

## Effect of a protein-free diet in the development of food allergy and oral tolerance in BALB/c mice

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(Submitted 31 May 2014 – Final revision received 1 October 2014 – Accepted 5 January 2015 – First published online 11 March 2015)

### Abstract

The aim of the present study was to investigate the effect of a protein-free diet in the induction of food allergy and oral tolerance in BALB/c mice. The experimental model used was mice that were fed, since weaning up to adulthood, a balanced diet in which all dietary proteins were replaced by amino acid diet (Aa). The absence of dietary proteins did not prevent the development of food allergy to ovalbumin (OVA) in these mice. However, Aa-fed mice produced lower levels of IgE, secretory IgA and cytokines. In addition, when compared with mice from control group, Aa-fed mice had a milder aversive reaction to the allergen measured by consumption of OVA-containing solution and weight loss during food allergy development. In addition, mice that did not have dietary proteins in their diets were less susceptible to induction of oral tolerance. One single oral administration was not enough to suppress specific serum Ig and IgG1 levels in the Aa-fed group, although it was efficient to induce suppression in the control group. The present results indicate that the stimulation by dietary proteins alters both inflammatory reactivity and regulatory immune reactivity in mice probably due to their effect in the maturation of the immune system.

**Key words:** Diets: Dietary proteins: Food allergy: Oral tolerance

The gut mucosa is the major route for foreign antigens to contact the immune system. A large and regular amount of dietary antigens reach the gut daily, and a continuous exposure to the autochthonous microbiota provides additional stimulation to the abundant lymphoid tissue located in the intestinal mucosa<sup>(1)</sup>. This daily antigenic contact results in a continuous capture, processing and presentation of antigens that play an important role in the development of the immune system after weaning<sup>(2,3)</sup>. Germ-free mice present a poorly developed gut-associated lymphoid tissue with smaller Peyer's patches without germinative centres<sup>(4)</sup>, reduced frequency of IgA-secreting plasma cells and cluster of differentiation (CD)4<sup>+</sup>T cells as well as diminished number of intraepithelial lymphocytes (IEL)<sup>(5)</sup>. It is well documented that microbiota and dietary proteins influence the proliferation of IEL and lamina propria (LP) lymphocytes<sup>(6)</sup>. Germ-free mice also have lower levels of serum IgG and IgA<sup>(6)</sup>. Previous

studies from our group have shown that conventional C57BL/6 mice fed exclusively a protein-free diet show a reduction in the levels of secretory IgA (SIgA), serum IgG and IgA; diminished numbers of T and B lymphocytes in many lymphoid sites; and a cytokine profile similar to that found in neonates. These results suggest that not only the microbiota but also dietary proteins are relevant for the maturation of the immune system after weaning<sup>(2)</sup>.

The continuous antigenic exposure through the intestinal mucosa leads to local production of SIgA that neutralises toxins, blocks the adherence of bacteria to the epithelium and reduces the penetration of antigens across the mucosa<sup>(7)</sup>. In addition, antigenic contact initiated by oral route is known to induce oral tolerance, a state of systemic suppression of specific immune responses to subsequent parenteral injections of the same antigen<sup>(8,9)</sup>. Once oral tolerance is induced, many aspects of specific immune responses such as Ig production,

**Abbreviations:** Aa, amino acid diet; Cas, casein diet; CD, cluster of differentiation; IEL, intraepithelial lymphocytes; LP, lamina propria; MLN, mesenteric lymph nodes; OVA, ovalbumin; SIgA, secretory IgA.

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delayed-type reaction and production of several pro-inflammatory cytokines are suppressed<sup>(8,10–12)</sup>. Many subsets of lymphocytes with regulatory properties as well as IEL have been described to be important for oral tolerance induction due to their ability to produce non-inflammatory and regulatory cytokines such as transforming growth factor  $\beta$  and IL-10, which play a major role in the gut homeostasis<sup>(13,14)</sup>.

One of the consequences of impairment of oral tolerance is the induction of hypersensitivity to dietary antigens that occurs in food allergy<sup>(15)</sup>. Food allergic process depends on specific IgE production triggered by total or partially digested proteins that cross the gut mucosa<sup>(16)</sup>. Allergic reaction is initiated by triggering Th2 cells able to secrete IL-4, IL-5, IL-10 and IL-13 that are involved in B-cell differentiation to IgE-producing plasma cells and in increased mucus production<sup>(17)</sup>. Eosinophil accumulation is another key player of allergic reaction, although many resting eosinophils are already present in the normal gut mucosa. Activation and recruitment of eosinophil to the inflammatory sites are dependent on stimulation by eotaxin and IL-5. These mediators induce differentiation and proliferation of local eosinophils in addition to the recruitment of circulating cells to the inflammatory sites<sup>(18,19)</sup>.

In the present study, we evaluate the impact of whole protein withdrawal from the diet in the induction of these two antagonistic and important phenomena, i.e. food allergy and oral tolerance.

## Materials and methods

### Animals

Female BALB/c mice (3- to 4-week-old) were obtained from CEBIO (Rodent Experimental Facility of Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Belo Horizonte, Brazil) and maintained in our experimental animal facility throughout the experiments. All animal procedures were approved by the local ethics committee for animal research (CETEA-UFGM, Brazil).

### Diets and consumption measurement

BALB/c mice (3- to 4-week-old) were fed *ad libitum* either a control diet containing 15% casein as a protein source (casein diet (Cas)-fed mice) or an experimental diet containing 15% of a mixture of amino acids (amino acid diet (Aa)-fed mice) at the same proportion found in casein. Both diets were iso-energetic and equivalent in all nutrients, and were fed from weaning for at least 8 weeks (when mice reached adulthood). Mice were individually caged, and food was daily weighted for consumption measurement. Diets were prepared in accordance with the American Institute of Nutrition<sup>(20)</sup> and modified as described by Menezes *et al.*<sup>(2)</sup>. All the ingredients are expressed as g/kg of diet as follows: 454.32 g of maize starch (Amphora Inc.); 150 g of casein; 151.17 g of dextrinised starch; 50 g of cellulose; 35 g of mineral mix; 10 g of AIN-93M vitamin mix; 2 g of L-cystine; 2.5 g of choline bitartrate; 0.009 g of BHT (Rhoder); 100 g of sucrose (União); 45 g of soyabean oil (Liza).

### Antigen

Crystallized egg albumin of hen (ovalbumin (OVA), grade III or V; Sigma-Aldrich Co.) was used as an antigen. Al(OH)<sub>3</sub> (Sigma-Aldrich Co.) was used as an adjuvant.

### Food allergy induction and aversion test

Food allergy to OVA was induced by the protocol previously described by Saldanha *et al.*<sup>(21)</sup>. Mice were immunised intraperitoneally with 10  $\mu$ g OVA plus 1 mg Al(OH)<sub>3</sub> in 0.2 ml saline. A booster with 10  $\mu$ g OVA in 0.2 ml saline was given 14 d thereafter. After 7 d, mice were fed daily with a 20% OVA solution for seven consecutive days. Mice were individually caged, and OVA solution was daily exchanged and measured.

### Oral tolerance induction

Oral tolerance was induced by intragastric (orally) administration, using a ballpoint needle, of a single dose of 20 mg grade III OVA in 0.2 ml saline (0.15 M-NaCl) 7 d before primary immunisation.

### Analysis of total and specific Ig by ELISA

Levels of OVA-specific and total Ig were determined by ELISA. Briefly, 96-well plates (Nunc™) were coated with 2  $\mu$ g/well OVA or 0.1  $\mu$ g goat anti-mouse unlabelled antibody in coating buffer, pH 9.8, overnight. Wells were washed and blocked with 200  $\mu$ l PBS containing 0.25% casein for 1 h at room temperature. Serum samples were added to the plate and incubated for 1 h at room temperature; plates were washed and then peroxidase-conjugated goat anti-mouse antibodies (1:15 000; Southern Biotechnology Associate, Inc.) were added. Plates were incubated for 1 h at 37°C. Colour reaction was developed at room temperature with 100  $\mu$ l/well of orthophenylenediamine (1 mg/ml), 0.04% H<sub>2</sub>O<sub>2</sub> substrate in Na citrate buffer. Reaction was interrupted by addition of 20  $\mu$ l/well of 1 M-H<sub>2</sub>SO<sub>4</sub>. Absorbance was measured at 492 nm by an ELISA reader (Bio-Rad Model 450 Microplate Reader).

### Measurement of serum albumin and total proteins

Serum albumin levels were measured using maximum absorptive peak deviation of Bromocresol green dye (Commercial kit; Diagnostic Labtest SA). Total serum protein concentrations were determined by biuret method using a kit (Diagnostic Labtest SA).

### Cell preparations and cytokine assays

Mesenteric lymph nodes (MLN) were removed, cell suspensions were prepared using a tissue homogeniser and were gently centrifuged. Isolated cells were cultured at  $1 \times 10^6$  cells/well for cytokine secretion analyses in 96-well plates (Nunc™) in complete RPMI with or without 1 mg/ml grade V OVA. Supernatants were collected after 72 h to measure IL-4, IL-5, IL-10 and interferon- $\gamma$ . Plates were then coated with

purified monoclonal antibodies to each cytokine overnight at 4°C. On the following day, wells were washed and supernatants were added and left overnight at 4°C. On the 3rd day, biotinylated monoclonal antibodies were added and left for 1 h at room temperature. Detection solution, containing streptavidin–peroxidase conjugate (50 µl/well; Southern Biotechnology Associate, Inc.) diluted 1:15 000, was added and incubated for 1 h at room temperature. Colour reaction was developed at room temperature with 100 µl/well of orthophenylenediamine (1 mg/ml), 0.04% H<sub>2</sub>O<sub>2</sub> substrate in Na citrate buffer. Reaction was interrupted by addition of 20 µl/well of 1 M-H<sub>2</sub>SO<sub>4</sub>. Absorbance was measured at 492 nm by an ELISA reader (Bio-Rad Model 450 Microplate Reader).

### Histology, eosinophil and intestinal mucus evaluation

The small intestines were removed and immersed in 10% formaldehyde buffer. Then, they were dehydrated in alcohol solutions using an automatic tissue processor Titertek. Gut tissue was included in paraffin, and 4 µm transverse sections were obtained by Spencer microtome. Tissues were stained with haematoxylin and eosin, and the morphological features were determined using an Olympus microscope. Eosinophil counting was performed in haematoxylin and eosin-stained histological slides. A total of ten fields were evaluated randomly using an optical microscope (40×), and results were expressed as eosinophils/field. Mucus-producing cells (goblet cells) in the intestinal mucosa were determined using a periodic acid–Schiff dye method. Slides were mounted and images of each portion of the small intestine were captured using a miniature camera JVC TK-1270/RGB. The images were analysed using the ImageJ software. For the determination of goblet cell volume, all pixels were selected for the creation of a binarised image and subsequent calculation of the total area. The result was expressed as percentage.

### Isolation of lymphocytes from lamina propria and flow cytometry analysis

Small intestines were removed from killed mice and placed in Hanks' balanced salt solution + 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) medium (2% HEPES buffer, 1% penicillin/streptomycin and 0.05% gentamicin). For the isolation of LP cells, the small intestine tissue was washed, diced and incubated in IEL medium (RPMI, 2% fetal bovine serum, 2% HEPES buffer, 1% penicillin/streptomycin and 0.5% gentamicin) for 40 min at 37°C in a shaker (150 rpm). Cells were filtered through a 70 µm cell strainer and the supernatant was discarded. The remaining fractions were incubated with collagenase II (100 units/mg) for 40 min at 37°C in a shaker (150 rpm). They were then smashed, passed through a 70 µm cell strainer and resuspended in IEL medium. LP cells were centrifuged at 300 g (5 min at 4°C), resuspended in 4.5 ml of 44% Percoll, and 2.3 ml of 67% Percoll were underlaid in 15 ml tubes (Falcon). Cells were centrifuged at 600 g for 20 min and the interface layer was harvested and separated as the lymphocyte population. Cells were stained with fluorescently conjugated antibody (BD Pharmingen) in

1% bovine serum albumin (antibodies for CD4, CD25 and CD45RB) and fixed in 4% formaldehyde. Cells were analysed by a FACSCalibur cytometer (30 000 events were acquired) and data were analysed by FlowJo (TreeStar).

### Statistical analyses

Statistical analyses were performed using GraphPad Prism 5.0 software. Differences were determined by one-way ANOVA and Tukey's *post hoc* test when more than two groups were compared or by Student's *t* test when only two groups were compared. Results are expressed as means with their standard errors with a significance level of 5% ( $P < 0.05$ ).

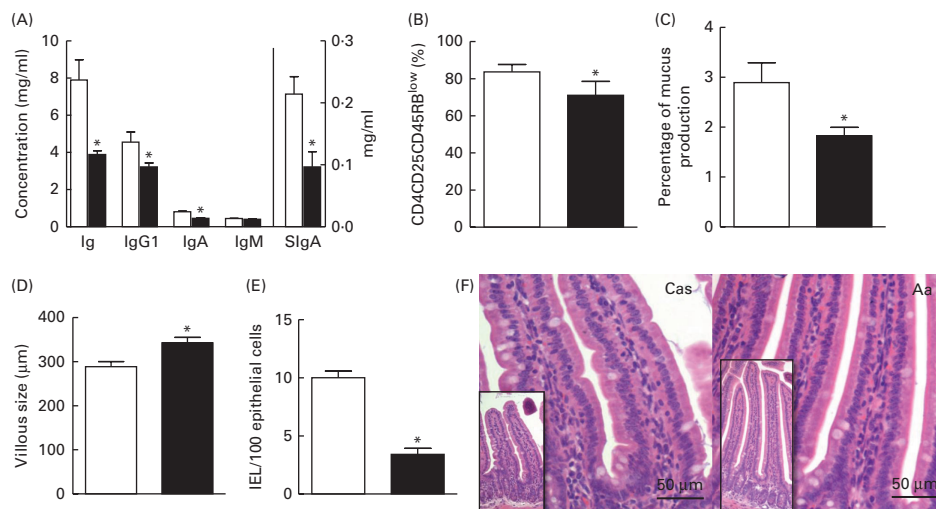
## Results

### Food proteins provide stimulation for the maturation of the immune system

Our group has previously reported that adult C57BL/6 mice that were fed a protein-free diet containing amino acids since weaning present an immature immunological profile resembling newborn and germ-free mice. This profile includes reduction in the levels of SIgA, serum IgG and IgA, Th1 cytokines as well as a poorly developed lymphoid gut-associated tissue<sup>(2)</sup>. In order to confirm these data in BALB/c mice, we evaluated serum Ig production, small intestine morphology and mucus secretion by goblet cells in mice that were fed either an Aa or Cas diet. Aa-fed mice presented diminished levels of SIgA, serum Ig, IgG and IgA when compared with Cas-fed mice. Serum concentrations of IgM are not statistically different between Aa-fed mice when compared with Cas-fed mice (Fig. 1(A)), confirming that only the production of post-switch Ig is altered in Aa-fed mice. The frequency of activated CD4<sup>+</sup>T cells (CD25<sup>+</sup>CD45RB<sup>low</sup>) was reduced in LP of Aa-fed mice (Fig. 1(B)) consistent with the low activation state of the intestinal mucosa. Interestingly, we observed that MLN cells from OVA-immunised Aa-fed mice had a poor ability to mount a recall response *in vitro* when stimulated with the antigen. Levels of all cytokines measured in the culture supernatants of OVA-stimulated cells from Aa-fed mice were lower than those found in the supernatants of Cas-fed mice (Fig. 3(F)). We also observed histological alterations in the small intestine of Aa-fed mice, namely increase in villous size (Fig. 1(D) and (F)) with reduced numbers of intra-epithelial lymphocytes (Fig. 1(E)) as well as reduction in mucus production by goblet cells (Fig. 1(C)).

### Absence of dietary proteins in the diet does not alter the nutritional status of mice

Since the present study involves diet manipulation, nutritional control of Aa-fed and Cas-fed mice was performed to determine whether nutritional changes would be interfering with the present results. Both groups were nutritionally normal according to albumin concentration (Fig. 2(A)), total serum protein (Fig. 2(B)) and food intake (Fig. 2(C)). In spite of the fact that Aa-fed mice presented lower body weight at



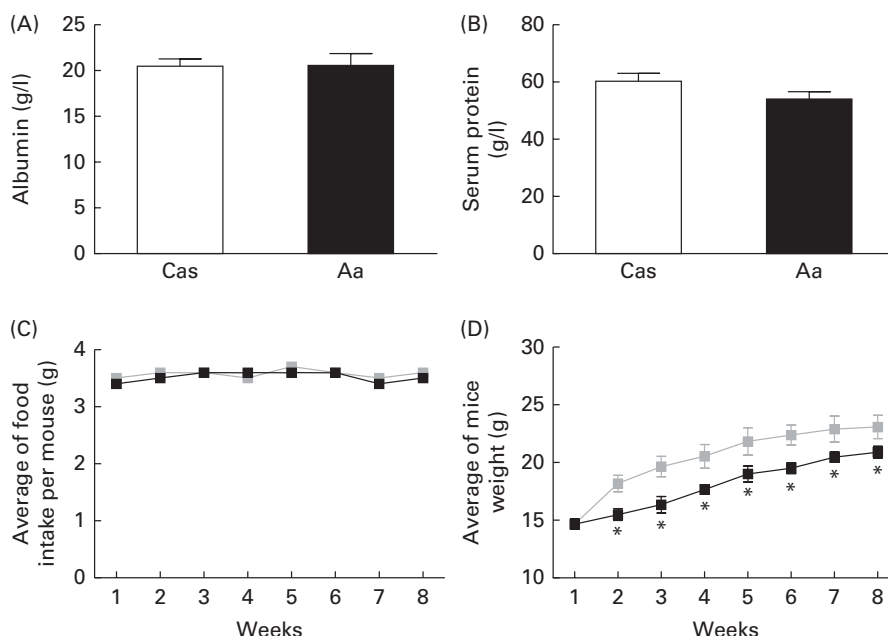
**Fig. 1.** Immature profile of amino acid diet (Aa)-fed mice (■). (A) Serum concentrations of total Ig, IgM, IgG1, IgA and secretory IgA (SIgA) determined by quantitative ELISA. (B) Flow cytometry of cells isolated from the small intestine (lamina propria, LP) stained for cluster of differentiation (CD)4, CD25 and CD45RB, expressed as percentage of all CD45RB<sup>low</sup> cells, gated on CD4<sup>+</sup>CD25<sup>+</sup>T cells. (C) Percentage of mucus production. Histological analyses of small intestine. (D) Villous size, (E) number of intraepithelial lymphocytes and (F) LP cells and villous morphology visualisation of casein diet (Cas)-fed mice (□) and Aa-fed mice at 12 weeks of age, presented at  $\times 100$  magnification in haematoxylin and eosin stain. Values are means and standard deviations represented by vertical bars ( $n = 5$ ). \* Mean value was significantly different from that of Cas-fed mice group ( $P < 0.05$ ). IEL, intraepithelial lymphocytes. A colour version of this figure can be found online at <http://www.journals.cambridge.org/bjn>

time point 2–8 weeks, when compared with Cas-fed mice, their growth curve was upward (Fig. 2(D)).

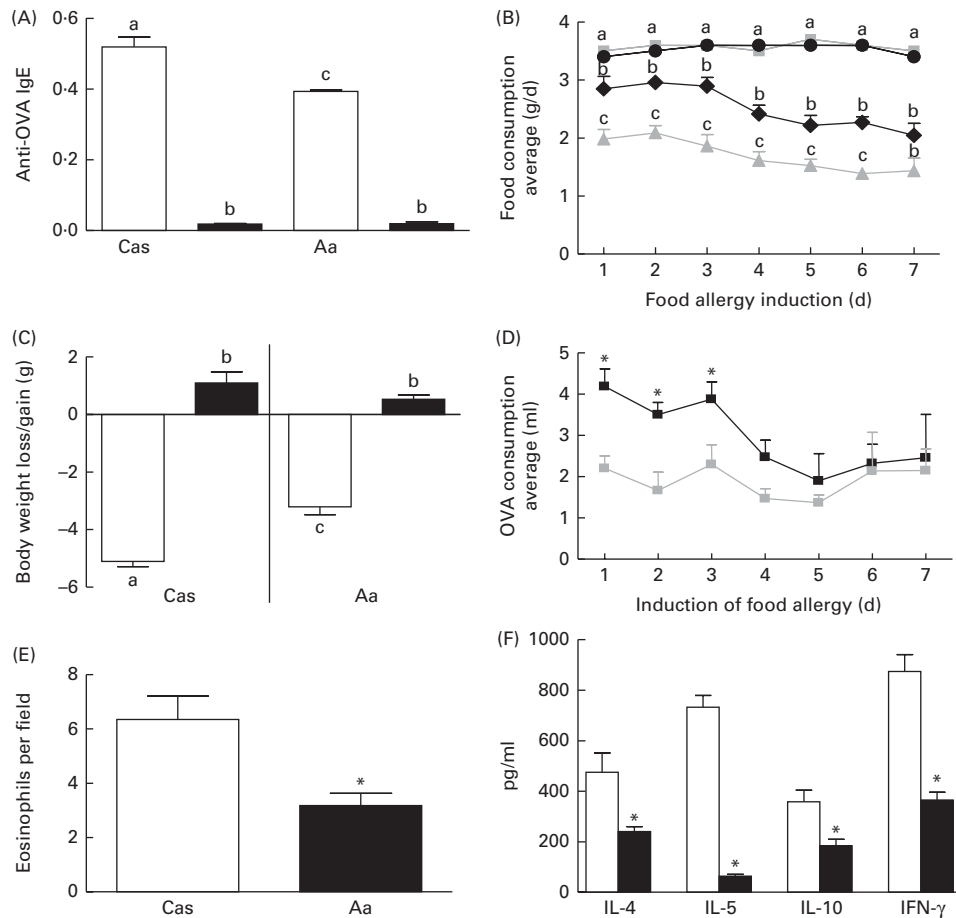
#### Dietary proteins interfere with the immune responses associated with food allergy

To analyse whether the immature profile seen in Aa-fed mice would interfere with inflammatory reactions, we also evaluated the susceptibility of these animals to allergy. Food allergy

was induced as described in the Materials and methods section. Several parameters related to food allergy development were used to measure this inflammatory reaction. We observed that Aa-fed mice had lower levels of serum anti-OVA antibodies (Fig. 3(A)) and a more discrete intestinal inflammation with lower number of eosinophils in the gut mucosa when compared with Cas-fed mice (Fig. 3(E)). Levels of all measured cytokines (IL-4, IL-5, IL-10 and interferon- $\gamma$ ) in the supernatants of MLN cells stimulated *in vitro*



**Fig. 2.** Nutritional control of casein diet (Cas)-fed (□) and amino acid diet (Aa)-fed (■) mice. (A) Serum albumin measurement made by using dye-binding technique with bromocresol green, (B) total serum protein measurements made by using dye-binding technique with biuret reagent, (C) food intake and (D) body weight average weekly measured in Cas- and Aa-fed mice. Values are means and standard deviations represented by vertical bars ( $n = 10$ ). \* Mean value was significantly different from that of Cas-fed mice group ( $P < 0.05$ ).



**Fig. 3.** Immunological immaturity status interferes with the development of food allergy. Mice (12-week-old) were intraperitoneally immunised with 10  $\mu$ g ovalbumin (OVA) + 1 mg Al(OH)<sub>3</sub>. A booster with OVA was given 14 d afterwards. After 7 d, mice were fed 20% OVA solution for seven consecutive days for food allergy induction. (A) Levels of serum anti-OVA IgE before (naïve, ■) and during allergy (allergic, □), (B) consumption of food before (casein diet (Cas) C (■) and amino acid diet (Aa) C (●)) and during allergy (Cas A (▲) and Aa A (◆)), (C) weight loss/gain before (naïve, ■) and during allergy (allergic, □), (D) ingestion of OVA-containing solution during allergy (■, Cas; ■, Aa), (E) eosinophil counting in the small intestine (lamina propria) stained by haematoxylin and eosin in allergic mice and (F) cytokine production (IL-4, IL-5, IL-10 and interferon (IFN)- $\gamma$ ) by cells from mesenteric lymph nodes of allergic mice, measured by capture ELISA from cell culture supernatants stimulated with OVA (1 mg/ml) (□, Cas; ■, Aa). Values are means and standard deviations represented by vertical bars. <sup>a,b,c</sup> Mean values with unlike letters are significantly different ( $P < 0.05$ ). \* Mean value was significantly different from that of Cas-fed mice group ( $P < 0.05$ ).

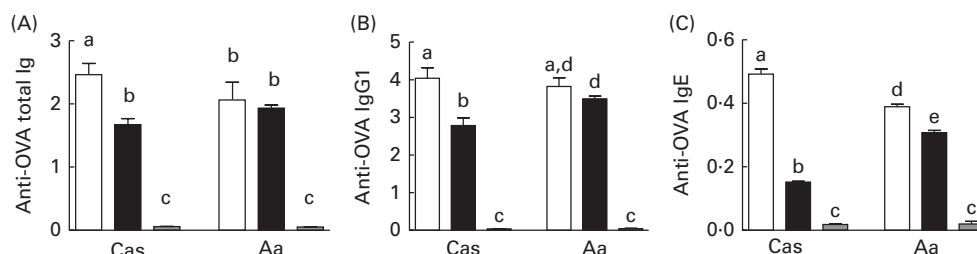
with OVA (Fig. 3(F)) were also reduced in Aa-fed mice, confirming their immature immunological profile. Allergic mice presented a deficient consumption of food that leads to weight loss, when compared with naïve group<sup>(22)</sup>. We also observed that Cas-fed mice presented decreased food ingestion and greater weight loss following food allergy induction when compared with Aa-fed mice (Fig. 3(B) and (C)). Since food allergy is associated with aversion to the ingestion of the allergen<sup>(21)</sup>, we measured the consumption of a solution containing 2% OVA by all group of mice. As expected, Cas-fed allergic mice avoid the OVA-containing solution, whereas Aa-fed mice ingested the solution at a higher rate (Fig. 3(D)).

#### Dietary proteins play an important role in oral tolerance induction

Oral tolerance is an important phenomenon that is initiated in the gut by the contact of antigens in the intestinal lumen. It is a natural event usually triggered by dietary antigens and

microbiota components<sup>(13,23,24)</sup>. Since the absence of food proteins impairs the development of the gut-associated lymphoid tissue and the immunological maturation, we next evaluate whether protein-free diet would interfere with the development of oral tolerance. Oral tolerance was induced as described in the Materials and methods section. Control Cas-fed mice but not Aa-fed mice could be rendered tolerant by the oral administration of OVA. OVA-specific serum Ig (total Ig) and IgG1 (Fig. 4(A) and (B)) levels were reduced only on Cas-fed mice orally treated with OVA before immunisation. Although a reduction in anti-OVA IgE levels in the OVA-treated Aa-fed mice could be observed, only a partial tolerance was achieved when compared with Cas-fed control group (Fig. 4(C)). These results suggest that there is a reduction in oral tolerance susceptibility in Aa-fed mice. It, therefore, seems that mucosal inflammatory and regulatory activities are underdeveloped in Aa-fed mice. Interestingly, these two phenomena were T-cell dependent, and the frequency of activated CD4<sup>+</sup>T cells (CD25<sup>+</sup>CD45RB<sup>low</sup>)<sup>(25)</sup> in





**Fig. 4.** Immunological immaturity status interferes with the induction of oral tolerance. Mice (12-week-old) were treated by oral administration of 20 mg OVA (ovalbumin; oral OVA, ■) and PBS (oral PBS, □) or untreated and non-immunised mice were used as control (Naive, ■) and intraperitoneally immunised 7 d afterwards with 10 µg OVA + 1 mg Al(OH)<sub>3</sub>. A booster with OVA was given 14 d afterwards. After 7 d, mice were fed 20% OVA solution for seven consecutive days. (A, B and C) Anti-OVA total Ig, IgG1 and IgE levels, respectively, were measured by ELISA. Values are means and standard deviations represented by vertical bars (n 10). <sup>a,b,c,d,e</sup> Mean values with unlike letters are significantly different ( $P < 0.05$ ).

the small intestine LP compartment was also reduced (Fig. 1(B)). These T-cell population comprises not only effector-activated T cells but also a population of T cells with regulatory properties that are important for gut homeostasis, as described earlier by Powrie *et al.*<sup>(25)</sup>. Many subtypes of regulatory T cells and cytokines have been described as important for oral tolerance induction<sup>(23)</sup>. It is noteworthy that production of the regulatory IL-10 by MLN cells upon stimulation was also diminished in Aa-fed mice (Fig. 3(F)).

## Discussion

Protein breakdown into amino acids in the gut has an essential nutritive role; however, it generates important peptides that interact and activate the immune cells that are present in the gut mucosa. To study the immunological relevance of dietary proteins, we used a model that replaces intact proteins by amino acids that have been previously described by our group<sup>(2,26)</sup>. It is important to notice that, in the present study, neither Cas-fed nor Aa-fed mice displayed nutritional or behavioural disorders, showing normal growth curve, blood cell counts, and serum albumin and total serum protein concentrations (Fig. 2), demonstrating that the diet is well balanced and that this is a suitable model to study the direct impact of dietary proteins in the immune system development. The immunological effects of microbiota antigens have been addressed by several studies. There are many reports showing dramatic immunological changes in germ-free mice including a poorly developed gut-associated lymphoid tissue, low levels of SIgA, reduced serum Ig levels and reduced inflammatory responses<sup>(27–31)</sup>. More recent studies on the impact of microbiota on Ig production have shown that it includes down-regulation of transcription factors involved in early B-cell activation steps and up-regulation of genes and proteins involved in later stages of B-cell response<sup>(32)</sup>.

We have also observed similar immunological alterations in mice that were reared from weaning to adulthood in a diet free of intact protein (Aa)<sup>(2,26)</sup>. The introduction of normal diet after weaning leads to fast changes in the gut mucosa characterised by the maturation of its associated lymphoid tissue followed by appearance of germinative centres in Peyer's patches and increase in SIgA production<sup>(33)</sup>. In a

previous study<sup>(2)</sup>, we demonstrated that C57BL/6 adult mice fed a protein-free diet since weaning present elongated intestinal villous, lower number of intraepithelial and LP lymphocytes, reduced SIgA and serum Ig production as well as a predominant production of Th2 cytokines by cells from peripheral lymphoid organs. These features resemble the profile found in germ-free and neonate mice. In the present study, we confirmed these data showing that BALB/c mice fed an Aa presented lower levels of serum Ig, elongated intestinal villous, reduced production of SIgA (Fig. 1) as well as reduced ability of MLN cells to produce cytokines (Fig. 3(F)) when compared with Cas-fed control mice. Therefore, Aa-fed BALB/c mice also had an immature profile at adulthood. A plausible explanation for the elongation of the villi observed in Aa-fed mice is the reduction in stimulation provided by lymphocytes that are associated either with the epithelia (intraepithelial lymphocytes) or the LP compartment. Indeed, Aa-fed mice presented a lower frequency of activated CD4<sup>+</sup>CD25<sup>+</sup>CD45RB<sup>low</sup> T cells in the gut LP and reduced numbers of intraepithelial lymphocytes (Fig. 1(B) and (E)). SCID mice, which bear few lymphocytes, present the same elongated villi observed in neonates, germ-free mice and Aa-fed mice<sup>(5,6)</sup>. This result demonstrates a direct connection between epithelial cells and intestinal lymphocytes. The morphological alterations and reduced number of lymphocytes may also explain the diminished number of mucus-secreting cells (goblet cells) seen in Aa fed mice (Fig. 1(C)). It is already reported that the lactation period represents a critical period of intense stimulation and consequent development of gut-associated lymphoid tissue in mice<sup>(34)</sup>. In contrast, the present data clearly indicate that the developmental period during lactation is followed by profound immunological changes triggered by the introduction of food proteins at weaning.

We next evaluated the impact of these immunological alterations in two important phenomena initiated at the gut mucosa, food allergy and oral tolerance. In the present model, we observed that in the absence of food proteins, there was a reduction in the susceptibility of both. Mice fed a Cas diet produced higher amounts of OVA-specific IgE and SIgA, and secreted more IL-4, IL-5, IL-10 and interferon-γ; they also had reduced consumption of chow and OVA during food allergy induction, increased weight loss and

elevated number of eosinophils in the LP compartment (Fig. 3). In contrast, Aa-fed mice display reduction in clinical parameters associated with food allergy development in the experimental models such as weight loss and aversion to ingest the allergen<sup>(35)</sup>. Curiously, germ-free and antibiotic-treated mice exhibit increased food-allergen sensitivity<sup>(36)</sup>, high serum IgE levels and increased circulating basophils<sup>(37)</sup>. However, interaction of host immunity and commensal microbiota is very complex and depend upon the species of bacteria involved. Several lines of evidence support, for instance, that some specific bacteria and their products are associated with protection against allergic sensitisation, probably through sustaining generation of regulatory T cells in the intestinal mucosa<sup>(38)</sup>. Rodriguez *et al.*<sup>(39)</sup> also demonstrated that a transplanted healthy infant microbiota mainly composed of *Bifidobacterium* and *Bacteroides* had a protective impact on sensitisation and food allergy in mice. Other studies have shown that the presence of gut microbiota is required for the development of systemic specific immune responses in mice and infants<sup>(40)</sup>. In the conditions tested by the present study, the absence of whole dietary proteins was associated with a dramatic reduction in antigenic stimulation in the gut mucosa and, as a consequence; impairment of immunological functions related to activation of CD4<sup>+</sup>T cells, production of post-switch Ig such as IgG, IgA and IgE (Fig. 1); and release of inflammatory cytokines including the ones associated with food allergy development<sup>(35,41)</sup> (Fig. 3(F)).

Other studies using diets containing low amounts of protein have also shown that both food allergy and oral tolerance are affected by the dietary protein content<sup>(42,43)</sup>. Nevertheless, these studies have addressed the effect of nutritional status on immunological responses. Two reports have shown that low-protein diets favour oral tolerance induction to OVA and  $\beta$ -lactoglobulin, whereas IgE responses were negatively affected by the reduction in dietary protein. These studies have focused on the nutritional effects of dietary proteins, and the immunological impact observed was indirect since the proteins were withdrawn from the diets. In our study, the protein-free diet is balanced in terms of its amino acid content, and mice did not present nutritional deficiency as shown here by the concentration of albumin and total protein (Fig. 2(A) and (B)) and in a previous study using the same diet<sup>(2)</sup>. The effects were, therefore, directly related to the immunological stimulation by intact proteins acting as antigens. Another distinct aspect of the present study is that mice received the protein-free Aa since weaning up to adulthood covering the post-lactation period of the immune system maturation.

In addition to the interference in food allergy induction, ingestion of the Aa diet resulted in a deficient ability to generate oral tolerance (Fig. 4). Previous studies from our group have shown that mice fed the same protein-free diet present reduced expression of co-stimulatory molecules such as CD40 and CD80 in antigen-presenting cells<sup>(44)</sup>. This deficient signalling provided by antigen-presenting cells could impair the expansion of T and B cells as well as Ig class switch<sup>(45)</sup>, and it may explain the reduced antigen-specific immune response observed in the present study. Regulatory CD4<sup>+</sup>T cells (Tregs) and regulatory cytokines

such as transforming growth factor  $\beta$  and IL-10 are described as important mediators of oral tolerance induction<sup>(23,24,46,47)</sup>. In addition, some reports have demonstrated that Tregs can modify allergic responses in mice<sup>(48)</sup>. The present results suggest that the absence of protein stimuli favour the maintenance of T cells with a naïve phenotype CD4<sup>+</sup>CD25<sup>+</sup>CD45RB<sup>high</sup> (data not shown) as opposed to activated CD4<sup>+</sup>CD25<sup>+</sup>CD45RB<sup>low</sup> T cells that comprise a mixture of regulatory and effector CD4<sup>+</sup>T cells (Fig. 1(B)). Production of pro-inflammatory cytokines (interferon- $\gamma$  and IL-4) as well as regulatory cytokines (IL-10) was reduced in Aa-fed mice (Fig. 3). Neonatal mice also exhibit a naïve T-cell profile<sup>(49)</sup>, and they are deficient in the development of oral tolerance<sup>(50)</sup>. The immature immune system presented by Aa-fed mice can explain the inability of these mice to generate inflammatory as well as regulatory events such as food allergy and oral tolerance. We have previously shown that Aa-fed mice display a similar impairment of developing a systemic protective immune response against *Leishmania major* infection probably due to the immature state of their antigen-presenting cells<sup>(44)</sup>. In the present study, we demonstrate that dietary protein ingestion since weaning is important for the differentiation of inflammatory and regulatory cells at the gut mucosa. These results show that the systemic immunological activities initiated at the mucosal sites are highly modulated by the stimulation of gut antigens such as dietary proteins.

## Acknowledgements

The authors thank Ilda Marçal de Souza for the excellent care of our animal facility, Frankcinéia A. Assis and Dairton Miranda for technical support, and the following sources of support: FAPEMIG (Foundation for Research Support of Minas Gerais), CAPES (Coordination of Personal Improvement of Higher Education) and CNPq (National Council of Technological and Scientific Development). All the authors read and approved the final manuscript.

The present study was supported by grants from FAPEMIG and CAPES, and some of the authors (A. M. C. F., J. P.-S., A. F. S. and R. P. O.) received scholarships and fellowships from CNPq, Brazil.

The authors' contributions are as follows: J. P.-S. conducted the research, analysed the data and wrote the paper; A. F. S. performed flow cytometry assays and helped in writing the paper; R. P. O. and M. L. P. R. helped in the material processing and cell culture assays; C. R. C. and J. F. A. co-supervised the research; A. M. C. F. designed and supervised the research.

None of the authors has any conflict of interest to declare.

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