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Modulation of mucosal immunity in a murine model of food-induced intestinal inflammation

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Clinical and Experimental Allergy

Summary

Background Hypersensitivity or uncontrolled responses against dietary antigens can lead to inflammatory disorders like food allergy and current models reflect a variety of causes but do not reveal the detailed modulation of gut immunity in response to food antigens after breakdown in mucosal tolerance.

Objective To develop and characterize a murine model for food-induced intestinal inflammation and to demonstrate the modulation of gut immune response by dietary allergenic antigens.

Methods C57BL/6 mice were sensitized with peanut proteins, challenged with peanut seeds and their sera and gut segments were collected for subsequent analyses.

Results Sensitization and challenged with peanut seeds led to alterations in gut architecture with inflammatory response characterized by oedema in lamina propria and cell infiltrate composed mainly by eosinophils, mast cells, phagocytes, natural killer and plasma cells, together with low percentage of $\gamma\delta^+$ and $CD4^+CD25^+Foxp3^+$ cells in Peyer's patches. These animals also presented high levels of specific IgE and IgG1 in sera and modulation of mucosal immunity was mediated by increased expression of GATA-3, IL-4, IL-13 and TNF- α in contrast to low IFN- γ in the gut.

Conclusion A murine model for food-induced intestinal inflammation was characterized in which modulation of gut immunity occurs by peanut antigens in consequence of T-helper type 2 (Th2) allergic response and failure of regulatory mechanisms necessary for mucosa homeostasis, resembling food allergy. This work shed some light on the understanding of the pathogenesis of gastrointestinal disorders and intolerance in the gut and supports the development of therapies for food-related enteropathies like food allergy, focusing on gut-specific immune response.

Keywords animal model, food allergy, intestinal inflammation, mucosal immune response, peanut

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Introduction

The physiological access of dietary macromolecules into the organism through the gut mucosa may result in an abnormal response to ingested nutrients and lead to several detrimental immunological consequences, like food allergy. Allergies are described as the ability of some proteins to induce immune responses, in particular IgE production, which is considered one of the major effectors

in food allergic reactions [1, 2]. There is no doubt that food allergy is an important cause of life-threatening hypersensitivity responses and it is likely that, in common with other forms of atopic allergic disease, both the incidence and prevalence are increasing [3, 4]. Adverse reactions to food may result in a large number of clinical signs involving skin, circulatory system and the gastrointestinal tract, and can lead to fatal final consequences like hypotension and ultimately anaphylaxis [5, 6]. Fatal

anaphylactic reactions induced by peanut seeds are not uncommon [7, 8] and peanut allergy is one of the most severe food allergies due to its persistency, accidental ingestion and life-threatening character [4].

Mucosal immune responses to ingested food antigens are generally based on antigen exclusion along with suppressive immunity leading to oral tolerance [9] that is abrogated in food allergy [10]. In this case, the initiation of an immune response requires interactions between non-degraded proteins and gut-associated lymphoid tissue, and the resulting active immunity against such non-pathogenic matters leads to hypersensitivity responses and allergic reaction [11, 12]. However, the mechanism by which the normal intestinal immune system responds to food antigens and its involvement in the development of food-related enteropathies remains essentially unresolved.

Besides the relevance of using animal models to provide information regarding the potential allergenicity of foods derived from biotechnology [13], several animal studies have been performed to clarify mechanisms underlying hypersensitivities or allergies caused by food antigens and to enhance progress towards novel therapies against them [10, 14, 15]. However, to our knowledge, there is no current animal model that markedly resembles human food allergy and describes in depth intestinal alterations of mucosal immunity that occur after breakdown of oral tolerance to ingested nutrients. Therefore, an important advance in the study of the modulation of gut immune system associated with intolerance to food antigens could be the development and subsequent analysis of novel animal models that better illustrate the gastrointestinal immunological manifestations that are raised after exposure to some allergenic food antigens.

Accordingly, in this study we developed a murine model for food-induced intestinal inflammation that resembles food allergy, and characterized the relevant gut mucosal alterations that could be associated to the outcome or prevention of food allergic reactions in susceptible individuals.

Methods

Peanut protein extraction and electrophoresis

The extraction of peanut proteins was conducted according to Landry and Moureaux [16], with modifications. Briefly, commercially available raw peanuts were crushed and mixed with 0.0125 M sodium borate buffer containing 1% SDS and 2% β -mercaptoethanol. After shaking for 30 min at 4 °C, the contents were centrifuged for 30 min at 4 °C, 1600 g. The supernatant was collected and proteins were quantified by Coomassie Blue assay (Pierce, Rockford, IL, USA). Electrophoresis was conducted on 12.5% SDS-PAGE under reducing conditions. Proteins were visualized with Coomassie Blue staining (Bio Rad Laboratories, Hercules, CA, USA).

Mice sensitization and development of food-induced intestinal inflammation

Preliminary experiments were performed with the utilization of a wide range of sensitization protocols and the best procedure to induce food allergic inflammation was chosen as follows: C57BL/6 mice ($n = 5$ animals/group) were age (6–8 weeks old) and gender matched and sensitized twice, 3 weeks apart, with 100 μ g of peanut proteins extract (PPE) in the presence (first sensitization) or absence (second sensitization) of 1 mg of aluminium hydroxide by subcutaneous injection. One week after the last sensitization, mice were fed peanut seeds *ad libitum* for 30 days uninterruptedly, in the absence of the conventional chow (S+Peanut group). Control mice were divided into three groups: non-sensitized (NS) group, received the first sham sensitization with phosphate-buffered saline (PBS) plus aluminium hydroxide, followed by a second sensitization with PBS only and was not submitted to the diet containing peanut seeds; Peanut group was submitted to same protocol of sensitization as NS group, but the animals were fed peanut seeds for 30 days, beginning 1 week after the last sensitization and S group (sensitized), was sensitized twice with PBS containing PPE but was not challenged with the peanut diet. Water was available and animals were weighed continuously during all the experiment. All mice were anaesthetized, bled and killed on day 30 after introduction of the peanut diet. The experimental procedures followed the guidelines of the Brazilian Council for Use of Animals in Research.

Western blot assays

Proteins (15 μ g) from PPE were resolved in a 12.5% SDS gel by electrophoresis and transferred to a polyvinylidene fluoride membrane (GE Healthcare, Sao Paulo, Brazil). The blots were blocked with 5% fat-free dry milk in 5% Tween-20 PBS buffer for 1 h at room temperature and then washed with 5% Tween-20 PBS. The membranes were incubated with 1 : 800 dilution of mice sera, washed and then incubated with an alkaline phosphatase-conjugated goat anti-mouse IgG (Miles-Yeda Laboratories, Rehovot, Israel) for 1 h at 37 °C. The reactions were developed with BCIP/NBT phosphatase substrate (KPL, Gaithersburg, MD, USA).

Histopathological analysis and morphometry

Jejunum segments were collected, formalin-fixed and paraffin-embedded. Sections were stained with haematoxylin and eosin (HE), toluidine blue (connective tissue mast cells) or Congo Red (eosinophils) for histopathological analysis of mucosal inflammation. For eosinophil and mast cell counts an area equivalent to 20 jejunum villous (mucosa and submucosa) in each animal, was evaluated.

Transmission electron microscopy

For transmission electron microscopy (TEM), jejunum segments were collected and immediately fixed in 4% sodium cacodylate-buffered glutaraldehyde, pH 7.4 at 4 °C for 24 h. The segments were post fixed in 1% osmium tetroxide at 4 °C for 1 h. Tissues were dehydrated in ascending concentrations of acetone and embedded in Araldite[®] 502 resin (Polysciences Inc., Warrington, PA, USA). Ultrathin sections were stained with uranyl acetate, lead citrate and examined in a Zeiss EM 109 electron microscope at an 80 kV accelerating voltage (Carl Zeiss, Oberkochen, Germany).

FACS (fluorescence-activated cell sort)

The small gut inflammatory infiltrate was evaluated as Peyer's patches (PP), intraepithelial lymphocytes (IEL) and lamina propria leucocytes (LPL), as described previously [17, 18]. Viability was assessed by Trypan blue exclusion and cells were counted in a Neubauer chamber. Leucocytes obtained were incubated with anti-CD16/CD32 mAb (Fc block, Clone 2.4G2-Pharmingen, San Jose, CA, USA) followed by incubation with the following antibodies: anti-CD8, anti-CD19 and anti-CD25 FITC-conjugated (BD Biosciences Pharmingen), anti-Pan NK, anti-CD4, anti-TCR γ δ , anti-CD11b, anti-Gr1 (Southern Biotechnologie Associates Inc., Birmingham, AL, USA) and anti-Foxp3 PE-conjugated (eBioscience, San Diego, CA, USA), anti-CD3 and anti-CD4 PerCP-labelled antibodies (BD Biosciences Pharmingen). Leucocytes were acquired (FACScan[™] and CELLQuest[™] software; BD Biosciences Pharmingen) according to size (FSC) and granularity (SSC) scatter dot plot. Single or two-colour staining was used to identify T lymphocytes (CD3⁺, CD3⁺CD4⁺, CD3⁺CD8⁺), B lymphocytes (CD19⁺), phagocytes (CD11b⁺), granulocytes (Gr1⁺) and natural killer cells (Pan NK⁺). The isotype controls used were rat IgG2a-PerCP, rat IgG2b-FITC and rat IgG1-PE (BD Biosciences Pharmingen). Results demonstrate the mean $\pm$ SEM of the percentage of each antibody specific stained subpopulation within the gated cells, obtained from small gut of five different animals per group.

Enzyme-linked immunosorbent assay measurements

PPE specific IgG, IgG1, IgG2a and IgE serum levels were measured by ELISA in an *inhouse* assay, on 96-well polystyrene ELISA plates (low binding, Corning, New York Costar, Acton, MA, USA) coated with PPE (20 μ g/well). To measure the samples at more than one concentration, sera were plated in serial twofold dilutions (1 : 100–1 : 6400), incubated with anti-IgG, anti-IgG1, anti-IgG2a or anti-IgE biotinylated antibodies (Southern Biotechnologie Associates), followed by streptavidin-HRP-specific conjugates (Vector Laboratories Inc., Burlingame, CA, USA). Reactions were developed with 3,3',5,5'-tetramethylbenzidine (TMB;

KPL). The absorbance was read at 450 nm in a microplate spectrophotometer (Microplate ELISA Reader, SoftMax program, Molecular Devices, Sunnyvale, CA, USA). All samples were analysed for a minimum of two to three times.

Real-time polymerase chain reaction

Total jejunum RNA was extracted using RNA extraction kit (Promega, Madison, WI, USA), according to the manufacturer's instructions. The RNA concentration was determined (Biomate 3 spectrophotometer Thermospectronic, Rochester, NY, USA) and complementary DNA (cDNA) was synthesized using 1 μ g 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 SYBR green fluorescence (Applied Biosystems, Warrington, UK). The standard PCR conditions were 95 °C for 10 min, 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 Primer Express software (Applied Biosystems) using nucleotide sequences present in the GenBank database and were depicted in Table 1. SYBR Green PCR Master Mix (Applied Biosystems), 0.1–0.2 μ g/ μ L specific primers, and 2.5 ng of cDNA were used in each reaction. Threshold for positivity was determined based

Table 1. Primers sequences used in Real time PCR assays

Primers	Sequences
β -actin	Sense AGC TGC GTT TTA CAC CCT TT Antisense AAG CCA TGC CAA TGT TGT CT
T-Bet	Sense CCC CTG TCC AGT CAG TAA CTT Antisense CTT CTC TGT TTG GCT GGC T
GATA-3	Sense AGG AGT CTC CAA GTG TGC GAA Antisense TTG GAA TGC AGA CAC CAC CT
IL-4	Sense CTG ACG GCA CAG AGC TAT TGA Antisense TAT GCG AAG CAC CTT GGA AGC
IL-5	Sense GAG GTT ACA GAC ATG CAC CAT T Antisense TCA GTT GGT AAC ATG CAC AAA G
IL-13	Sense ACC AAC ATC TCC AAT TGC AA Antisense ATG CAA TAT CCT CTG GGT CC
TNF- α	Sense TGT GCT CAG AGC TTT CAA CAA Antisense CTT GAT GGT GGT GCA TGA GA
IL-12 p40	Sense AGC ACC AGC TTC TTC ATC AGG Antisense GCG CTG GAT TCG AAC AAA G
IFN- γ	Sense GCA TCT TGG CTT TGC AGC T Antisense CCT TTT TCG CCT TGC TGT TG
TGF- β	Sense GCT GAA CCA AGG AGA CGG AAT Antisense GCT GAT CCC GTT GAT TTC CA
IL-10	Sense TGG ACA ACA TAC TGC TAA CC Antisense GGA TCA TTT CCG ATA AGG CT
mMCP1	Sense AGGACAGATGTGGTGGGTTT Antisense TGCAGCAGTCAACACAAATTG

mMCP1, mucosal mast cell protease-1.

on negative controls. The results were demonstrated as mRNA expression of the NS, Peanut, S and S+Peanut animals, relative to a naïve non-sensitized, non-exposed to alumen and non-challenged group. Calculations to determine the relative level of gene expression were made according to the instructions from Applied Biosystems User's Bulletin #2 (P/N 4303859), by reference to the β -actin in each sample, using the threshold cycle (C_t) method. Negative controls without RNA and without RT were also performed. Results show one experiment representative of three.

Statistical analysis

Results were submitted to the one-way ANOVA, followed by Tukey's post test. Values of $P < 0.05$ were considered statistically significant.

Results

Peanut proteins extraction and gel electrophoresis: establishment of the sensitization protocol

To establish a novel model for food-induced intestinal inflammation, the seeds of peanut and their proteins were the eligible antigens due to their high consumption by world-wide population and mainly because of the largely known peanut allergenic properties, besides being a natural source of feeding for rodents like mice. The electrophoretic profile of PPE showed a large variety of proteins, with molecular weight ranging from 10 to 90 kDa (Fig. 1a). This whole PPE and feeding on peanut seeds were used in C57BL/6 mice sensitization and challenge protocol, as described before.

Sensitization leads to specific immune recognition of peanut proteins extract

To verify if the sensitization protocol induced specific immune response to PPE, sera were collected at the end of the challenge period and used for western blot assays. Sensitized animals from both S and S+Peanut groups recognized the majority of proteins ranging from 10 to 60 kDa with the exception of a band of approximately 25 kDa (arrow) which was not recognized by S group but instead was present in the blots incubated with sera of peanut-fed mice (Peanut and S+Peanut). As expected, NS group did not recognize any of the proteins present in PPE (Fig. 1b).

Exposure to peanut seeds leads to weight loss in C57BL/6 mice

To verify if sensitization followed by challenge with peanut seeds could lead to any clinical manifestation of allergy or gastrointestinal disorder, mice were weighed

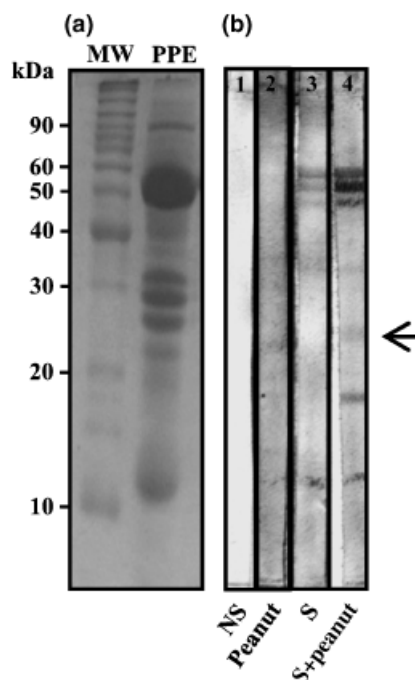


Fig. 1. Peanut proteins extract (PPE) electrophoretic profile. (a) Peanut proteins were resolved on 12.5% SDS-PAGE and visualized after Coomassie Blue staining. Lane 1, PPE; lane 2, markers of molecular weight (MW). (b) Western blot assays with immune and non-immune sera collected at the end of the 30-day challenge. Reactions were developed after incubation with anti-IgG alkaline phosphatase antibodies and BCIP/NBT phosphatase substrate. (1) Non-sensitized (NS), (2) Peanut, (3) sensitized (S) and (4) S+Peanut sera.

weekly and followed for the development of diarrhoea, intestinal bleeding or rectal prolapse. Results showed that animals from both S+Peanut and Peanut group presented weight loss throughout the experimental period, especially 30 days after introduction of peanut seeds in their diet (Fig. 2a). This loss was statistically significant when compared with the other control groups NS and S that were fed on conventional chow during the same period. In addition, there was a reduction in peanut consumption by both groups after the second week of exposure, as showed in Fig. 2b. No signs of diarrhoea, intestinal bleeding or rectal prolapse were found during the experimental period.

Gut damage in mice sensitized and challenged with peanut seeds

To determine if weight loss was related to any intestinal damage caused by ingestion of peanut seeds, small gut segments from experimental and control mice were collected and processed for histopathology. As demonstrated in Fig. 3a (upper panel), only S+Peanut mice developed intestinal inflammation, characterized by leucocyte infiltrate, epithelial exulceration and oedema in lamina propria, along with disruption of small gut architecture.

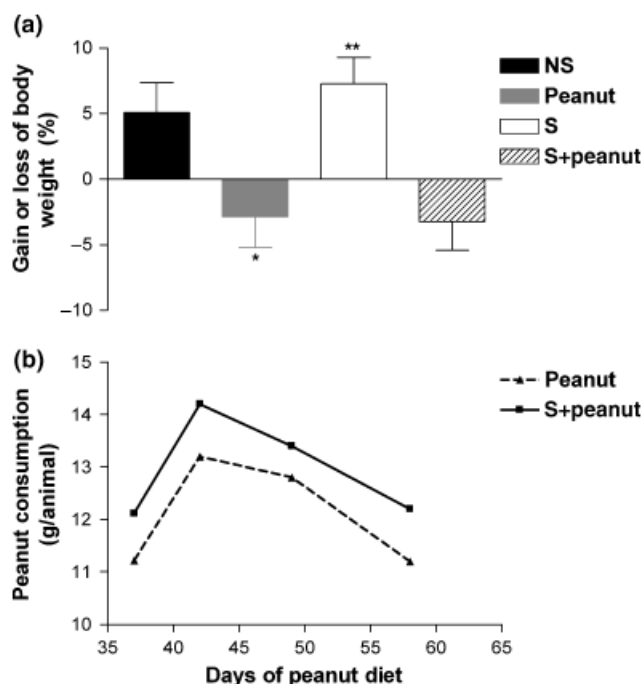


Fig. 2. Variation in body weight and peanut consumption. The gain or loss of weight after the 30-day challenge is visualized as percentage of the animals' weight in the first day of peanut diet (a). (b) Peanut consumption decreases throughout the chronic exposure to the seeds. Non-sensitized (NS), Peanut and sensitized (S), control groups. Results are representative of three to five independent experiments, * $P < 0.05$.

These alterations were found in duodenum, jejunum, ileum and no marked changes were visualized in control mice (Fig. 3a, upper panel). Inflamed gut also demonstrated an increase in eosinophils (Fig. 3b, S+Peanut vs. NS and S+Peanut vs. Peanut, $P < 0.05$), although sensitized (S) mice also presented elevated counts when compared with the non-sensitized groups NS and Peanut (S vs. NS and S vs. Peanut, $P < 0.05$). It is of note that oral exposure to peanut seeds in the absence of systemic sensitization also led to an increase in eosinophils in comparison with non-exposed NS group. Connective tissue mast cells were shown to be augmented in the jejunum segments of inflamed mice too (Fig. 3c, S+Peanut vs. NS, S+Peanut vs. Peanut and S+Peanut vs. S, $P < 0.05$). In addition, we observed an increase in mucosal mast cell protease-1 (mMCP-1) mRNA in the jejunum of S+Peanut mice, although not significant when compared with the other groups.

Then, in order to have a more accurate visualization of the ultrastructural damages induced after sensitization and peanut consumption, jejunum segments were also processed for TEM analysis. No specific alterations in enterocyte structures neither in its microvillus surface were found in both experimental or control groups (data not shown). On the other hand, sensitization followed by long-term ingestion of peanut seeds led to oedema and a

great influx of plasma cells in the interior of lamina propria of the majority of S+Peanut mice. Such cells presented abundant and notably dilated cisterns of rough endoplasmic reticulum, suggesting a role for these cells or their products in the pathogenesis of the intestinal inflammation observed (Fig. 3a, lower panel).

Inflammation is associated with differential accumulation of leucocytes in the gut

To have a more specific and quantitative characterization of the inflammatory infiltrate in the damaged tissue of S+Peanut mice, the entire small gut was collected, enzyme digested and the viable leucocytes analysed by flow cytometry. Results showed a significant increase in cellularity in the gut of mice sensitized and challenged with peanut seeds when total cell counts were compared with NS, Peanut and S groups. After separation of leucocytes from LP, PP and intraepithelial compartments, we observed that mice sensitized and fed peanut seeds presented an evident decrease in the percentage of intraepithelial $\gamma\delta^+$ T lymphocytes when compared with the NS, Peanut and S groups (Fig. 4a, $P < 0.05$). In addition, we could observe a tendency towards an accumulation of T ($CD3^+$) and B ($CD19^+$) lymphocytes in the lamina propria of the same animals (Fig. 4b and c, $P > 0.05$), although B cells were also shown to accumulate in the jejunum of non-sensitized peanut-fed mice, demonstrating the association between B cells infiltrate and peanut consumption. Inflamed tissues presented increased frequency of NK (Fig. 4d) and $CD11b^+$ cells (Fig. 4e) in LP when compared with the control NS (Fig. 4d, NS vs. S+Peanut, $P < 0.05$) or NS, Peanut and S, respectively (Fig. 4e, $P < 0.05$). The Peanut group also presented increased accumulation of NK cells, which was statistically significant when compared with NS and S animals (Fig. 4d, $P < 0.05$), suggesting that NK accumulate in the gut preferentially after peanut consumption. We did not observe any particular accumulation of $CD3^+CD4^+$, $CD3^+CD8^+$ or $Gr1^+$ cells in the lamina propria of S+Peanut group. Furthermore, exposure to peanut proteins in Peanut, S and S+Peanut groups led to diminished percentage of $CD4^+CD25^+$ cells expressing Foxp3 (Fig. 4f–h) in PP, although only relevant when sensitized and challenged mice were compared with NS animals ($P < 0.05$). These results suggest that down modulation of regulatory mechanisms in the gut of allergic mice might be involved in the development of peanut-induced food allergy.

Mice sensitization induces a T-helper type 2 profile of Immunoglobulin production

The presence of B and plasma cells in small gut inflammatory infiltrate highlighted the hypothesis of the

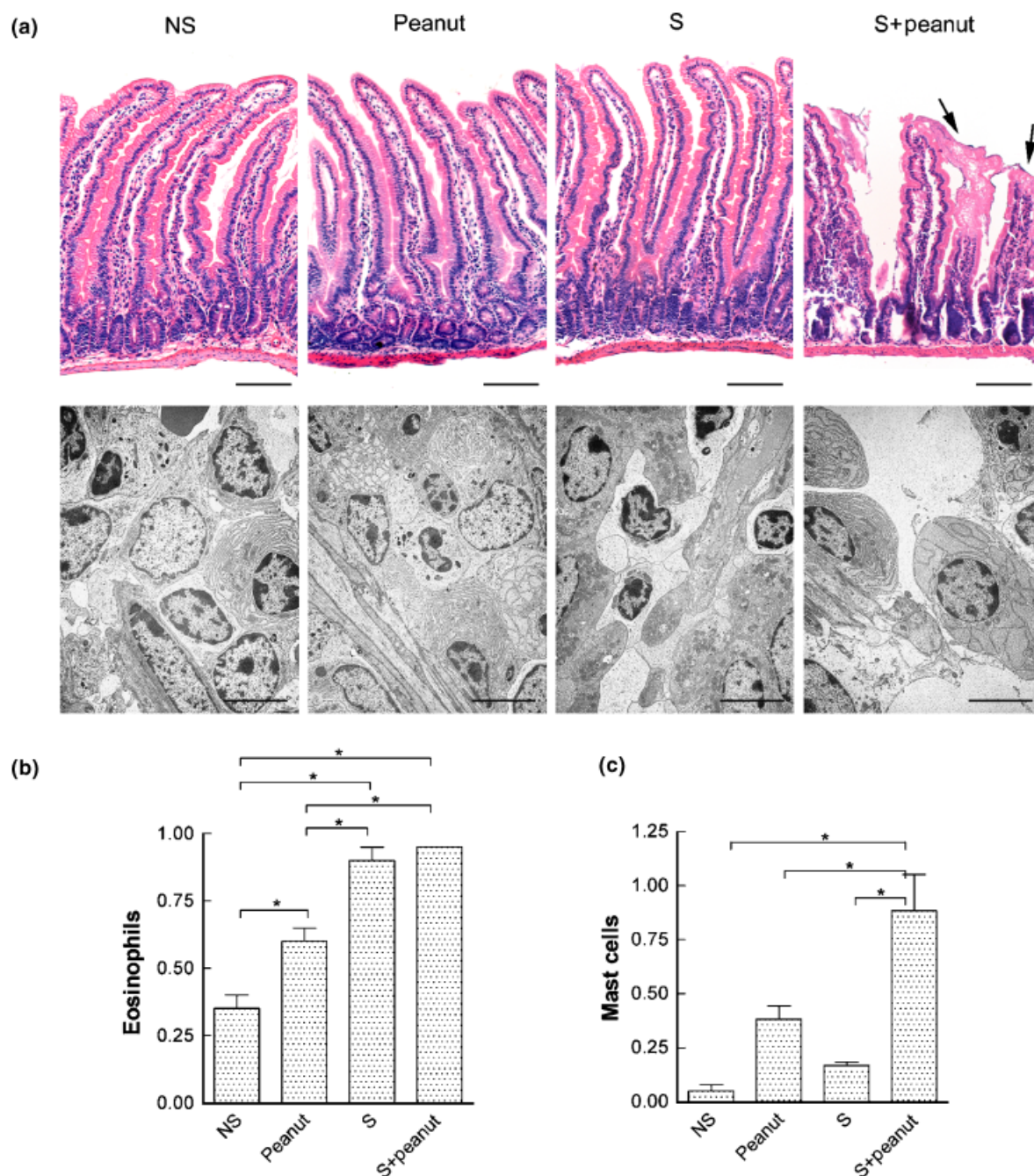


Fig. 3. Peanut challenge induces jejunum inflammation. Jejunum segments were collected and processed for optical (upper panel) or transmission electron microscopy (TEM, lower panel) analysis at the end of the 30-day challenge with peanut seeds. In (a) S+Peanut, note inflammatory infiltrate, tissue destruction, villous enlargement and epithelial exulceration (upper panel, arrow, scale bar = 100 μ m). TEM shows oedema and plasma cells in lamina propria, with dilated cisterns of the rough endoplasmic reticulum (lower panel, scale bar = 5 μ m). (b, c) Eosinophil and mast cell counts, respectively. Non-sensitized (NS), Peanut and sensitized (S), controls. Results are representative of three to five independent experiments.

participation of Igs in the pathogenesis of the food-induced intestinal inflammation. We found that both S and S+Peanut mice presented higher production of PPE-specific IgG antibodies than the controls NS and Peanut (Fig. 5a, S+Peanut vs. NS; S+Peanut vs. Peanut; S vs. NS and S vs. Peanut, $P < 0.05$), especially in elevated sera

concentrations, demonstrating that sensitized mice respond to PPE by means of Ig production. As peanut proteins are often described to be one of the most common antigens able to induce allergic inflammatory responses in susceptible individuals, we then asked if the intestinal inflammation observed could be related to a T-helper type 2

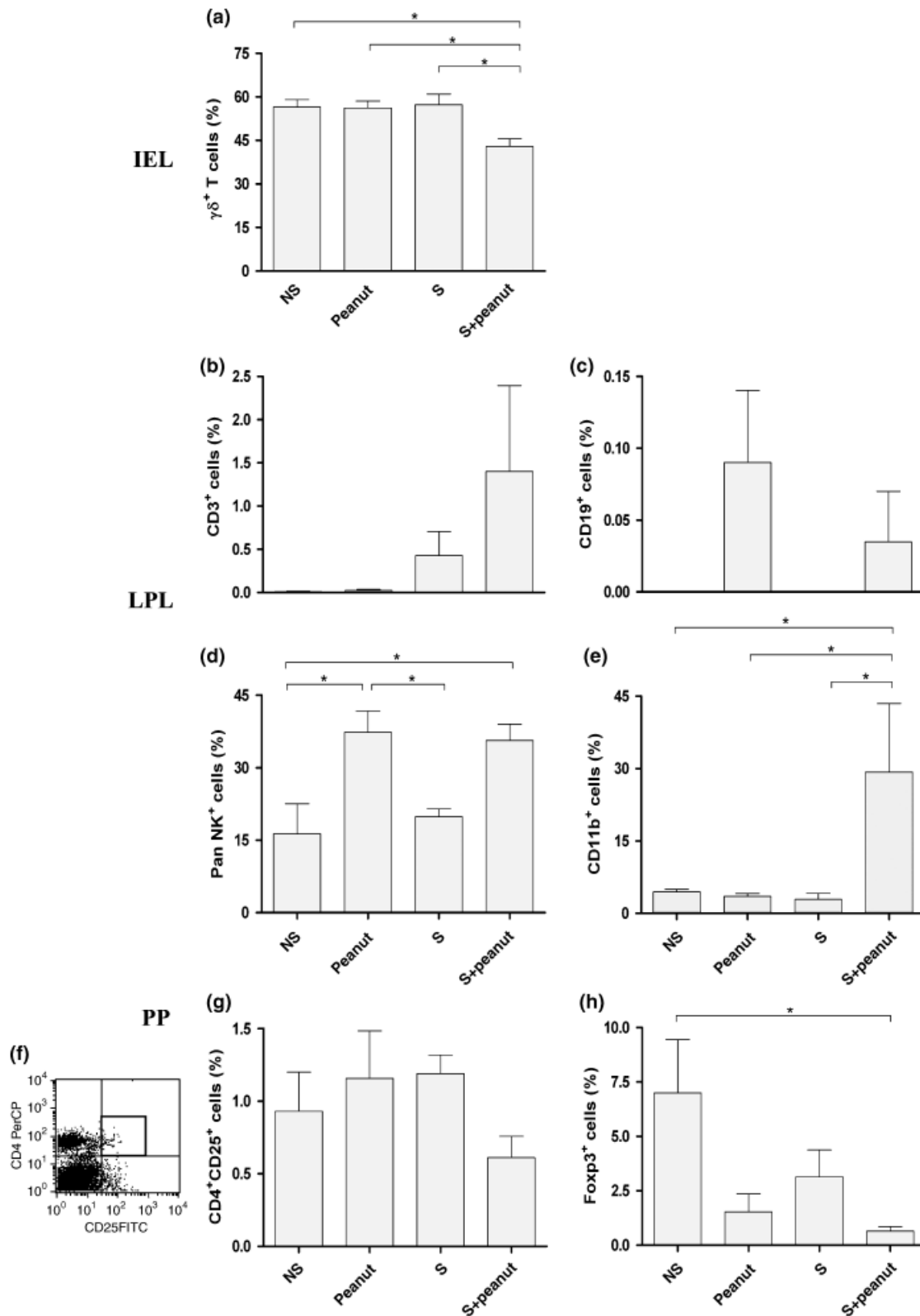


Fig. 4. Inflammatory infiltrate in food-induced enteropathy. Leucocytes obtained from small gut of experimental and control mice at the end of the 30-day challenge protocol were processed for FACS, as described in 'Materials and methods'. (a) Percentage (mean $\pm$ SEM) of $\gamma\delta^+$ IEL, (b) CD3⁺, (c) CD19⁺, (d) Pan NK⁺ and (e) CD11b⁺ LPL. (f, g) PP CD4⁺CD25⁺ analysis where Foxp3⁺ cells were gated and quantified (h). Results are representative of two independent experiments. Non-sensitized (NS), Peanut and sensitized (S), control groups. * $P < 0.05$. IEL, intraepithelial lymphocytes; LPL, Lamina propria leucocytes; PP, Peyer's patches.

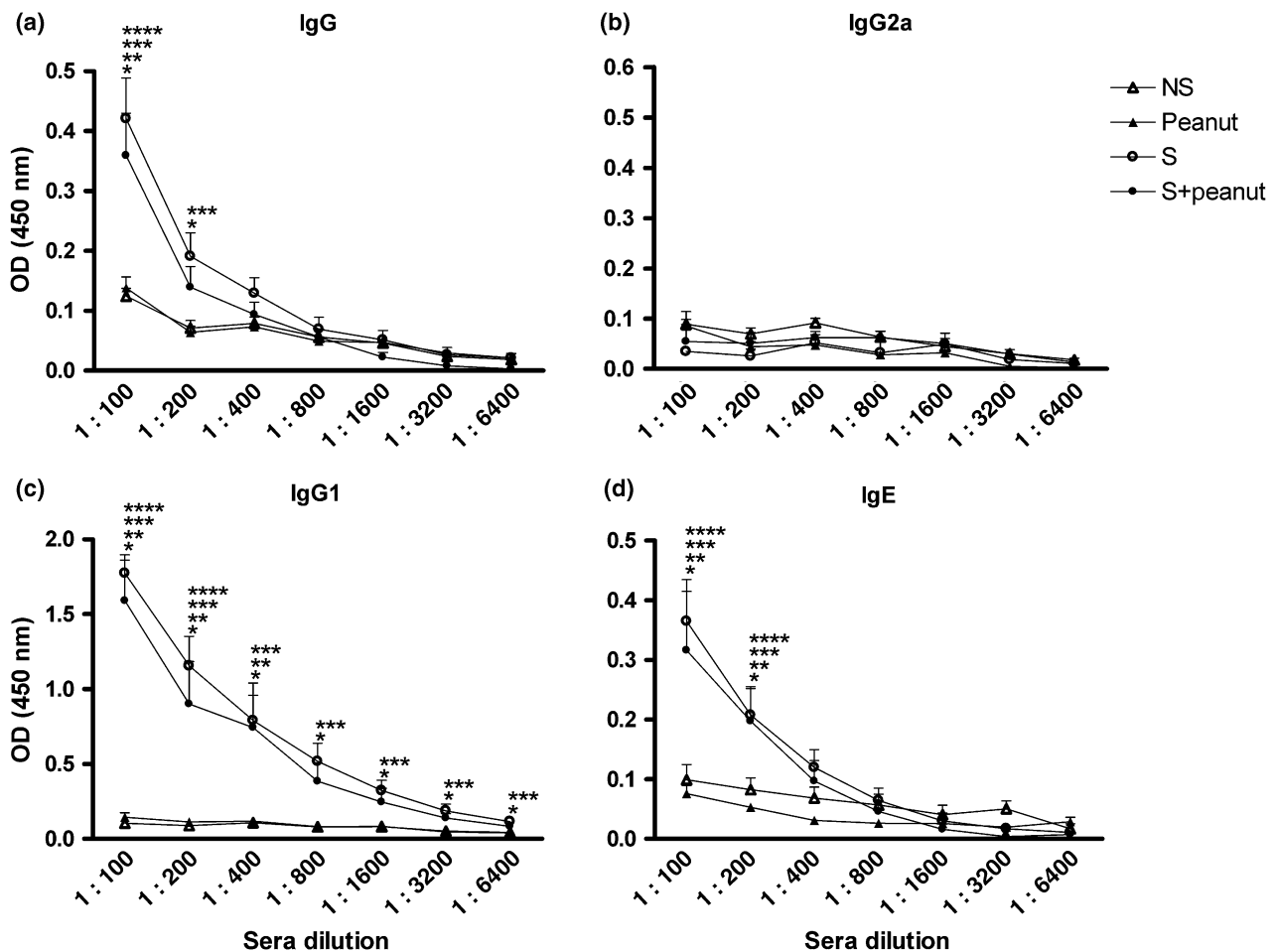


Fig. 5. Mice sensitization leads to T-helper type 2 (Th2) Ig production. Animals were sensitized and challenged with peanut seeds for 30 days, when sera were collected for Ig detection, as described in 'Materials and methods'. (a) IgG, (b) IgG2a, (c) IgG1 and (d) IgE. The results (mean + SEM) represent values from one experiment representative of three. Non-sensitized (NS), Peanut and sensitized (S), control groups. *NS vs. S; **NS vs. S+Peanut; ***S vs. Peanut; ****S+Peanut vs. Peanut, $P < 0.05$.

(Th2) or allergic response raised against PPE. As demonstrated in Fig. 5b, there was no difference in the detection of IgG2a among the groups tested. Otherwise, we observed a vast increase in sera IgG1 in S and S+Peanut mice, statistically significant when compared with NS and Peanut groups (Fig. 5c, S+Peanut vs. NS; S+Peanut vs. Peanut; S vs. NS and S vs. Peanut, $P < 0.05$) especially in high sera concentrations too. In accordance, both sensitized groups presented a significant increase in IgE levels when compared with the NS and Peanut animals (Fig. 5d, $P < 0.05$).

Differential gene expression modulates gut inflammation

To understand the modulation of the gut immune response in the pathogenesis of food allergy, we further investigated the involvement of cytokines and transcription factors in the development of the intestinal inflammation. While we could not detect any difference in

mRNA expression of the transcription factor T-Bet (Fig. 6a), S+Peanut mice exhibited high levels of messages for GATA-3, which is important for Th2 cytokines transcription. Such differences were statistically significant when compared with the NS, Peanut and S groups (Fig. 6b, $P < 0.05$). The increase in GATA-3 mRNA expression was accompanied by a significant elevation in IL-4 and IL-13 messages in the inflamed gut, when compared with controls (Fig. 6c and e, respectively), while no differences in IL-5 mRNA expression was found among the groups tested (Fig. 6d). In addition, we observed an increase in TNF- α mRNA expression in the groups Peanut and S+Peanut, although this difference was only statistically significant when the groups S+Peanut vs. NS (Fig. 6f, $P < 0.05$) and S+Peanut vs. S (Fig. 6f, $P < 0.05$) were compared. In contrast, experimental S+Peanut mice were demonstrated to have low mRNA expression for the cytokines IL-12p40 (Fig. 6g, S+Peanut vs. S, $P < 0.05$) and IFN- γ (Fig. 6h, S+Peanut vs. Peanut, $P < 0.05$),

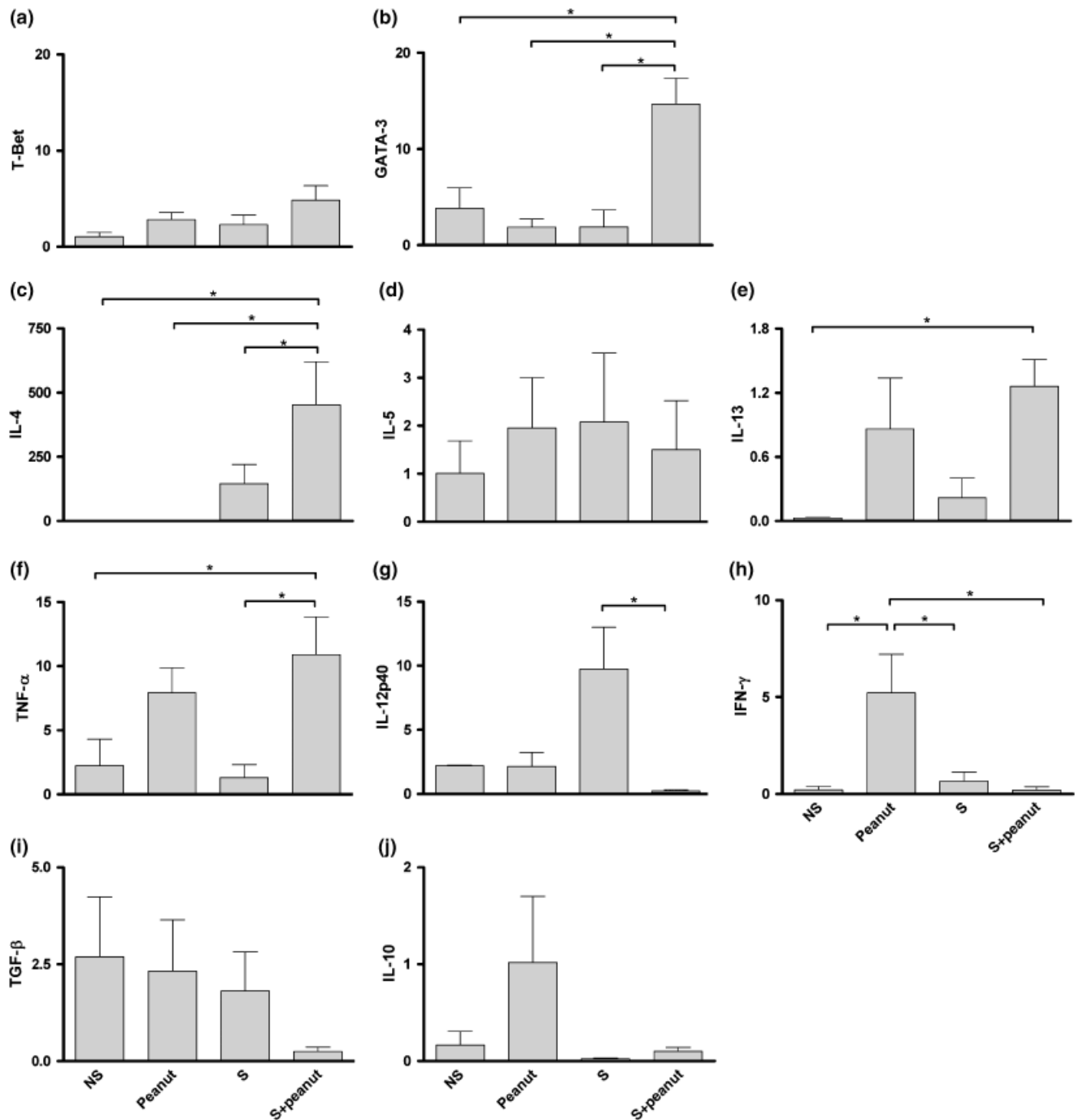


Fig. 6. Modulation of intestinal immunity in sensitized (S)+Peanut mice. T-Bet (a), GATA-3 (b), IL-4 (c), IL-5 (d), IL-13 (e), TNF- α (f), IL-12p40 (g), IFN- γ (h), TGF- β (i) and IL-10 (j) mRNA was quantified by Real-time PCR in jejunum segments collected at the 30th day of peanut challenge. Results are demonstrated as mRNA expression of non-sensitized (NS), Peanut, S and S+Peanut animals, relative to a naïve non-sensitized, non-exposed to alumen and non-challenged group. The relative levels of gene expression were calculated by reference to the β -actin in each sample, using the threshold cycle (C_t) method. Data (mean+SEM) represent values from one experiment representative of three. NS, Peanut and S, controls. * P <0.05.

especially when compared with the controls S and Peanut, respectively. We also detected low levels of message for the regulatory cytokines TGF- β and IL-10 in the experimental group, although not statistically significant (Figs 6i and j, respectively).

Discussion

The expanded surface of the intestinal epithelium allows for efficient secretory, absorptive and digestive processes representing vital functions for the body. In the gut, an

aggressive immune response against commensals or non-self peptides like food antigens can be wasteful and lead to destructive inflammatory disorders. In this study, the characterization of a novel murine model of food-induced enteropathy allowed the evaluation of gut inflammatory response where the breakdown in mucosal tolerance was induced by the allergenic peanut proteins [19].

Elimination diet is still the mainstay of IgE-dependent food allergy therapy [20, 21]. However, with the advent of manufactured food, trace amounts of allergens can still be risky to some atopic patients that appear to be continuously exposed. In these patients, continuous unwilling exposure to a food allergen may induce a persistent allergic condition. To try to mimic the latter clinical situation, we developed an experimental model in which sensitized mice were persistently given peanut seeds. As the long-term exposure of mice to peanuts together with other sources of feeding could lead to refusal of peanut seeds in benefit to chow, we decided to establish a controlled diet, in which animals were exclusively fed peanut seeds, a natural feeding for rodents like mice.

Inducing breakdown of mucosal tolerance and eliciting intestinal inflammation in response to food antigens is an extremely complex deal, as gut immune system is equipped with multifaceted mechanisms that prevent tissue injury. Even though it was not observed a massive intestinal inflammation like that seen in inflammatory bowel diseases (IBD), it is possible that the non-tolerogenic antigens modulated the mucosal immunity by triggering an exacerbated immune response against peanut proteins, visualized through the development of a mild but relevant inflammation with oedema in LP and loss of gut architecture in allergic mice. In this model, animals sensitized and challenged with peanut seeds showed a differential recognition of peanut proteins, demonstrating that they were capable of mounting efficient immune responses to the antigens used to induce inflammation.

The first clinical sign of intestinal immune modulation by peanut ingestion was the loss of weight, associated with refusal of peanut diet after the second week of exposure. The precise reasons for such behaviour are not known, but the diminished consumption of peanut seeds coupled with the probable absence of a nutritionally equilibrated diet could, at first, explain the weight loss observed in both groups fed peanut seeds, as sensitization did not have any impact on weight loss. However, in contrast to the animals only fed peanut seeds, the absence of gain of weight in S+Peanut mice was accompanied by intestinal inflammation, specifically in small bowel, essential for nutrients absorption. In a similar model of food sensitization [22], prolonged exposure to ovalbumin (OVA) followed by oral challenge resulted in histological changes in the intestine of experimental mice, as well as elevated plasma histamine levels.

In spite of a large variety of mechanisms for host protection [23], the breakdown in mucosal tolerance was

possibly induced by peanut sensitization and challenge and led to an influx of differential leucocytes that, upon activation could be releasing mediators involved in the pathogenesis of the food-related enteropathy. Considering that $\gamma\delta^+$ T cells are one of the most important cell population within the IEL [24] and may be critical effector lymphocytes preventing intestinal lesions [25], we suggest that the decreased percentage of these cells in epithelia of allergic mice might be related to the inflammation observed, as IEL are the first line of defence against non-tolerogenic antigens in the gut [24]. Surprisingly, we did not observe any particular accumulation of CD4⁺ or CD8⁺ cells in the lamina propria of S+Peanut mice although we believe that T cells might be involved in the early development of the peanut enteropathy. While a study reported the relevance of CD4⁺ T lymphocyte migration to rat small gut mucosa [26] another described little infiltration of inflammatory cells other than eosinophils and mast cell hyperplasia in the intestine of long-term OVA-exposed mice, thus corroborating with our results [27]. It is interesting to note the prominent presence of CD11b⁺ cells and there are several evidences that indicate these leucocytes as the APCs involved in our model. It is also clear that the way by which antigens are captured and presented to the immune system is a critical factor that controls whether it results in tolerance or immunity [28]. In the present study, we propose that the increased frequency of phagocytes associated with low TGF- β and IL-10 mRNA expression is in accordance with a predominant 'inflammatory' milieu in the gut [28]. Once more, our results are consistent with earlier reports where mice sensitized with peanut proteins showed induction of macrophages recruitment in the lungs after oral or nasal challenge with the same proteins [5]. Human NK cells can differentiate into NK2 subsets and produce the 'type 2' cytokines IL-5 and IL-13 [29]. Although little is known about the role of NK cells in allergy [30], in our study the elevated percentage of these leucocytes in LP together with high IL-13 expression suggests their involvement in the modulation of the intestinal immune response in both peanut-fed groups. Additionally, the presence of a significant infiltrate of plasma cells with abundant rough endoplasmic reticulum in LP highlighted the hypothesis of the importance of antibody producing cells in the pathogenesis of the peanut-induced enteropathy. Accordingly, mice sensitized to PPE developed a Th2 profile of Ig production, even in the absence of oral challenge. Similarly, we also realized that eosinophils accumulate in the jejunum of mice-exposed sub-cutaneously to peanut proteins (S and S+Peanut groups) and, together with connective tissue mast cells could modulate gut immunity during food-induced inflammation in allergic S+Peanut mice [31, 32]. Such modulation of intestinal immune response could occur either through release of mediators like TNF- α [33, 34] or mucosal mast cell degranulation [35], because the

protease mMCP-1 seemed to be elevated in the gut of S+Peanut animals in our model (data not shown).

The high expression of GATA-3, IL-4 and IL-13 corroborated with the hypothesis of the modulation of gut immunity towards a Th2 profile of cytokine production [36–38], although other studies had demonstrated mixed systemic Th1/Th2 response to peanut antigens in an oral sensitization model [10]. Future studies will be performed to identify and to measure the cytokines produced by specific antigen-activated cells.

The modulation of gut immune response and induction of inflammation by peanut proteins could also be a consequence of the loss of regulatory mechanisms involved in mucosa homeostasis, as demonstrated by the low expression of TGF- β and IL-10 mRNA and also Foxp3 mRNA (data not shown) in intestinal mucosa along with a significant decrease in the frequency of CD4⁺CD25⁺ cells expressing Foxp3 in PP of S+Peanut mice. Together with the Th2 profile of systemic (IgE) and local immune response, the inhibition of natural regulatory cell accumulation in the gut possibly accounted for the allergic profile [39] and the breakdown of mucosal tolerance observed in the presence of the food allergens [40].

Even though some alterations like increase in Th2 Ig production, eosinophil accumulation (S and S+Peanut groups), NK and B cells infiltrate, as well as Foxp3 reduction were seen concomitantly in both peanut-fed groups or PPE-sensitized animals, it is noteworthy that only mice sensitized and challenged with peanut seeds presented intestinal inflammation probably as a consequence of the sum of alterations observed. On the other hand, long feeding regimen may actually be inducing tolerance in non-sensitized peanut-fed animals, as observed by the high detection of IL-10 and TGF- β mRNA as well as absence of intestinal inflammation in these mice. The results described herein can answer intriguing questions raised about the participation of gut immune system in the development of food-related enteropathies and permit further understanding of the functional relevance of regulatory events triggered by chronic oral antigen exposure in allergic individuals.

The prevention of food-induced immune responses is complex and involves an intact mucosal barrier, an innate immune system able to neutralize some of the pathogens or food antigens and finally the adaptive immune system restrained in the state of oral tolerance. In the absence of one or some of these elements, the outcome of intestinal inflammatory disorders is eminent. Animal experiments have already documented many features of the development and modulation of food allergy in mice. Nevertheless, to our knowledge, none of them have detailed until now how the modulation of mucosal immune response occurs, involving elicitation of active immunity to food allergens in the intestine.

Finally, this work shed some light on understanding the pathogenesis of inflammatory gastrointestinal disorders

that involves allergic mechanisms and food intolerance in the small gut. Our results support the idea of developing novel therapies for food-related enteropathies, focusing on gut specific immune responses. These procedures should rely, for example, on altering the polarized Th2 immune response in food allergy into a more balanced mucosal immune regulation or re-establishment of the oral tolerance to food antigens.

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