IL-4 regulates susceptibility to intestinal inflammation in murine food allergy

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FOOD ALLERGY IS AN IMPORTANT cause of life-threatening hypersensitivity responses, and it is likely that, in common with other forms of atopy, both the incidence and prevalence are increasing (26, 27). Adverse reactions to food may result in a large number of clinical presentations involving skin, circulatory system, and the gastrointestinal tract and can lead to severe, potentially life-threatening anaphylactic reactions (7, 17, 29).

The capacity of T cells to produce an inflammatory reaction is largely controlled by their repertoire of cytokines (23, 24), and allergies have traditionally been considered to be Th2 cell-derived immunopathologies. In these atopic diseases, IL-4, IL-5, IL-9, and IL-13 are strongly implicated through their enhancement of immunoglobulin E (IgE)-mediated and eosinophilic immune responses (25). In accordance, the involvement of T lymphocytes and their type 2 products were already described as well as the enhanced allergen-specific IgE serum levels in atopic patients (11). IgE mediates allergic responses by binding to its high-affinity receptor, FceRI, expressed on target cells and the initiation of the epsilon (ε) heavy chain gene transcription that is induced by IL-4 and IL-13, whereas IFN-γ inhibits it (21).

The development and control of inflammatory reactions in allergy is mediated by antigen presentation and the resulting T helper (mainly Th2) or regulatory responses. Whereas, in allergic sensitization to food antigens, gastrointestinal dendritic cells are able to induce Th2 responses via OX40L (1), CD4+CD25+ T cells are crucial for the induction of tolerance to peanut proteins by regulating the IgE antigen-induced responses (37). Then, through complex interactions, cytokines not only cooperate but also antagonize each other’s functions (5). In accordance, IL-4 is a pleiotropic cytokine with regulatory effects on B cell growth, T cell growth and function, immunoglobulin switching, hematopoietic cells, and tumor cells (20, 31) and, as described above, is a critical factor for the development of type 2 immune responses (10, 12). There is evidence to support both anti- and counterinflammatory roles for IL-4 in experimental models of intestinal inflammation. Previous studies have reported the expression of ε germ-line and IL-4 transcripts in human intestinal mucosa as well as enhanced expression in patients with food allergy (6). Additionally, the involvement of IL-4 in other forms of atopic diseases, like allergen-induced airway remodeling was also described, demonstrating the crucial role for this cytokine in the development of Th2 pathological responses (13).

With the advent of manufactured food, trace amounts of allergens can still be present in food, and some atopic patients appear to be continuously exposed to these antigens. In these patients, continuous unwilling exposure to a food allergen may induce a mild and persistent allergic condition, besides unexpected and fatal anaphylactic reactions. To mimic this clinical situation, we recently developed an experimental model in which mice sensitized with peanut proteins extract (PPE) were given peanut seeds as the only feeding source to develop chronic allergic intestinal inflammation (4). This study suggested a role for allergenic food antigens in the modulation of mucosal immunity, probably by inducing IL-4-dependent allergic responses.

However, although it is clear that Th2 responses play a pivotal role in the development of allergic responses, it remains
unclear which mechanisms are involved in the development of the intestinal damage that underlies the onset of food allergy. Accordingly, in the present work, we elucidated the role of IL-4 in the modulation of intestinal immunity during the development of food allergy in vivo.

MATERIALS AND METHODS

Mice sensitization and induction of food allergy. For the induction of food allergy, C57BL/6 wild-type (WT) and IL-4−/− (Il4tm1Nnt/J) mice, from the same genetic background (6–8 wk old, n = 5 animals/group), were sensitized with PPE and challenged with peanut seeds, as described previously (4). Control mice were divided in the nonsensitized (NS) group, which received the first sensitization with PBS plus aluminum hydroxide followed by a second sensitization with PBS only and was not submitted to the diet containing peanut seeds; the peanut group, the group submitted to the same protocol of sensitization as the NS group, but animals were fed peanut seeds for 30 days beginning a week after the last sensitization; and the sensitized group (S), the group sensitized twice with PBS containing PPE but not challenged with the peanut diet. Water was available, and animals were weighed continuously throughout the experiment. All mice were anesthetized, bled, and euthanized on day 30 after introduction of the peanut diet. The experimental procedures followed the guidelines of APS guiding principles in the care and use of animals, and the protocols used were approved by the School of Medicine of Ribeirão Preto Institutional Animal Care and Use Committee.

Histopathological analysis. Small and large gut segments were collected, formalin fixed, and paraffin embedded. Sections (5 μm thick) were stained with hematoxylin and eosin and inspected for the presence of mucosal inflammation. Grading of intestinal inflammation was determined in a blinded fashion, as described previously (19). Briefly, sections of mice jejunum were scored individually, as follows: epithelium: 0-normal morphology, 1-exulceration; infiltration: 0-no infiltrate, 1-mild infiltrate, 2-moderate infiltrate, 3-extensive infiltrate; edema: 0-no edema, 1-mild edema, 2-moderate edema, 3-severe edema; damage in gut architecture: 0-no damage, 1-mild damage, 2-severe damage, 3-severe damage. The total histological score represents the sum of all features evaluated and thus ranges from 0 to 10.

Myeloperoxidase activity assay. The extent of granulocyte accumulation in the intestinal mucosa was measured by assaying myeloperoxidase (MPO) activity, as previously described (32). Briefly, 50–100 mg of small gut tissue were homogenized in two volumes of ice cold buffer (0.1 M NaCl, 20 mM NaPO4, 15 mM Na EDTA, pH 4.7, and centrifuged at 600 g for 15 min. The pellet was then subjected to hypotonic lysis (900 μl of 0.2% NaCl solution followed 30 s later by addition of an equal volume of a solution containing 1.6% NaCl and 5% glucose). After further centrifugation, the pellet was resuspended in 50 mM NaPO4 buffer, pH 5.4, containing 0.5% hexadecyl trimethylammonium bromide, and rehomogenized. The homogenate was then frozen and thawed three times and centrifuged again at 3,000 g for 15 min at 4°C. MPO activity in the supernatant was assayed by 10.220.33.3 on July 8, 2017 http://ajpgi.physiology.org/ Downloaded from the threshold (Ct) method, and the results were depicted as arbitrary units. ELISA measurements. ELISA-specific IgG, IgG1, IgG2a, and IgE serum levels were measured by ELISA on a 96-well polystyrene ELISA plates (low binding, Corning; New York Costar, Acton, MA) coated with PPE (20 μg/well). Samples were measured in serial twofold dilutions (1:100-1:6,400), incubated with anti-IgG, anti-IgG1, anti-IgG2a, or anti-IgE biotinylated antibodies (Southern Biotechnology Associates, Birmingham, AL), followed by streptavidin-horseradish peroxidase-specific conjugates (Vector Laboratories, Burlingame, CA). Reactions were developed with TMB (KPL), and results were interpreted as ELISA indexes (EI) on the basis of previous description (30). Briefly, liquid optical density (OD) on optimal sera dilution (1:100) was obtained by the subtraction of the sample’s average OD value and blank wells. Cut-off titers were set on the basis of mean liquid OD of negative controls with the addition of three standard deviations. EI were acquired by the formula sample liquid OD/cut off. EI values under one were considered negative results. All samples were analyzed for a minimum of two to three times.

Real time PCR. Total RNA from jejunum segments was extracted using the TRIZOL reagent (Invitrogen, Carlsbad, CA) and Promega RNA extraction kit (Promega, Madison, WI), according to the instructions supplied by the manufacturer. The concentration of RNA was determined, and complementary DNA was synthesized using 1 μg of RNA through a reverse transcription reaction (M-MLV reverse transcriptase, Promega). Real-time PCR quantitative mRNA analyses were performed on the ABI Prism 7000 Sequence Detection System using the SYBR-green fluorescence quantification system (Applied Biosystems, Warrington, UK) for quantification of amplification. The standard PCR conditions were 95°C for 10 min, followed by 40 cycles for 1 min at 94°C, 56°C (1 min), and 72°C (2 min), followed by the standard denaturation curve. The sequences of murine primers were designed using the PrimerExpress software (Applied Biosystems, Warrington, UK) using nucleotide sequences present in the GenBank database and were as follows: β-actin: sense AGC TGC GTT TTA CAC CCT TT, antisense AAG CCA TGC CAA TGT TGT CT; TNF-α: sense TGT GCT CAG AGC TTT CAA CAA, antisense CTT GAT GGT GGT GCA TGA GA; IFN-γ: sense GCA TCT TGG CTG AGC T, antisense CCT TTT TCG CCT TCG TGT TG; IL-12 p40: sense AGC ACC AGC TTC TTC ATC AGG, antisense GCC CTG GAT TCG AAC AAA G; IL-5: sense GAG GAT GTC GGT GTG TT, antisense GCC GTA GCC GTA CAC AAC AA; TGF-β: sense GCT GAA CCA AGG AGA CGG AAT, antisense GCT GAT CCC GTT TTC TTG CA; IL-10: sense TGG ACA ACA GAC ATG CAC CAT T, antisense TCA GTT GGT AAC ATG CAC AAA G; TGF-β: sense GCT GAA CCA AGG AGA CGG AAT, antisense GCC CAC CTT TTC TIG GTT TTG. PCR conditions for each target gene were optimized with regard to primer dimmer formation, efficiency of amplification of target genes, and housekeeping gene control. Threshold for positivity of real-time PCR was determined on the basis of negative controls. The results were demonstrated as mRNA expression of the NS, Peanut, S, and IL-4−/− groups.

RESULTS

Exposure to peanut seeds leads to weight loss in C57BL/6 WT but not in IL-4−/− mice. To verify whether IL-4 was involved in the development of systemic or intestinal manifestation of allergy or even in any gastrointestinal disorder, mice were sensitized, challenged with peanut seeds, weighed weekly, and followed for the development of diarrhea, intestinal bleeding, or rectal prolapse. Results showed that, although WT animals from both experimental (S + Peanut) and the control group (Peanut) submitted to peanut diet presented an apparent loss of weight throughout the experimental period, IL-4−/− mice...
gained weight, especially 30 days after introduction of peanut seeds in their diet, as visualized in Fig. 1A (S + Peanut vs. S + Peanut knockout (KO), P < 0.05). The increase in body weight of IL-4−/− animals was accompanied by an increase in peanut consumption by the S + Peanut KO group, especially in the second week of peanut diet, in contrast to diminished ingestion of the seeds by WT animals in the same period (Fig. 1B). No signs of diarrhea, intestinal bleeding, or rectal prolapse were found during the experimental period in any group analyzed.

**Absence of inflammation and maintenance of gut integrity in IL-4−/− mice sensitized and challenged with peanut seeds.** To determine whether the gain of weight in IL-4−/− animals in contrast to weight loss in WT mice was related to any kind of gut histopathological alteration in food allergy, segments from WT and IL-4−/− mice were evaluated for the development of inflammation. As demonstrated in Fig. 2, A and B, only WT mice sensitized and challenged with peanut seeds developed intestinal inflammation, characterized by epithelial exudation, edema, and leukocyte infiltrate in lamina propria, along with disruption of small gut architecture. These changes were found in duodenum, jejunum, or ileum, and no marked changes were found in large bowel. On the other hand, intestinal segments from experimental and control IL-4−/− mice were absent of any mucosal inflammation, with no signs of edema or disruption of small or large bowel architecture. The jejunum segments were scored for microscopic inflammation, and the results also demonstrated absence of intestinal alterations in IL-4−/− mice in contrast to a huge increase in the inflammatory score in WT S + Peanut animals (Fig. 2B, P < 0.05), suggesting a major role for IL-4 in the development of the intestinal pathology in WT allergic mice. Moreover, to address the involvement of mucosal mast cells in the inflammatory infiltrate of allergic mice, we performed real-time PCR assays for MMCP-1. The results showed an increase in mRNA expression for this protease especially in the WT S + Peanut group compared with diminished detection in the IL-4−/− counterparts, demonstrating that these cells must also be involved in the allergic intestinal inflammation (Fig. 2C, P < 0.05). In addition, we evaluated the neutrophil influx by MPO activity in the gut tissues, and the results also demonstrated elevated MPO detection in WT S + Peanut compared with S + Peanut IL-4−/− animals (Fig. 2D, P < 0.05). We did not find differences in eosinophil peroxidase activity, as observed in eosinophil peroxidase assays (data not shown).

**Peanut sensitization and challenge induce a shift toward Th1 immunoglobulin response in IL-4−/− mice.** To evaluate the involvement of IL-4 in immunoglobulin production and isotype switching toward Th2 responses and food allergy, we next investigated the production of PPE-specific antibodies in IL-4−/− submitted to the protocol of development of food allergy. We found that WT and IL-4−/− mice sensitized (S) or sensitized and challenged with peanut proteins (S + Peanut) presented a higher production of PPE-specific IgG antibodies compared with the nonsensitized controls (Fig. 3A). Since peanut proteins are frequently described as antigens able to induce allergic responses in susceptible individuals, we then asked whether the intestinal inflammation could be related to a Th2/IL-4-dependent allergic response raised against PPE, as suggested by the data obtained from WT mice. As demonstrated in Fig. 3, C and D, there was a significant decrease in IgG1 and IgE antibodies in sera from IL-4−/− animals sensitized or sensitized and challenged with peanut seeds, compared with the related WT mice (P < 0.05). Interestingly, sensitized IL-4−/− mice presented an increase in IgG2a antibodies production in sera in contrast to WT ones, especially after peanut challenge, as demonstrated in Fig. 3B (P < 0.05). These results are in agreement with previous data that showed the participation of Th2 responses in peanut allergy, suggesting an important role for IL-4-dependent antibody production in the modulation of mucosal allergic responses.

**IL-4 modulates gut cytokine and Foxp3 expression in response to peanut sensitization.** To investigate the role of Th2/IL-4-dependent responses in the modulation of gut mucosal immunity in food allergy, we further characterized the expression of cytokines and the regulatory factor Foxp3 in the small gut of WT and IL-4−/− animals. Exposure to peanut seeds induced a marked reduction in TNF-α mRNA expression in the jejunum of IL-4−/− mice compared with WT animals (Fig. 4A; Peanut vs. Peanut KO and S + Peanut vs. S + Peanut KO; P < 0.05). On the contrary, in the absence of IL-4 expression, the levels of the Th1 cytokines IFN-γ and IL-12p40 were substantially increased in KO mice sensitized and challenged with peanut seeds (Fig. 4, B and C, respectively, P < 0.05). Moreover, the induction of Th1 responses was accompanied by significant elevated expression of the regulatory cytokines TGF-β and IL-10, especially in the gut of S + Peanut mice compared with the related WT animals (Fig. 4, E and F, respectively, P < 0.05), besides diminished detection of Foxp3 messages in WT compared with IL-4−/− animals (Fig.

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**Fig. 1.** Variation in body weight and peanut consumption in wild-type (WT) and IL-4−/− sensitized and challenged (S + Peanut) mice. The gain or loss of weight after the 30-day challenge is visualized as percentage of the animals' weight in the first day of peanut diet (A). Peanut consumption throughout the chronic exposure to the seeds (B). Results are representative of 3 to 5 independent experiments, *P < 0.05, NS, nonsensitized group; KO, knockout.
These results demonstrated the relevant role for IL-4 in the induction of Th2 pathological responses and in the modulation of cytokine interplay and regulatory factors in the gut of allergic mice.

**DISCUSSION**

IL-4 directly induces the differentiation of T cells playing a central role in the development of committed effector Th2 lymphocytes (34). However, it remains unclear whether this immune response induced by ingested allergens could explain the development of the enteropathy observed in patients. Therefore, in this work we investigated the role of Th2 responses in the pathogenesis of food allergy using IL-4−/− mice and a model of allergic enteropathy induced by dietary allergens. In this model, mice were fed peanut seeds after a first encounter with the antigens by sensitization with PPE.

The mucosal immune system is usually equipped with complex mechanisms that prevent tissue injury in the gut. In this study, the relevant alterations observed in WT but not in IL-4−/− mice were of great importance to allow the elucidation of the role of IL-4 in the induction of pathological responses and in the modulation of cytokine interplay and regulatory factors in the gut of allergic mice.

Fig. 2. Sensitization and challenge with peanut seeds do not induce gut inflammation in IL-4−/− mice. At the end of the food allergy-inducing protocol, gut segments were collected and processed for histopathology, PCR, or myeloperoxidase (MPO) analysis. A: in jejunum from WT S + Peanut mice, note the intense edema, tissue destruction, inflammatory infiltrate, and villous enlargement, accompanied by enterocyte epithelial exulceration, in contrast to the absence of histopathological alterations in animals deficient for IL-4 B: microscopic inflammatory score of jejunum damage in WT and IL-4−/− animals. C: mucosal mast cell protease (MMCP)-1 mRNA expression (arbitrary units). D: MPO activity in small gut segments. Results are representative of 3 to 5 independent experiments. Scale bar = 250 μm. *P < 0.05.
and characterization of the extent of mucosal damage and immune modulation induced by allergic food antigens in the intestine. Such immune activation probably occurred after antigen recognition and loss of oral tolerance to peanut following the continuous exposure to the seeds with the relevant involvement of Th2 responses, as observed by the absence of intestinal inflammation in IL-4-deficient animals.

The first clinical sign of mucosal modulation by peanut ingestion was the loss of weight, associated with diminished consumption of peanut seeds by WT mice in contrast to IL-4−/− ones, which gained weight. Although the precise reasons for such behavior are not known, the higher consumption of peanut seeds associated with the gain of weight in IL-4−/− mice could, at first, explain the weight loss observed in WT animals sensitized and challenged with peanut seeds. Although the probable absence of a nutritionally equilibrated diet and an expected intestinal inflammation could also account for the weight loss, there is no doubt that IL-4-dependent/allergic mechanisms are involved in the variation of body weight observed in WT but not in IL-4−/− animals. In the gut, an aggressive immune response against food antigens can be wasteful and lead to destructive inflammatory immune disorders. Together with weight loss, the features of gut inflammation in WT mice were contrasting to those found in animals deficient in IL-4, suggesting the pathogenic role of Th2 reactions, mucosal mast cells, and neutrophils in the development of intestinal damage in food allergy. Our data agree with previous studies (19), which demonstrated that allergic diseases rely on IL-4-dependent responses, besides the influx of polymorphonuclear cells (7) and the involvement of mast cell mediators released after IgE-dependent degranulation (2). Moreover, it is clear that IL-4 is also important to the induction of colitis in IL-10 KO mice, another model of breakdown of mucosal tolerance, by promoting a deleterious Th2 effector response (33).

It is known that intestinal anaphylaxis in mice is accompanied by augmented permeability in the gut (2), similarly to patients with food allergy who present increased intestinal permeability (38). Although we did not perform permeability assays, we believe that the allergic phenotype observed in WT but not in IL-4−/− mice could also be a result of increased mast cells and a defect in the intestinal barrier function. Together, these factors probably led to a predisposition to peanut sensitization and intestinal inflammation since mast cell-mediated intestinal permeability seems to promote oral antigen sensitization that results in intestinal anaphylaxis (8).

The central feature in allergic responses is the enhanced allergen-specific IgE production in atopic patients in whom IL-4 initiates the epsilon (ε) heavy chain gene transcription (21). Therefore, we hypothesized that the Th2 deficiency could also account for lowering the production of allergic antibodies and consequently diminished immunopathology in the gut. As expected, IL-4−/− animals presented higher IgG2a and diminished IgG1 and IgE production in sera, confirming the relevant role of this cytokine in immunoglobulin isotype switching and Th2 antibody-mediated food allergy. These results agree with the high levels of peanut-specific IgE in patients with peanut allergy (15).

The modulation of mucosal immune responses is crucial to the maintenance of gut homeostasis and prevention of destructive enteropathies. As reported (4), our previous study is relevant in showing that allergenic protein consumption induces an altered intestinal immune response resulting in the reduction of the local suppressor/tolerance pathways. Then, since IL-4-deficient animals did not present weight loss, intestinal inflammation, or high Th2 antibody production in response to peanut proteins, we therefore evaluated the gut cytokine and regulatory factor interplay in the modulation of local immune response and development of the intestinal pathology. The lower expression of TNF-α in the IL-4−/− gut in contrast to high levels in WT animals agreed with an ileocolitis model in which the imbalanced production of this cytokine led to abnormal adaptive immune response and intestinal pathology (14). In addition, in our study, the diminished TNF-α expression was accompanied by a shift toward a Th1 profile in the intestine of IL-4−/− mice sensitized and challenged with peanut seeds. We believed that this increase in IFN-γ and IL-12p40...
levels might have accounted for a counter regulation in the balance between the Th2 pathogenic and Th1 protective immune responses in this scenario of food allergy. Indeed, production of IL-12 by Peyer’s patch-dendritic cells during antigen presentation is critical for the resistance to food allergy (35), whereas, in another murine model of peanut allergy, treatment with a Chinese herbal medicine preparation reestablished oral tolerance through upregulation of IFN-γ production in mesenteric lymph node cells (22). Additionally, the essential regulators of abnormal inflammatory processes, TGF-β and IL-10 (9, 28), besides the regulatory cell transcription factor Foxp3 (16), were also substantially altered in the absence of IL-4, demonstrating that this cytokine profoundly interferes with the induction of a regulatory phenotype and control of pathogenic immune responses in the peanut allergic enteropathy. In fact, it was recently demonstrated that IL-4 inhibits the induction of Foxp3 and then the generation of inducible regulatory T cells (16). Subsequently, although in some other experimental inflammatory disorders IL-4 functions as anti-inflammatory cytokine (3), our data corroborated once more with the hypothesis of the modulation of gut immune response toward inflammation and a Th2 profile of cytokine production in the presence of peanut allergenic antigens (36, 39). The therapy for IgE-dependent food allergy is based mainly in the elimination of potential harmful diets (29); however, trace amounts of allergens...
present in industrialized foods can be of difficult avoidance and become fatal to allergic individuals. Therefore, the inhibition of Th2 cytokines or induction of regulatory mechanisms in the gut may be a relevant therapeutic approach to prevent gut inflammation and food allergy. The results presented here fit within the idea of the contradictory role and the need of balance among regulatory, Th2, and Th1 responses to the modulation of pathogenic allergic responses (18), especially in food enteropathy. Furthermore, the clarification of the mechanisms underlying the development of food-sensitive intestinal inflammation will provide an important clue to combating food allergies.

Finally, this study highlighted some of the complex mechanisms involved in the pathogenesis of the allergic responses to food antigens in the intestine. The exploitation of regulatory pathways, reestablishment of the oral tolerance to food antigens, and understanding the factors responsible for Th2 sensitization at sites of allergen exposure, such as airway, skin, and, in this case, the gut, are crucial for directing novel therapeutic or preventive strategies to controlling food allergy and the consequent enteropathy in susceptible patients.

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