared with the tolerized group. These important controls demonstrate that the adjuvant itself has an effect on mucosal mast cells; whether this reflects a direct action of CT on mucosal mast cells or is a result of a sensitization towards CT remains open [20]. It is clear, however, that only the shift between MMCP-1 concentrations quantified before and after the challenge reflects direct OVA-mediated degranulation.

Beyond these markers of allergy, it is known that patients suffering from food allergy have an increased intestinal permeability when exposed to the allergen [21]. Such a feature would make our model of mucosal sensitization a valuable mimic of what happens in humans. We thus set up a sensitive ELISA to quantify translocated OVA in mouse sera. The results presented in Fig. 3 show that intestinal permeability towards the allergen was increased in sensitized mice exclusively, and that, globally, microgram amounts of immunoreactive OVA reached the blood 1 h post-challenge. Next, to investigate whether such serum OVA was responsible for the allergic manifestations observed, systemic i.v. challenges with 100 µg of lipopolysaccharide-free OVA were performed. Compared with an intra-gastric challenge, the i.v. challenge triggered much stronger allergic manifestations in sensitized mice, leading to death within 30 min. In contrast, tolerized and CT control mice remained unaffected by the i.v. challenge (data not shown). This further confirms that mice were selectively sensitized, and suggests that the quantity of OVA that crossed the intestinal barrier is related to the severity of the allergic reactions. Altogether, these data indicate that the sensitization protocol restricted to the mucosal route can lead to both systemic and intestinal anaphylaxes.

Numbers of mucosal mast cells are only mildly increased in sensitized mice

The presence of increased MMCP-1 and the enhanced intestinal permeability prompted us to examine intestinal mast cells, which are key players in the development of allergic diarrhoea [9] and in the regulation of paracellular permeability in the rat [22]. Jejunum morphology was preserved, indicating that no major tissular alterations

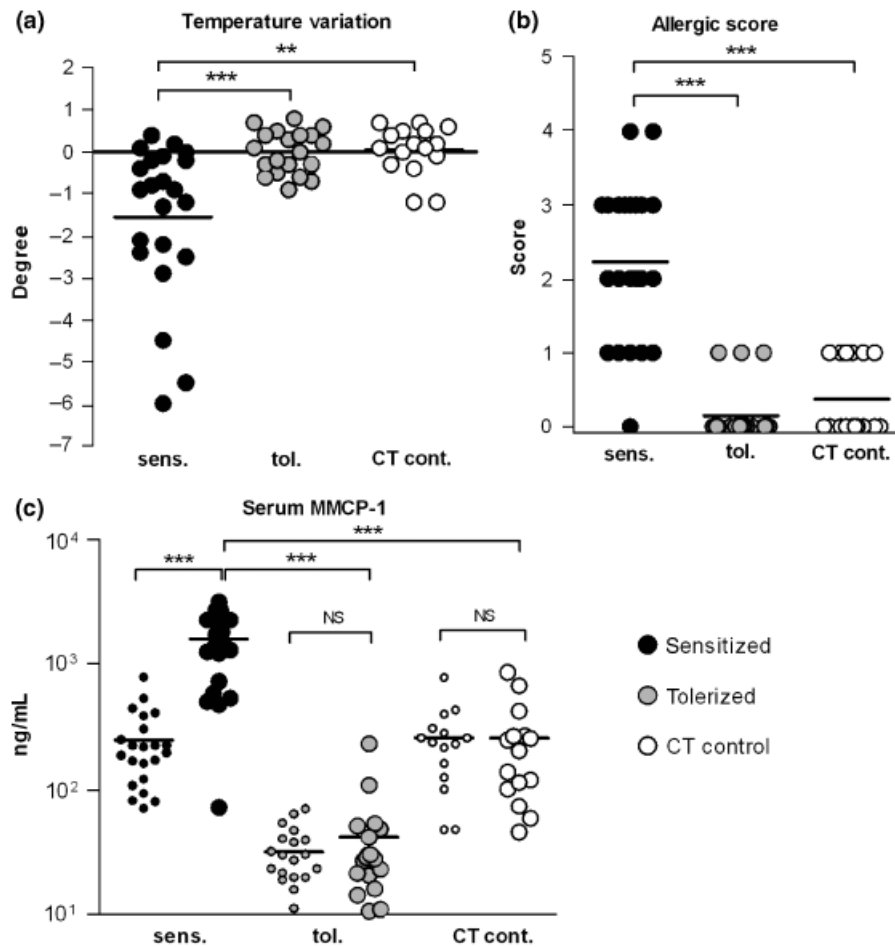


Fig. 2. Allergic manifestations. Sensitized, tolerized and cholera toxin (CT) control groups were challenged intra-gastrically with 100 mg ovalbumin (OVA) and allergic signs were monitored. (a) Temperature variation observed 30 min after challenge. (b) Allergic scores (see 'Methods'). (c) Concentration of mouse mast cell protease-1 (MMCP-1) before (small circles) and after (large circles) challenge.

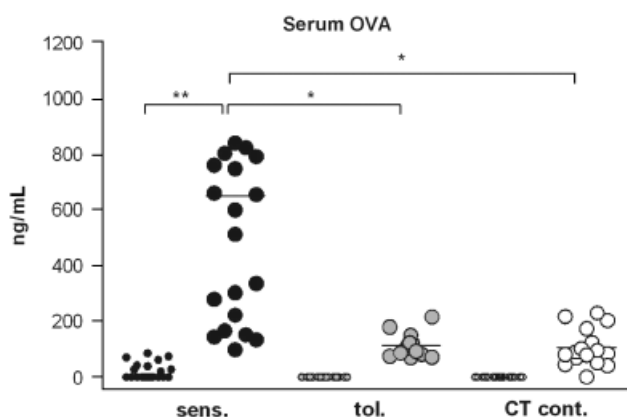


Fig. 3. Intestinal permeability. Intestinal permeability is reflected by the concentrations of immunoreactive ovalbumin (OVA) present in the sera of mice 1 day before (small circles) and 1 h after challenge (large circles). Sensitized mice (sens.) are shown in black, tolerized (tol.) in grey and cholera toxin (CT) control (CT cont.) in white. Data presented are a pool of two independent experiments, each performed with the three experimental groups.

occurred (Fig. 4a). By examining LPMC, and CTMC, we found that both populations were only mildly increased in the sensitized group compared with tolerized and CT control groups (Figs 4b and c). Thus, even though intestinal mast cells were only moderately recruited, it did, however, result in important intestinal manifestations (occurrence of diarrhoea in 50% of sensitized mice) upon mucosal challenge.

Cytokine profile of mesenteric lymph nodes and spleen cells

Because cytokines are important mediators of sensitization and tolerization mechanisms, we next examined the cytokine secretion profile of cells isolated from MLN and the SPL of all groups of mice. Following *in vitro* stimulation with OVA, we first measured the concentration of the allergy-associated T-helper type 2 (Th2) cytokines IL-5, IL-13 and IL-10, and observed a strong production by MLN and SPL cells from sensitized mice exclusively

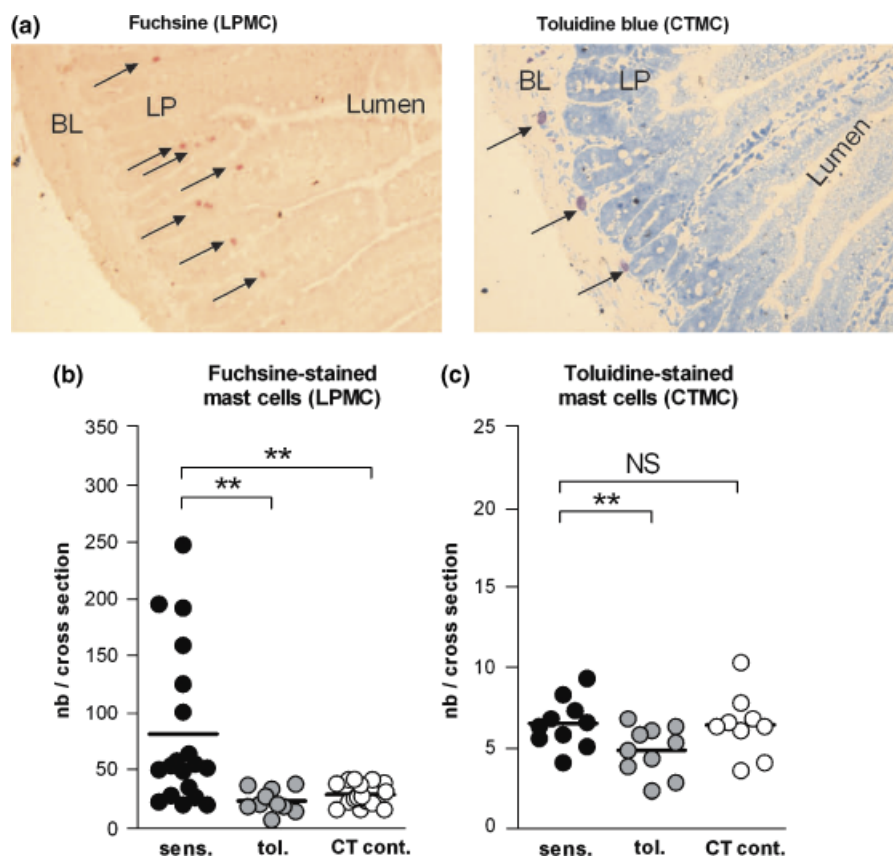


Fig. 4. (a) Pictures of jejunum cross-sections showing two different populations of mast cells (arrows). Mast cells stained with fuchsin (left panel) are detected within the lamina propria (LP) and are referred to as LPMC. Toluidine blue staining (right panel) highlights mast cells residing in connective tissue (CTMC) within the basal lamina (BL). (bc) Numbers of LPMC and CTMC detected per jejunum cross-section. Counts were obtained from four cross-sections per mouse and are depicted as the average value in dots.

(Figs 5a–c). As IL-10 is considered as a regulatory cytokine in other models, especially in the presence of TGF- β , we also quantified the latter, and found that it remained below the detection limit (<1 ng/mL; data not shown). As allergic reactions might not be restricted to a Th2-type of response, we further analysed the Th1 prototype cytokine IFN- γ , as well as the Th17-associated cytokine IL-17. A peculiarity of this model is that cells from sensitized mice also secreted these two cytokines in large amounts (Figs 5c and d). This indicates that despite being reported in many models as a negative modulator of allergic reactions, IFN- γ , however, did not counteract the production of Th2 cytokines and the onset of allergic reactions early after challenge. Furthermore, the presence of IL-17 suggests that the Th17 lineage might be associated with allergic reactions. Of note, concentrations of cytokines were almost equal in the supernatant of MLN and SPL cells, indicating that the sensitization process affected the mucosal and systemic compartments equally. In contrast, upon stimulation with OVA, cells from tolerized mice did not produce (or only very weakly produced) the various cytokines tested, and cells from CT controls

did not produce any cytokines. If the absence of an OVA-specific response of CT control cells was expected, the lack of any cytokine produced by cells from the majority of the tolerized mice was more surprising. Together with the absence of antigen-specific antibodies (Figs 1b–f), this suggests that the majority of OVA-specific cells were indeed actively suppressed and/or selectively depleted at the end of the tolerization protocol. Alternatively, we can also postulate that mice were ignorant of the OVA antigen (i.e. that OVA was never perceived by immunological cells), rather than specifically tolerized. We thus aimed to clarify this last question in the next experiments.

Tolerized mice are protected from subsequent sensitization and allergic manifestations

To determine whether tolerized mice were actually tolerized or simply ignorant, groups of mice were first tolerized (i.e. with seven intra-gastric administrations of OVA alone) and subsequently sensitized (i.e. with seven intra-gastric administrations of OVA+CT). Analyses of OVA-specific antibodies and allergic parameters triggered upon

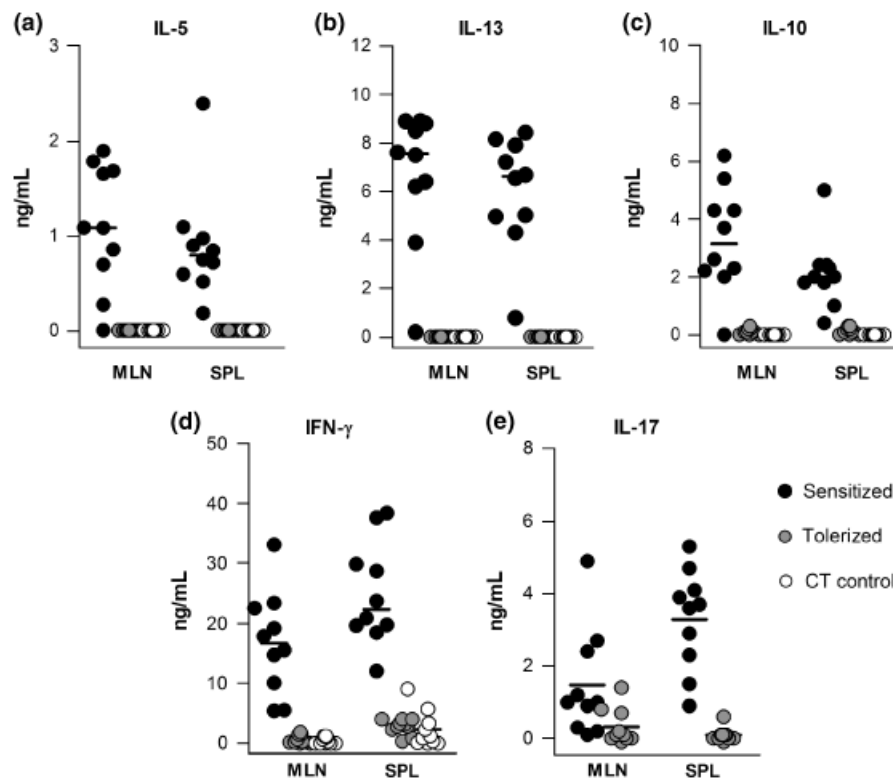


Fig. 5. Cytokine production by mesenteric lymph nodes (MLN) and spleen (SPL) cells. MLN and SPL cells from sensitized, tolerized and cholera toxin (CT) control groups were collected post-challenge and stimulated *in vitro* with 250 μ g/mL ovalbumin (OVA) for 5 days. Cytokine concentrations were determined in the culture supernatants by ELISA. (a) IL-5, (b) IL-13, (c) IL-10, (d) IFN- γ and (e) IL-17A. Stimulated cells from naïve mice resulted in undetectable concentrations of all cytokines tested, as well as cells kept in a plain medium (data not shown). The limits of detection were 10 pg/mL for each cytokine, except for IFN- γ , which was 100 pg/mL.

challenge are presented in Table 1. These data demonstrate that tolerized mice were protected from subsequent sensitization to OVA. OVA-specific IgE were undetectable in all mice, as well as most other antibody isotypes (IgG1, IgG2a, IgA and SIgA). Upon challenge, mice previously tolerized displayed no obvious allergic manifestations (no allergic scores or decline of temperature) and no degranulation of mast cells. Finally, stimulation with OVA of MLN and SPL cells collected at the end of the sensitization procedure did not result in substantial amounts of any cytokines. In contrast, mice first treated with PBS and then sensitized displayed strong allergic parameters, as in sensitized mice described in Figs 1, 2 and 5.

We can thus conclude that, although barely detectable in terms of antigen-specific responses (antibodies and cytokines), active tolerization was triggered and prevented subsequent sensitization of mice.

Discussion

In this work, we characterized a model of OVA-mediated allergy in the murine BALB/c background sensitized by procedures restricted to the mucosal route. This model allowed the identification of many features related to food

allergies at the mucosal and systemic level, as they appear in human patients. The production of OVA-specific IgE and allergic manifestations upon challenge (allergic scores, decline of temperature and degranulation of mucosal mast cells) were restricted to the sensitized group. The allergic phenotype was accompanied by a moderate recruitment of mucosal mast cells and an increased intestinal permeability. Although a high variability was observed in the sensitized group for most of the read-outs, differences from the tolerized and CT control groups were statistically significant. Furthermore, it is interesting to note that no correlation was found between these markers of allergy, suggesting that multiple and complex mechanisms underlie allergic reactions. Importantly, this model was tested in two animal facilities and similar results were obtained with only minor variations in the read-outs, emphasizing the robustness of this sensitization procedure (C. Perrier and A. Zuercher, unpublished data).

The tolerization protocol developed in parallel allowed us to identify the natural immune response of BALB/c mice towards OVA when administered intra-gastrically. This allowed a direct comparison of physiological and humoral and cellular immune responses with sensitized mice. While sensitized mice developed strong

Table 1. Allergic parameters of tolerized mice subsequently sensitized

	Tolerized sensitized	PBS sensitized	Statistics
OVA-specific antibodies (day of challenge)			
IgE (μg/mL)	BD	2.7±1.9	*
IgG1 (titre range)	BD-200	50 000–200 000	***
IgG2a (titre range)	BD-300	2000–10 000	***
IgA (titre range)	40–100	300–500	***
SIgA (titre range)	BD-10	10–500	*
Allergic manifestations upon challenge			
Allergic scores	0–1	2–3	*
Temperature variation (°C)	0.6±0.3	−1.9±0.2	***
MMCP-1 before (ng/mL)	380±260	1230±250	NS
MMCP-1 after (ng/mL)	440±280	10 600±3200	
Cytokines (ng/mL)			
IL-5			
MLN	0.11±0.05	2.1±0.5	**
SPL	0.06±0.04	2.5±0.3	***
IL-13			
MLN	0.04±0.01	2.8±0.6	*
SPL	0.02±0.01	5.3±1.2	*
IL-10			
MLN	0.64±0.14	3.6±0.6	***
SPL	0.20±0.08	3.5±0.6	***
IFN-γ			
MLN	0.84±0.32	24.4±3.3	***
SPL	3.25±1.14	35.3±3.9	***

Groups of tolerized mice were subsequently sensitized (tolerized-sensitized) and challenged intragastrically with OVA. All major allergic parameters were recorded as previously described and compared with groups of mice treated with PBS and subsequently sensitized (PBS-sensitized). OVA-specific antibodies were quantified by ELISA and expressed as $\mu\text{g/mL}$ (IgE) or as titres yielding an OD of 0.2. Data are presented either as lowest and highest ranges measured in individual mouse, or as means $\pm$ standard deviation (SD).

BD, below the limit of detection; OVA, ovalbumin; PBS, phosphate-buffered saline; OD, optical density; SPL, spleen; MLN, mesenteric lymph nodes; MMCP-1, mouse mast cell protease-1.

* $P < 0.05$, ** $P < 0.001$, *** $P < 0.0001$.

OVA-specific antibody responses at seric and mucosal levels, such responses remained below the limit of detection or very weak in tolerized mice. This observation differs from a previous study in which C3H/HeOuJ mice tolerized to β -lactoglobulin (BLG) presented a higher titre of allergen-specific SIgA in feces than sensitized mice [14]. Although the tolerization protocols used in these two studies are different (i.e. low dose of BLG *ad libitum* for 4 weeks vs. seven intra-gastric gavages with 20 mg OVA), this does not fully justify the dichotomy in the antibody response. Indeed, BALB/c mice tolerized with a low dose of OVA (0.8 mg/mL) in feeding bottles for 7 weeks displayed no OVA-specific antibody response throughout the protocol (data not shown). Therefore, the differences observed most likely reflect genetic susceptibilities to defined allergens. This demonstrates that antigen-specific secretory antibodies are not mandatory for efficient oral tolerance. These results add to the controversial role of allergen-specific SIgA, whose absence in patients might be associated with the onset of autoimmune disorders [23, 24].

The analysis of cytokines produced by immune cells from sensitized mice revealed that not only Th2 cytokines

(IL-5, IL-13 and IL-10) were produced but also the Th1 cytokine IFN- γ and the Th17 cytokine IL-17. This is of major interest as several reports have shown that the production of IFN- γ was protective against allergic reactions [13, 25, 26]. In our model, although produced in large amounts by MLN and SPL cells upon allergen stimulation, IFN- γ did not prevent the development of strong allergic reactions in sensitized mice. Thus, although IFN- γ might be associated with and even responsible for protection of mice in specific contexts, its mere presence cannot be considered as a universal protective mechanism. Such findings were also recently observed in a comparable model of sensitization to shrimp tropomyosin [27], confirming our results. Moreover, human studies demonstrated that allergen-stimulated IFN- γ^+ cells are present in allergic individuals [28, 29]. In this sense, the model described herein appears to reflect the situation in humans. The detection of substantial amounts of IL-17 raises novel perspectives in the understanding of food allergy, as it suggests that the Th17 lineage, associated with the production of IL-17, might contribute as effector cells in allergic reactions. Indeed, although Th17

cells were initially implicated in pulmonary host defense [30] and asthma [31], increasing evidences demonstrate their importance in various additional autoimmune diseases such as experimental autoimmune encephalomyelitis [32], multiple sclerosis [33] and inflammatory bowel disease [34]. Interestingly, Th17 cells and their associated cytokines (the IL-17 family, IL-21, IL-22 and IL-23) have paradoxical effects at the intestinal level, as they display protective/homeostatic and pathogenic/inflammatory functions [35]. This dual function was also observed at the level of the airways-associated immune system [36]. Altogether, the prevailing concept of the deleterious Th2 and protective Th1 and Th17 pathways does not apply in our model of gastrointestinal food allergy. In contrast, we observed a combination of the three types of responses (Th1/Th2/Th17) in sensitized animals as documented recently in models of autoimmunity and infection [37]. Further work is required to determine more precisely the contribution of Th17 cells in food allergy.

The absence of a cytokine response in MLN and SPL cells isolated from tolerized mice suggests that a large majority of OVA-reactive cells were depleted. We can thus speculate that in our model, oral tolerance preferentially relies on depletion mechanisms due to the high dose administered [38]. However, it cannot be strictly ruled out that suppressive mechanisms mediated by regulatory T cells took place. This work demonstrates that oral tolerance can take place without detectable allergen-specific SIgA in secretions, together with a very weak (detectable in some cases only) seric IgG response. However, the question of whether the immune system perceives, and subsequently induces active tolerance to every single ingested antigen, or only a small proportion of them, remains open. In a recent study, Turcanu et al. [39] showed that, although not allergic, children present a peanut-specific antibody response. Whether this is also true for other allergens, or any food protein needs to be further investigated.

Mast cells play a pivotal role in the development of allergic diarrhoea [9]. In our model, we observed that even a limited recruitment of mucosal mast cells was associated with strong intestinal symptoms (diarrhoea, a high concentration of MMCP-1 and increased intestinal permeability). It remains unclear whether such mucosal mast cells are mandatory for the systemic manifestations observed. It can be postulated that other mast cells distributed throughout the body, or basophils or even macrophages, might also participate in the allergic reactions. In the latter case, IgE-independent mechanisms might also occur [40, 41] via Fc γ -RIII and IgG, which reach high titres in our model. Nonetheless, degranulation of a few mucosal mast cells was sufficient to directly impact on the intestinal permeability of OVA, consistent with the observation that components released by mast cells increase paracellular permeability [22, 42, 43]. Together with the experiment of i.v. challenge, these data suggest that the more

OVA crosses the intestinal barrier, the stronger the allergic reaction. Together with a recent study demonstrating that increased intestinal permeability can predispose to sensitization towards an orally administered allergen [44], this highlights that altered intestinal permeability is not only a consequence but also a cause of food allergy. In support of this, it has been shown that CT increases intestinal permeability [45], a mechanism that certainly contributes to sensitization in our model. Collectively, this further argues that strengthening the intestinal barrier towards defined allergens can be considered as a preventive and therapeutic approach to treat food allergy.

We believe that the collection of parameters and the comparison with control groups underscore the validity of the model with respect to the complexity of the allergic disease, as it occurs in patients. We are confident that the mucosal model described herein will represent a solid alternative to explore the potential of orally delivered anti-allergic compounds in the most adequate context, before being tested in clinical trials.

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