



Prolonged ingestion of ovalbumin diet by Ova sensitized mice suppresses mBSA-induced arthritis



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ARTICLE INFO

Article history:

Received 19 October 2012

Accepted 10 July 2013

Available online 18 July 2013

Keywords:

Food allergy

Mice

Antigen-induced arthritis

Oral tolerance

Ovalbumin

Oral immunotherapy

ABSTRACT

Concomitant chronic diseases are a common finding in clinics and may consist in a major issue in therapeutics. Here, we investigated whether prolonged ingestion of ovalbumin (Ova) by sensitized mice would reduce the severity of an associated concurrent immunomediated condition such as antigen-induced arthritis (AIA). AIA was induced by administration of methylated bovine albumin (mBSA) into the knee joints of previously immunized mice, and evaluated by articular leukocyte trafficking and levels of cytokines (TNF- α , IL-1 β) and chemokine (CXCL-1) in the periarticular tissue. Continuous Ova feeding by Ova sensitized mice decreased serum levels of anti-Ova IgE, and led to a significant suppression of leukocyte adhesion and infiltration into synovial tissue and cavity. Also, a marked cytokine reduction was observed, suggesting that prolonged ingestion of ovalbumin by sensitized mice suppresses specific IgE production with concomitant reduction in peripheral T cells, which may impact in the pathogenesis of AIA, a non-related condition.

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1. Introduction

Dietary proteins and products from gut microbiota constantly activate physiological interactions among immune cells and these molecules [1–3]. It is estimated that 100 kg of proteins may pass through the gut of an adult human being every year without causing detectable adverse reactions [4]. Such untroubled relationship may be explained by a phenomenon named “oral tolerance to dietary proteins”, and it usually refers to how a prior ingested antigen modulates the systemic immune responses to an antigenic challenge given by a parenteral route [5]. On the other hand, it is well

accepted that the breakdown or the absence of oral tolerance might trigger food allergy reactions [4].

Food allergies are disorders that affect about 20–30% of the human population in developing countries, making them one of the most common chronic diseases [6]. It is generally accepted that 6–8% of all children below three years old present food allergy reactions [7,8], specially IgE-mediated hypersensitivities [9]. The most common foods associated to allergy episodes are milk, egg, peanut, chestnut and shrimp [7,10]. In atopic patients, involuntary exposure to a food allergen may induce a mild and persistent allergic condition involving disorders of skin, gastrointestinal and respiratory tracts, or trigger a multi-organ systemic reaction with cardiovascular collapse [11]. Therefore, there is a considerable interest in identifying therapies directed to prevent or reduce food allergy-related morbidity.

Oral immunotherapy (OIT) is one of these interventions and involves the regular administration of small amounts of allergen by the oral route in order to first rapidly induce desensitization and then induce tolerance to the allergen [12]. Recently, it has been shown that the OIT protocols are efficient as treatment of allergies to cow's milk, egg, fish and fruits [13,14]. However, little is known regarding the effect of OIT in non related pathological conditions. For example, homing of immune cells from the gut to the inflamed

Abbreviations: AIA, antigen-induced arthritis; CFA, Freund's complete adjuvant; ELISA, enzyme-linked immunosorbent assay; H&E, hematoxylin and eosin; mBSA, methylated bovine albumin; MPO, myeloperoxidase; OIT, oral immunotherapy; Ova, ovalbumin; PBS, phosphate buffered saline; RA, rheumatoid arthritis.

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synovium may represent a link between intestinal immunity and arthritis [15] and it is reasonable to propose that the immune regulation of one pathological condition may affect the other one.

Rheumatoid arthritis (RA) is an autoimmune inflammatory disease that affects the synovial tissue within joints, typically the small joints of the hands and feet [16]. RA affects between 0.3% and 1% of the general population and it is more common in middle age. In general, RA results in persistent pain and stiffness, progressive joint destruction, functional decline and premature death [16], and the available therapies usually fail to promote satisfactory results [17]. The pathological outcome of inflammatory arthritis is governed by the presence of the cytokines TNF- α , IL-1 β and chemokine CXCL-1 in the synovium, which activate resident cells to attract neutrophils into the synovial cavity, amplifying the inflammatory response. In fact, these inflammatory events are observed in the murine model of antigen-induced arthritis (AIA) in a similar manner to human rheumatoid arthritis [18–22].

Considering that concomitant chronic diseases are a major challenge in therapeutics, the aim of this study was to investigate whether the continuous ingestion of ovalbumin (Ova) by Ova-sensitized mice could trigger oral tolerance and dampen the development of antigen-induced arthritis.

2. Materials and methods

2.1. Mice

Male BALB/c mice (6–8 weeks old) were used. Mice were obtained from the animal facility at ICB/UFMG. All mice received standard (Purina) mouse chow until the Ova diet consumption. The investigations were consistent with the Ethical Principles in Animal Experimentation, adopted by the Ethics Committee in Animal Experimentation of our Institution (protocol 191/2007-CETEA/UFMG).

2.2. Ova sensitization and oral ingestion

The Control group received 0.2 ml saline (0.9%) with adjuvant [1 mg Al(OH)₃] on day 0 and saline on day 14. The Ova group received 0.2 ml saline (0.9%) with adjuvant and 10 μ g Ova (five times crystallized hen's egg albumin; Sigma, St. Louis, MO, USA) on day 0 and saline with 10 μ g Ova on day 14. All injections were performed subcutaneously. Seven days after the booster (day 21), the standard chow of all groups was replaced with 14% Ova diet for a period of one or two weeks (from day 21 until the end of the experiment) (Fig. 1A). This diet was prepared using a lyophilized egg white (Salto's, Belo Horizonte, MG, Brazil) and nutrient contents were the same specified for AIN-93G [23]. During the period of Ova diet, food consumption was assessed daily by weighting the remaining chow, in the cage of five mice, and comparing its weight with the previous day. Data were reported as percentage of diet consumption (Ova group/Control group), considering diet consumption by Control group as 100%.

In another set of experiments, some mice received the same sensitization with Ova described above and were fed with Ova diet only for 24 h on day 21. Other groups were not sensitized or fed with the antigen. All these additional groups are specified in the related figures (Fig. 7A and B).

2.3. Arthritis induction

In another set of experiments mice were separated in four groups (Control, Arthritis, Ova, Ova/Arthritis). Ova and Ova/Arthritis groups received the Ova sensitization described before and the Control and Arthritis group received only the adjuvant [Al(OH)₃].

On day 21, the standard mouse chow (Purina, Belo Horizonte, MG, Brazil) was replaced with a 14% Ova diet for all groups until the end of the experiments.

Also on day 21, the Arthritis and Ova/Arthritis groups were intra-dermally immunized at the base of the tail with 500 μ g of methylated bovine serum albumin (mBSA; Sigma, St. Louis, MO) in 100 μ l of an emulsion of saline and an equal volume of Freund's complete adjuvant (CFA; Sigma). The Control and Ova groups received only 100 μ l of an emulsion of saline and an equal volume of Freund's complete adjuvant. Fourteen days later (day 35), each mouse in Arthritis and Ova/Arthritis groups received an injection of 10 μ g of mBSA (in 10 μ l sterile saline) in both knee joint and in Control and Ova groups they received only 10 μ l of sterile saline in both knee joint. Details of each experimental group are described in Fig. 2A.

2.4. Intravital microscopy of the synovial microcirculation

Intravital microscopy was performed in the synovial microcirculation of the mouse knee, as described previously [19]. Briefly, the left hind limb was placed on a stage, with the knee slightly flexed and the patellar tendon mobilized and partly resected. The intra-articular synovial tissue of the knee joint was then visualized for the determination of leukocyte rolling and adhesion.

A 20-fold objective was used to select 2–4 regions of interest in each mouse. To measure the leukocyte–endothelial cell interactions, the fluorescent marker rhodamine 6 G (Sigma) was injected intravenously as a single bolus of 0.15 mg/kg immediately before the measurements. Rhodamine epiillumination was achieved with a 150 W variable HBO mercury lamp in conjunction with a Zeiss filter set 15 (546/12 nm band-pass filter, 580 nm Fourier transforms, 590 nm late potentials; Zeiss, Wetzlar, Germany). The microscopic images were captured with a video camera (5100 HS; Panasonic, Secaucus, NJ) and recorded on S-VHS videotape, using both filter blocks consecutively. Data analysis was performed off-line.

Rolling leukocytes were defined as those cells moving slower than the cells moving at a regular flux in a given vessel. The flux of rolling cells was measured as the number of rolling cells passing by a given point in the venule per minute, with results expressed as cells/minute. A leukocyte was considered to be adherent if it remained stationary for at least 30 s, and total leukocyte adhesion was quantified as the number of adherent cells within a 100 μ m length of venule, with results expressed as cells/100 μ m.

2.5. Serum and tissue collection

At the end of the experimental protocol, all groups were deeply anesthetized by intraperitoneal injection (i.p.) of 10 mg/kg xylazine and 100 mg/kg ketamine hydrochloride and blood samples were obtained from the axillary plexus for anti-Ova IgE and anti-mBSA IgG assays. Next, the animals were euthanized by i.p. injection of more of the same anesthetic solution and knee synovial cavity lavage was performed. The periarticular tissue of the left knee was removed for evaluation of myeloperoxidase activity and the levels of cytokines (TNF-, IL-1) and chemokine (CXCL-1).

2.6. Determination of absolute peripheral T cell count

For quantification and *phenotyping* of T cells, heparinized anti-coagulated blood was lysed using ACKlysis buffer. Specimens were then counted and 10⁶ cells/mouse were labeled with anti-CD3 PE-Cy7, anti-CD4 FITC, anti-CD25 APC anti-CD8 PerCPor their respective isotype controls (BD Biosciences). Intracellular staining for Foxp3 was performed with fixation/permeabilization kit according to the manufacturer's protocol (BD Biosciences) and detected with anti-Foxp3 PE (BD Biosciences). Data were acquired with a BD

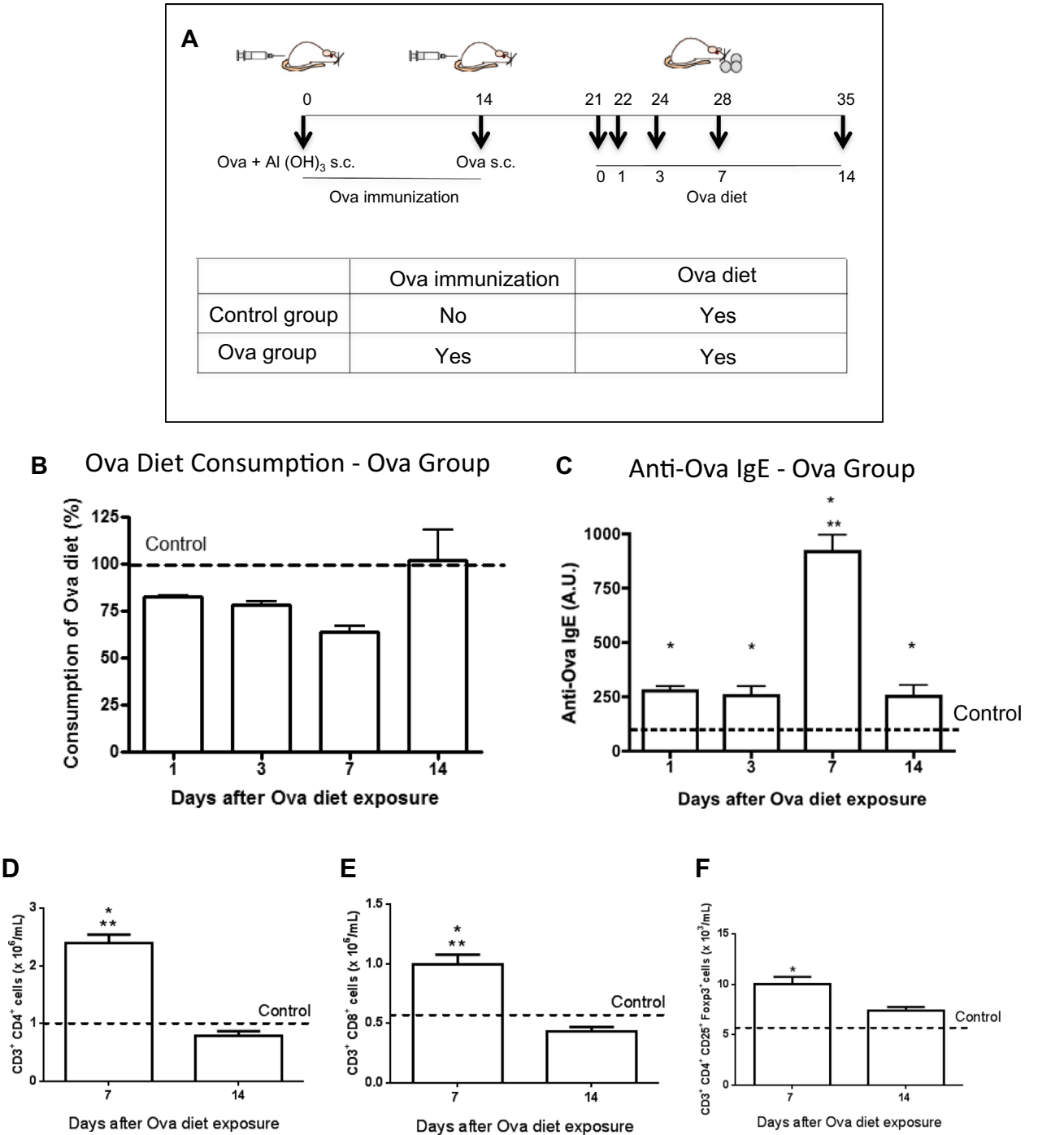


Fig. 1. Experimental protocol, kinetics of diet consumption, kinetics of specific serum IgE levels and quantification of peripheral T cells. (A) BALB/c mice of Ova group were sensitized with ovalbumin (Ova) in Al(OH)₃ on day 0 and with Ova on day 14. The Control group received Al(OH)₃ on day 0 and saline on day 14. On day 21, the standard mouse chow was replaced by Ova diet for both groups until the end of experiment (day 35). (B) Food intake was assessed every day from day 21 to day 35 and the data was reported as percentage of diet consumption (Ova group/Control group). (C) On days 1, 3, 7 and 14, serum of sensitized mice was collected for anti-Ova IgE measurement by ELISA. On days 7 and 14 whole blood of sensitized mice was collected for quantification of peripheral CD3⁺ CD4⁺ cells (D), CD3⁺ CD8⁺ cells (E) and CD3⁺ CD4⁺ CD25⁺ Foxp3⁺ cells (F). Values represent the mean ± SEM. **p* < 0.05 compared to Control group. ***p* < 0.05 compared to 1, 3 and 14 days of Ova consumption.

FACSCanto II flow cytometer and the proportions of CD3⁺ CD4⁺, CD3⁺ CD8⁺ and CD3⁺ CD4⁺ CD25⁺ Foxp3⁺ cells were determined in the lymphocyte gate, defined in terms of light-scattering properties, using FlowJo software (TreeStar, Inc. Ashland, OR). Absolute counts were calculated from the absolute blood count.

2.7. Knee synovial cavity lavage

The left knee synovial cavity was washed with phosphate buffered saline (PBS) (2 × 5 μl), and the total number of leukocytes in the cavity lavage was determined by counting the leukocytes

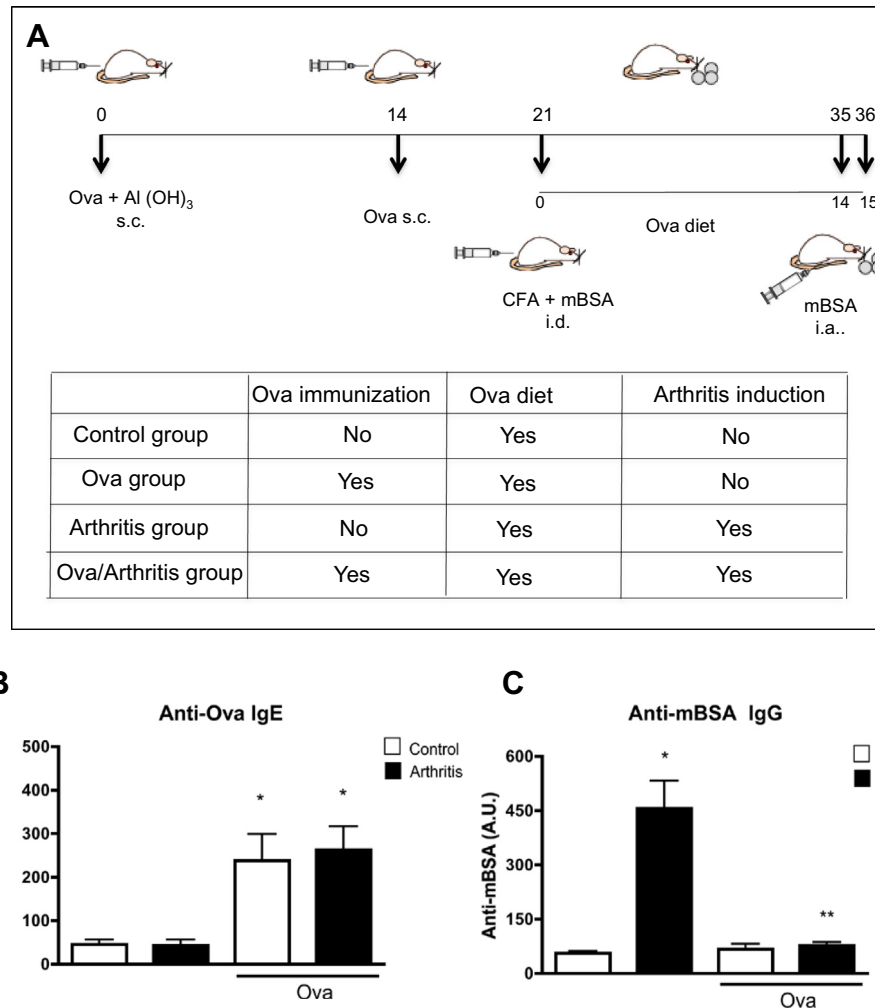


Fig. 2. Experimental protocol of arthritis induction and evaluation of specific serum IgE and IgG levels. (A) BALB/c mice of Ova and Ova/Arthritis groups were sensitized with ovalbumin (Ova) in Al(OH)₃ on day 0 and with Ova on day 14. The Control and Arthritis groups received Al(OH)₃ on day 0 and saline on day 14. On day 21 the Arthritis and Ova/Arthritis groups were immunized with mBSA in Freund's complete adjuvant (CFA) and the Control and Ova groups received only an emulsion of saline in CFA. On the same day (day 21), the standard mouse chow was replaced by Ova diet for all groups until the end of experiment (day 36). On day 35, the Arthritis and Ova/Arthritis groups received an injection of mBSA in saline in both knee joint while the Control and Ova groups received only saline. On day 36, serum was collected for anti-Ova IgE (B) and anti-mBSA IgG (C) measurements by ELISA. Values represent the mean $\pm$ SEM. * $p < 0.05$ compared to Control group. ** $p < 0.05$ compared to Arthritis group.

in a Neubauer chamber after staining cells with Turk's solution. Differential counts were obtained from cytospin preparations (Shandon III; Thermo Shandon, Frankfurt, Germany) by evaluating the percentage of each leukocyte on a slide stained with May–Grunwald–Giemsa stain.

2.8. Histological analysis

The right knee joint was removed and fixed for 48 h with 10% paraformaldehyde (pH 7.2). The joints were then incubated in 10% EDTA at pH 7.2 for 20 days at room temperature to decalcify the bone. Samples were washed with PBS and dehydrated. After being embedded in paraffin, the joints were sliced into 3 μ m-thick sections that were stained with hematoxylin and eosin (H&E). The slides were evaluated qualitatively using various parameters, as follows: severity of synovial hyperplasia (pannus formation), cellular exudate, cartilage depletion/bone erosion and extent of synovial infiltrate.

2.9. Determination of myeloperoxidase (MPO) activity

The periarticular tissue of the left knee joint was removed after knee cavity lavage and frozen at -70°C . Upon thawing of the sample, the tissue (0.1 mg of tissue per 1.9 ml of buffer) was

homogenized and processed for determination of MPO activity. The assay included 25 μ l of 3,3',5,5'-tetramethylbenzidine (Sigma) in PBS (pH 5.4) as the color reagent [22].

2.10. Enzyme-linked immunosorbent assay (ELISA) measurements

Levels of anti-Ova IgE were evaluated by capture-ELISA using plates coated with rat anti-mouse IgE, 50 μ l total serum and biotinylated Ova, as previously described [24]. The results for anti-Ova IgE antibodies were reported in arbitrary units (A.U.) using a highly positive reference serum determined as 1000 units. The ELISA test for anti-mBSA IgG was carried out using plates coated with 2 μ g/well of methylated bovine serum albumin (mBSA; Sigma, St. Louis, MO), 100 μ l of a 1:400 dilution of mouse sera, rabbit anti-mouse IgG (H + L)-HRP (Southern Biotechnology Associates, Birmingham, AL, USA). The reaction for anti-mBSA IgG was developed with *O*-phenylene-diamine and H₂O₂. The plates were read at 492 nm on an automated ELISA reader (Expert Plus, AsysHitech GmbH, Eugendorf, Austria). The concentrations of cytokines TNF- α and IL-1 β and the chemokines CXCL1 were measured in the periarticular tissue of left knee using a commercially available enzyme-linked immunosorbent assay, following the instructions supplied by the manufacturer (Duo-Set kits; R&D Systems, Minneapolis, MN). Briefly, 100 mg of tissue

was homogenized in 1 ml of PBS containing anti-proteases (0.1 mM phenylmethylsulfonyl fluoride, 0.1 mM benzethonium chloride, 10 mM EDTA, and 20 kallikrein inhibitor units of aprotinin A) and 0.05% Tween 20. The samples were then centrifuged for 10 min at 3000g and the supernatants were immediately assessed by ELISA at a 1:3 dilution in PBS. All samples were assayed in duplicate.

2.11. Statistical analysis

All values were expressed as mean $\pm$ SEM. Parametric data were evaluated using analysis of variance, followed by the Tukey test for multiple comparisons. Differences were considered statistically significant at $p < 0.05$. The GraphPad Prism software was used.

3. Results

3.1. Kinetics of Ova diet consumption, specific serum antibody levels and CD4⁺, CD8⁺, CD4⁺CD25⁺Foxp3⁺ blood cells in Ova sensitized mice after continuous Ova diet feeding

Analysis of Ova diet consumption and serum anti-Ova IgE levels were performed after 1, 3, 7 and 14 days of continuous and restricted Ova diet consumption by Ova sensitized (Ova group) and non-sensitized mice (Control group) (Fig. 1A). Ova immunized mice displayed a continuous decrease in Ova diet consumption comparing to the Control group until the seventh day of Ova diet exposure. In contrast after this time, the aversion of Ova diet was abrogated and mice from Ova group showed higher food consumption in comparison to the Control group (Fig. 1B). The serum anti-Ova IgE levels were increased in immunized mice in all days, despite the highest level was observed on day 7, in which the aversive behavior was more evident. On day 14, the serum anti-Ova IgE levels were similar to the first days of Ova consumption (Fig. 1C). In a similar way to the humoral response, the peripheral blood CD3⁺ CD4⁺, CD3⁺ CD8⁺ and CD3⁺ CD4⁺ CD25⁺ Foxp3⁺ cells of sensitized mice were increased after 7 days of Ova diet ingestion in comparison to Control group, with a significant decrease after 14 days of continued antigen ingestion (Fig. 1D–F).

3.2. Serum levels of anti-Ova IgE and anti-mBSA IgG

Antigen-induced arthritis was induced by intra-dermally immunization with mBSA in CFA, followed by injection of mBSA into synovial cavity 14 days thereafter (Arthritis and Ova/Arthritis groups); mice injected only with CFA (Control and Ova groups) served as controls (Fig. 2A). The protocol of arthritis induction did not interfere on anti-Ova IgE serum levels (Fig. 2B). On the other hand, the anti-mBSA IgG levels induced by mBSA immunization were totally abrogated in Ova sensitized mice after continuous ingestion of Ova diet (Fig. 2C).

3.3. Cytokine and chemokine concentrations in periarticular tissue

An increased level of the inflammatory cytokines TNF- α , IL-1 β , and the chemokine CXCL-1 were found in periarticular tissue from Arthritis group in comparison to Control group. Ova ingestion by Ova immunized mice greatly reduced the concentration of TNF- α , IL-1 β , and the CXCL-1 in periarticular tissue induced by arthritis protocol (Fig. 3A–C).

3.4. Leukocyte recruitment to periarticular tissue and knee synovial cavity

Leukocyte recruitment to periarticular tissue was evaluated by the visualization of leukocyte-endothelial cell interactions in the

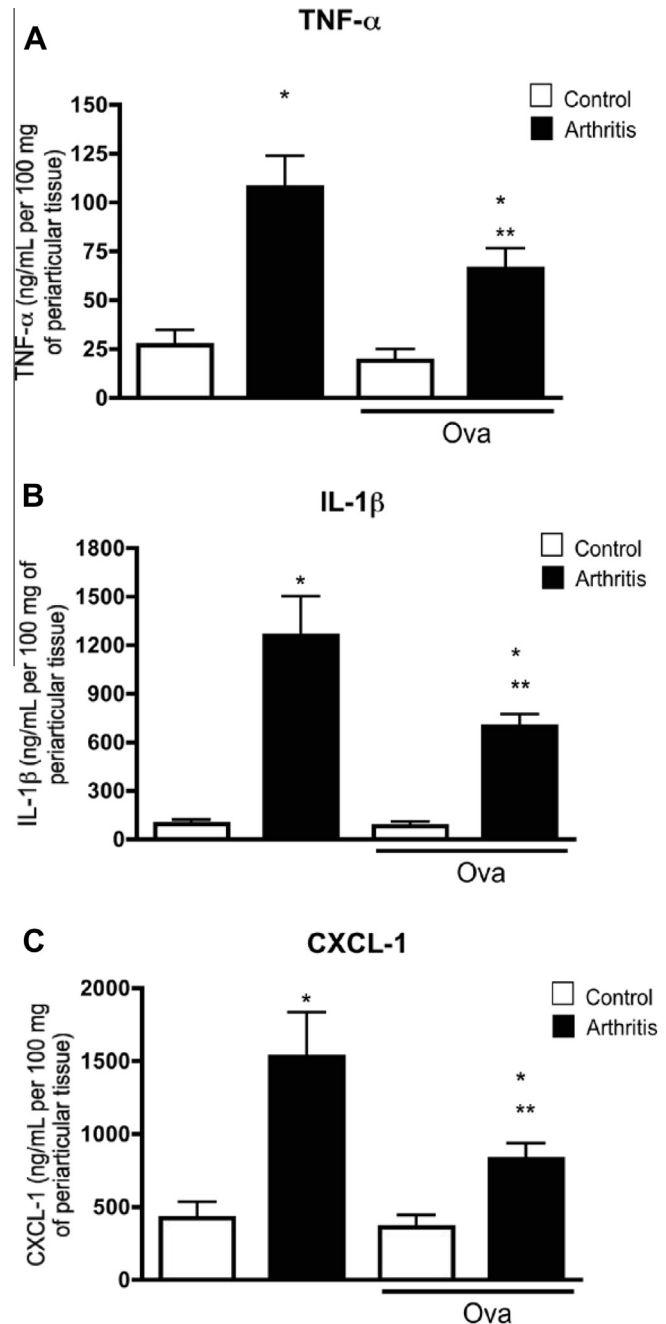


Fig. 3. TNF- α , IL-1 and CXCL-1 levels in periarticular tissue. BALB/c mice of Ova and Ova/Arthritis groups were sensitized with ovalbumin (Ova) in Al(OH)₃ on day 0 and with Ova on day 14. The Control and Arthritis groups received Al(OH)₃ on day 0 and saline on day 14. On day 21, the Arthritis and Ova/Arthritis groups were immunized with mBSA in Freund's complete adjuvant (CFA) and the Control and Ova groups received only an emulsion of saline in CFA. On the same day (day 21), the standard mouse chow was replaced by Ova diet for all groups until the end of experiment (day 36). On day 35, the Arthritis and Ova/Arthritis groups received an injection of mBSA in saline in both knee joint while the Control and Ova groups received only saline. On day 36, the periarticular tissue of left knee joint was collected and assayed for TNF- α (A), IL-1 β (B) and CXCL-1 (C) levels by ELISA. Values represent the mean $\pm$ SEM. $p < 0.05$ compared to Control group. $^{**}p < 0.05$ compared to Arthritis group.

synovial microcirculation and by histology of knee joint. As seen in Fig. 4 and in the Supplementary video 1, AIA induced an increase in leukocyte rolling (Fig. 4A) and adhesion (Fig. 4B) followed by migration towards the tissue (Fig. 5B) and synovial cavity (Fig. 6). Histological analysis of H&E-stained joint sections from

the mice with AIA demonstrated a synovial hyperplasia and a dense infiltration of neutrophils in the synovium (Fig. 5B and E), which was also confirmed by the presence of activated MPO in periarticular tissue (Fig. 5F). However, Ova/Arthritis group showed decreased number of adherent leukocytes in synovial microcirculation (Fig. 4B and Supplementary video 2) and decreased neutrophil infiltration to the tissue (Fig. 5F) and to the knee synovial cavity (Fig. 6B). The decreased neutrophil recruitment was associated with reduced joint damage, as observed on histopathological analysis (Fig. 5D).

In order to see whether only immunization with Ova could interfere on leukocyte recruitment, a new set of experimental groups were added to the study. As observed in Fig. 7A, the protocol of Ova sensitization, without further Ova ingestion, did not reduce the migration of leukocytes toward the knee synovial cavity. Also, in order to see if there was an antigen competition in the moment of mBSA immunization and Ova ingestion, other two groups were added. Both groups were sensitized with Ova on day 0 and 14. However, one of these groups was immunized with mBSA on

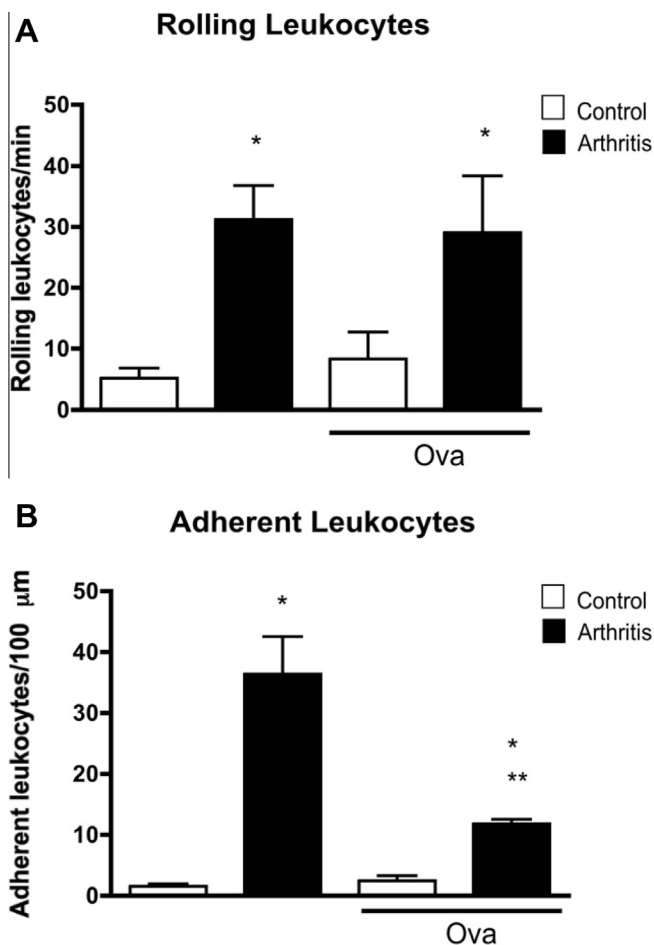


Fig. 4. Leukocyte recruitment to periarticular tissue and knee synovial cavity. BALB/c mice of Ova and Ova/Arthritis groups were sensitized with ovalbumin (Ova) in Al(OH)₃ on day 0 and with Ova on day 14. The Control and Arthritis groups received Al(OH)₃ on day 0 and saline on day 14. On day 21, the Arthritis and Ova/Arthritis groups were immunized with mBSA in Freund's complete adjuvant (CFA) and the Control and Ova groups received only an emulsion of saline in CFA. On the same day (day 21), the standard mouse chow was replaced by Ova diet for all groups until the end of experiment (day 36). On day 35, the Arthritis and Ova/Arthritis groups received an injection of mBSA in saline in both knee joint while the Control and Ova groups received only saline. On day 36, the leukocyte rolling (A) and adhesion (B) to the synovial microcirculation were assayed by intravital microscopy. Values represent the mean $\pm$ SEM. * $p < 0.05$ compared to Control group. ** $p < 0.05$ compared to Arthritis group.

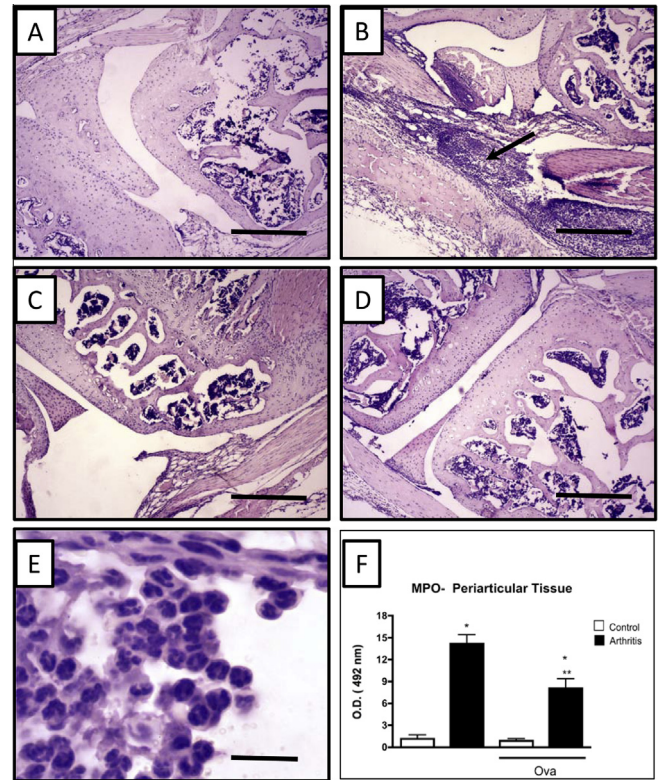


Fig. 5. Histological analysis and determination of MPO activity. BALB/c mice of Ova and Ova/Arthritis groups were sensitized with ovalbumin (Ova) in Al(OH)₃ on day 0 and with Ova on day 14. The Control and Arthritis groups received Al(OH)₃ on day 0 and saline on day 14. On day 21, the Arthritis and Ova/Arthritis groups were immunized with mBSA in Freund's complete adjuvant (CFA) and the Control and Ova groups received only an emulsion of saline in CFA. On the same day (day 21), the standard mouse chow was replaced by Ova diet for all groups until the end of experiment (day 36). On day 35, the Arthritis and Ova/Arthritis groups received an injection of mBSA in saline in both knee joint while the Control and Ova groups received only saline. On day 36, the right knee joint was collected for histological analysis (A–E) and the left knee joint for MPO determination (F). The histological slides were stained with hematoxylin-eosin and evaluated qualitatively. Scale bars mean 100 μm (A–D) and 25 μm (E). Arrow indicates the leukocyte infiltration in synovial cavity. Illustrative picture of the knee joint from Control group (A), Arthritis group (B and E), Ova group (C) and Ova/Arthritis group (D). Values represent the mean $\pm$ SEM. * $p < 0.05$ compared to Control group. ** $p < 0.05$ compared to Arthritis group.

day 21, was fed with Ova diet only for 24 h (day 21) and received mBSA intra-articular on day 35. The other group was immunized with mBSA on day 18, was fed with Ova diet only for from day 21–33, and received mBSA intra-articular on day 32. As shown in Fig. 7B, the ingestion of Ova on day 21 for 24 h did not reduce the antigen-induced leukocyte recruitment, discarding the occurrence of antigenic competition. Moreover, the continuous antigen ingestion was able to reduce leukocyte recruitment to periarticular tissue and cavity.

4. Discussion

We demonstrated that following 24 hours of mBSA injection into knee joint of immunized mice, an intense inflammatory infiltrate, predominantly neutrophilic, was observed. Also, higher levels of TNF- α , IL-1 β and CXCL-1 confirmed a marked inflammatory response. Interestingly, arthritic mice which were chronically fed with antigen had reduced articular leukocyte infiltration and lower serum levels of anti-mBSA IgG. Chronic ingestion of ovalbumin by Ova sensitized animals induced transitory high levels of anti-OVA

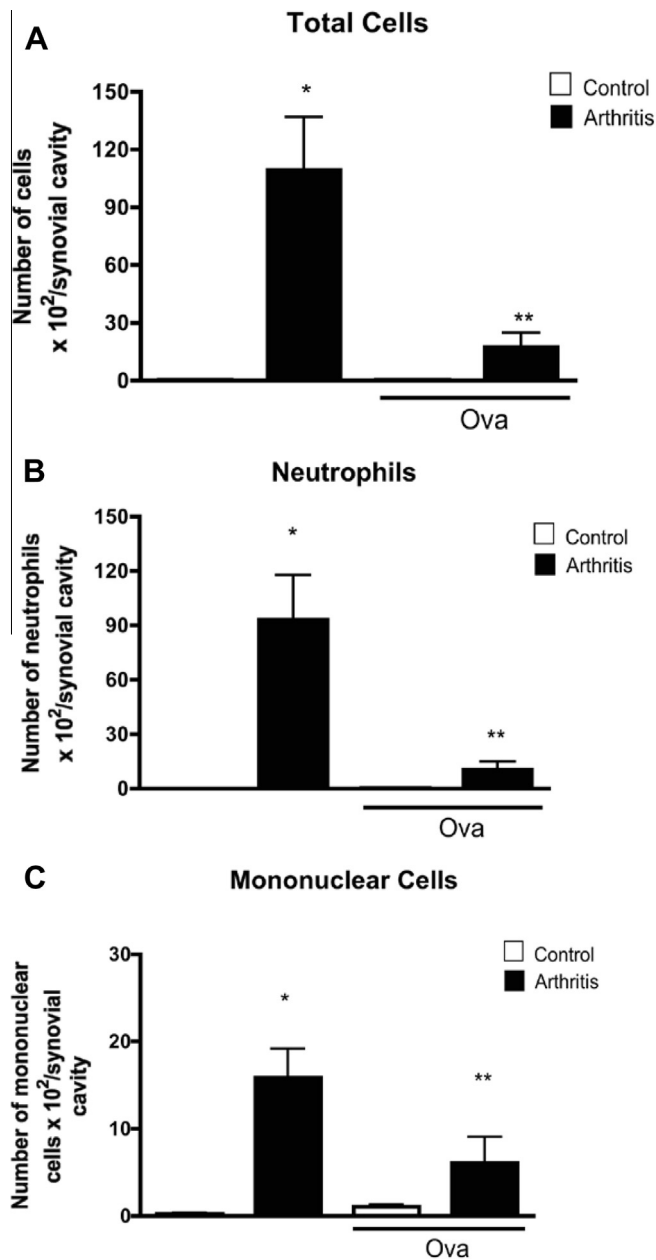


Fig. 6. Leucocytes in the synovial cavity. BALB/c mice of Ova and Ova/Arthritis groups were sensitized with ovalbumin (Ova) in $\text{Al}(\text{OH})_3$ on day 0 and with OVA on day 14. The Control and Arthritis groups received $\text{Al}(\text{OH})_3$ on day 0 and saline on day 14. On day 21, the Arthritis and Ova/Arthritis groups were immunized with mBSA in Freund's complete adjuvant (CFA) and the Control and Ova groups received only an emulsion of saline in CFA. On the same day (day 21), the standard mouse chow was replaced by Ova diet for all groups until the end of experiment (day 36). On day 35, the Arthritis and Ova/Arthritis groups received an injection of mBSA in saline in both knee joint while the Control and Ova groups received only saline. On day 36, the cavity of the left knee was washed and number of total leucocytes (A), neutrophils (B) and mononuclear cells (C) was counted. Values represent the mean $\pm$ SEM. * $p < 0.05$ compared to Control group. ** $p < 0.05$ compared to Arthritis group.

IgE, CD4^+ , CD8^+ and regulatory CD4^+ CD25^+ Foxp3^+ T cells that decreased after fourteen days, suggesting that a continuous feeding may induce oral tolerance to Ova [25]. Moreover, the prolonged ingestion of antigen after sensitization also showed anti-inflammatory effects on cellular as well as humoral events triggered in animals with arthritis induced by an unrelated antigen.

As primed (immunized) organisms are resistant to oral tolerance induction, its therapeutic use might be challenging. In this

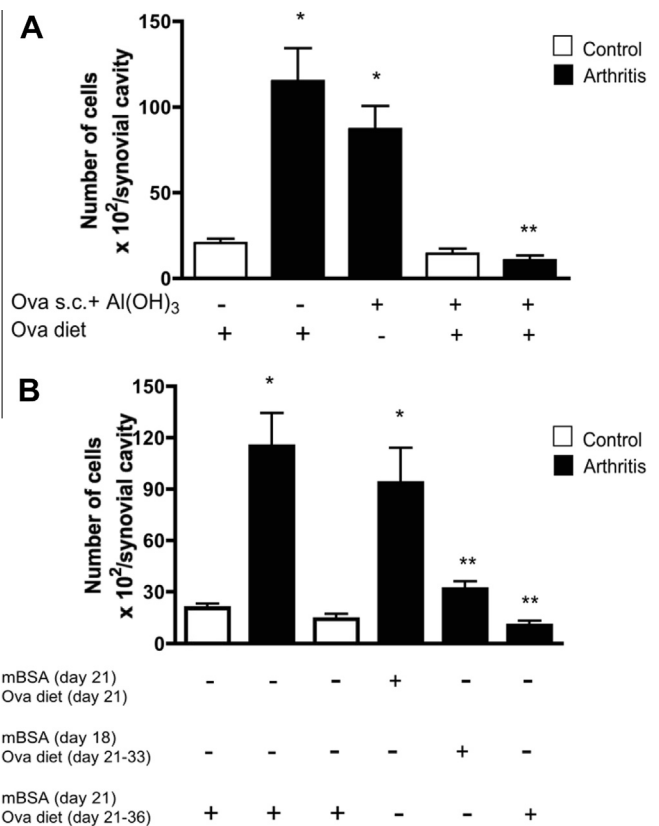


Fig. 7. Interference of immunization and different times of challenge with ovalbumin in the recruitment of leucocytes to the synovial cavity. BALB/c mice were separated in groups and submitted to different experimental protocols as specified in the figures. The Ova sensitized groups received the antigen in $\text{Al}(\text{OH})_3$ on day 0 and only the antigen on day 14. For some groups, the standard chow was replaced by Ova diet only on day 21, on days 21–33 or on days 21–36. On day 18 or 21, the Arthritis group was immunized with mBSA in Freund's complete adjuvant (CFA) and the Control group received only an emulsion of saline in CFA. On day 32 or 35, the Arthritis group received an injection of mBSA in saline in both knee joint while the Control group received only saline. On day 33 or 36, the cavity of the left knee was washed and number of total leucocytes was counted. Values represent the mean $\pm$ SEM. * $p < 0.05$ compared to Control group. ** $p < 0.05$ compared to Arthritis group.

case, oral exposures may actually induce secondary (booster) responses and anaphylactic reactions [26,27]. However, therapeutic application of oral tolerance in allergic diseases has gained increasing attention. In clinical trials, oral immunotherapy (OIT) and sublingual immunotherapy (SLIT) involving oral administration of allergenic foods have been applied as an allergen-specific immunotherapy for persistent food allergies [27–30]. Proposed mechanisms to explain oral tolerance include active suppression by regulatory T cells and clonal energy/deletion of lymphocytes in gut-associated lymphoid tissue [27]. In this sense, it was demonstrated that antigen-experienced CD4^+ T cells do not acquire regulatory phenotype, neither become anergic or undergo to an immune deviation, but die by apoptosis within the liver [31], contributing to antigen-specific systemic unresponsiveness upon oral antigen uptake. Also, the oral route favors a robust stabilization of specific antibody formation and may occur at low, medium or high serum antibody levels [32].

Our work not only demonstrated that the prolonged ingestion of antigen after immunization reduced specific IgE levels and specific blood T cells, but also showed anti-inflammatory effects on cellular as well as humoral events to unrelated antigen. According to these findings, we demonstrated that production of anti-mBSA IgG by animals immunized and fed with Ova diet was almost

completely suppressed. Additionally, reduced cytokine production and leukocyte recruitment to the tissue were concomitantly observed, which might be a result of decreased expression of adhesion molecules and chemokine release. Also, in turn, the reduced influx of neutrophils could decrease the local production of cytokines [33].

The previously indirect effect of oral tolerance described is a possible way to explain our results. Indeed, it has been demonstrated that the contact with a protein by oral route several days before an immunization containing adjuvant with the same protein and another unrelated antigen is able to block the formation of antibodies to this unrelated antigen. As a consequence, it reduces drastically the formation of granulomas around *Schistosoma mansoni* eggs, affects the eosinophil production in bone marrow and improves wound healing in skin. We can suggest, similarly to these authors, that it is much more general than suggested by innocent bystander suppression hypothesis, phenomenon in which regulatory cells and immunosuppressive cytokines activated by feeding suppress immune response in their vicinity [34–37]. However, contrary to our results Yoshino and Inada et al., using as oral treatment mBSA and Ova protein, during five days after immunization, failed to inhibit the establishment of synovial inflammation in antigen-induced arthritis [21,38]. This discrepancy may be attributed to the differences in the experimental animals and to the ingestion time of protein, considering that until the seventh day of Ova ingestion diet we did not observed reduced anti-Ova IgE levels in the serum of immunized animals.

It has been previously suggested that maintaining a balanced peripheral immune system may not be the only responsibility of a specialized subset of T cells dedicated to immune suppression, but rather a side effect of normal competition for shared resources within an intact immune system [39]. From this premises, we also investigated the suppressive effect of antigenic competition during immunization with mBSA by using two additional experimental groups. In one group, the immunization with mBSA was performed 4 days before the Ova diet consumption and we could observe that the cellular and humoral parameters of inflammation were similar to the Ova/Arthritis group. In another group, the ingestion of Ova was conducted only for 24 h on day 21, which did not interfere with the induction of arthritis, confirming that a continuous ingestion of antigen is necessary.

Although the phenomenon of oral tolerance needs to be clarified, in spite of intense basic and clinical research [27,29], important observations of our group have shown that chronic restricted and voluntary ingestion of the specific antigen by previously immunized animals, in addition to provoke mild inflammation in the gut [40], is able to suppress airway inflammation [41]. The oral tolerance and its indirect effects could explain the decreased levels of anti-Ova IgE in the serum and specific blood T cells of immunized mice after fourteen days of Ova diet consumption and its consequent impact on a non related pathological condition such as antigen-induced arthritis [32,34,35].

5. Conclusion

Our work, in addition to present a model to study the induction of oral tolerance in animals previously immunized, enables us to make important observations, that can be useful for future studies about the application of oral immunotherapy in allergic patients and new therapies for rheumatoid arthritis.

Acknowledgments

We are grateful to Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq/Brazil) and Fundação de Amparo a Pesquisa do Estado de Minas Gerais (FAPEMIG/Brazil) for financial

support. Some of the authors are also recipients of CNPq research fellowships (L.P.A.D. G.B.M. and D.C.C.), CAPES research fellowships (M.L.N.M. and N.V.B.) and FAPEMIG research fellowships (R.V.S.P.).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.cellimm.2013.07.005>.

References

- [1] P. Brandtzaeg, Food allergy: separating the science from the mythology, *Nat. Rev. Gastroenterol. Hepatol.* 7 (2010) 380–400.
- [2] P.J. Sansonetti, To be or not to be a pathogen: that is the mucosally relevant question, *Mucosal Immunol.* 4 (2011) 8–14.
- [3] P. Brandtzaeg, History of oral tolerance and mucosal immunity, *Ann. N.Y. Acad. Sci.* 778 (1996) 1–27.
- [4] S. Bischoff, S.E. Crowe, Gastrointestinal food allergy: new insights into pathophysiology and clinical perspectives, *Gastroenterology* 128 (2005) 1089–1113.
- [5] H.G. Wells, Studies on the chemistry of anaphylaxis. III. Experiments with isolated proteins, specially those of the hen's egg, *J. Infect. Dis.* 8 (1911) 147–171.
- [6] T. Matysiak-Budnik, M. Heyman, Food allergy and *Helicobacter pylori*, *J. Pediatr. Gastroenterol. Nutr.* 34 (2002) 5–12.
- [7] S.A. Bock, A critical evaluation of clinical trials in adverse reactions to foods in children, *J. Allergy Clin. Immunol.* 78 (1986) 165–174.
- [8] H.A. Sampson, Food allergy. Part 1: immunopathogenesis and clinical disorders, *J. Allergy Clin. Immunol.* 103 (1999) 717–728.
- [9] H.A. Sampson, Update on food allergy, *J. Allergy Clin. Immunol.* 113 (2004) 805–819, quiz 820.
- [10] D.D. Metcalfe, Food allergy, *Prim. Care.* 25 (1998) 819–829.
- [11] S.H. Sicherer, H.A. Sampson, Food allergy, *J. Allergy Clin. Immunol.* 125 (2010) S116–S125.
- [12] M.H. Land, E.H. Kim, A.W. Burks, Oral desensitization for food hypersensitivity, *Immunol. Allergy Clin. North. Am.* 31 (2011) 367–376, xi.
- [13] C. Patriarca, A. Romano, A. Venuti, D. Schiavino, V. Di Rienzo, E. Nucera, S. Pellegrino, Oral specific hyposensitization in the management of patients allergic to food, *Allergol. Immunopathol. (Madr.)* 12 (1984) 275–281.
- [14] G. Patriarca, E. Nucera, C. Roncallo, E. Pollastrini, F. Bartolozzi, T. De Pasquale, A. Buonomo, G. Gasbarrini, C. Di Campli, D. Schiavino, Oral desensitizing treatment in food allergy: clinical and immunological results, *Aliment. Pharmacol. Ther.* 17 (2003) 459–465.
- [15] P. Brandtzaeg, Review article: Homing of mucosal immune cells – a possible connection between intestinal and articular inflammation, *Aliment. Pharmacol. Ther.* 11 (Suppl. 3) (1997) 24–37, discussion 37–29.
- [16] G.S. Firestein, Immunologic mechanisms in the pathogenesis of rheumatoid arthritis, *J. Clin. Rheumatol.* 11 (2005) S39–S44.
- [17] R.F. van Vollenhoven, Unresolved issues in biologic therapy for rheumatoid arthritis, *Nat. Rev. Rheumatol.* 7 (2011) 205–215.
- [18] T. Kagar, H. Doi, T. Shimozato, The importance of IL-1 beta and TNF-alpha, and the noninvolvement of IL-6, in the development of monoclonal antibody-induced arthritis, *J. Immunol.* 169 (2002) 1459–1466.
- [19] F.M. Coelho, V. Pinho, F.A. Amaral, D. Sachs, V.V. Costa, D.H. Rodrigues, A.T. Vieira, T.A. Silva, D.G. Souza, R. Bertini, A.L. Teixeira, M.M. Teixeira, The chemokine receptors CXCR1/CXCR2 modulate antigen-induced arthritis by regulating adhesion of neutrophils to the synovial microvasculature, *Arthritis Rheum.* 58 (2008) 2329–2337.
- [20] S. Shiozawa, K. Tsumiyama, K. Yoshida, A. Hashiramoto, Pathogenesis of joint destruction in rheumatoid arthritis, *Arch. Immunol. Ther. Exp. (Warsz)* 59 (2011) 89–95.
- [21] S. Inada, O. Kohashi, Y. Hamasaki, Humoral immune-mediated acute, antigen-induced arthritis in rats is suppressed by the inducing antigen administered orally before, but not after, immunization, *Immunol. Invest.* 30 (2001) 47–56.
- [22] D.G. Souza, A.C. Soares, V. Pinho, H. Torloni, L.F. Reis, M.M. Teixeira, A.A. Dias, Increased mortality and inflammation in tumor necrosis factor-stimulated gene-14 transgenic mice after ischemia and reperfusion injury, *Am. J. Pathol.* 160 (2002) 1755–1765.
- [23] P.G. Reeves, F.H. Nielsen, G.C. Fahey Jr., AIN-93 purified diets for laboratory rodents: final report of the American Institute of Nutrition ad hoc writing committee on the reformulation of the AIN-76A rodent diet, *J. Nutr.* 123 (1993) 1939–1951.
- [24] M. Russo, M.A. Nahori, J. Lefort, E. Gomes, A. de Castro Keller, D. Rodriguez, O.G. Ribeiro, S. Adriaouch, V. Gallois, A.M. de Faria, B.B. Vargaftig, Suppression of asthma-like responses in different mouse strains by oral tolerance, *Am. J. Respir. Cell. Mol. Biol.* 24 (2001) 518–526.
- [25] A.M. Faria, R. Maron, S.M. Ficker, A.J. Slavin, T. Spahn, H.L. Weiner, Oral tolerance induced by continuous feeding: enhanced up-regulation of transforming growth factor-beta/interleukin-10 and suppression of experimental autoimmune encephalomyelitis, *J. Autoimmun.* 20 (2003) 135–145.
- [26] A.M. Faria, H.L. Weiner, Oral tolerance, *Immunol. Rev.* 206 (2005) 232–259.

- [27] A.W. Burks, S. Laubach, S.M. Jones, Oral tolerance, food allergy, and immunotherapy: implications for future treatment, *J. Allergy Clin. Immunol.* 121 (2008) 1344–1350.
- [28] A.M. Scurlock, A.W. Burks, S.M. Jones, Oral immunotherapy for food allergy, *Curr. Allergy Asthma Rep.* 9 (2009) 186–193.
- [29] A.T. Clark, S. Islam, Y. King, J. Deighton, K. Anagnostou, P.W. Ewan, Successful oral tolerance induction in severe peanut allergy, *Allergy* 64 (8) (2009) 1218–1220.
- [30] A. Nowak-Węgrzyn, A. Fiocchi, Is oral immunotherapy the cure for food allergies?, *Curr Opin. Allergy Clin. Immunol.* 10 (2010) 214–219.
- [31] F. Blumenthal-Barby, K. Eulenburg, A. Schrage, M. Zeitz, A. Hamann, K. Klugewitz, In vivo modulation of antigen-experienced cells in response to high-dose oral antigen: deletion but no evidence for alterations in the cytokine phenotype, *Int. Immunol.* 20 (2008) 893–900.
- [32] B.A. Verdolin, S.M. Ficker, A.M. Faria, N.M. Vaz, C.R. Carvalho, Stabilization of serum antibody responses triggered by initial mucosal contact with the antigen independently of oral tolerance induction, *Braz. J. Med. Biol. Res.* 34 (2001) 211–219.
- [33] D.G. Souza, D.C. Cara, G.D. Cassali, S.F. Coutinho, M.R. Silveira, S.P. Andrade, S.P. Poole, M.M. Teixeira, Effects of the PAF receptor antagonist UK74505 on local and remote reperfusion injuries following ischaemia of the superior mesenteric artery in the rat, *Br. J. Pharmacol.* 131 (2000) 1800–1808.
- [34] C.R. Carvalho, B.A. Verdolin, N.M. Vaz, Indirect effects of oral tolerance cannot be ascribed to bystander suppression, *Scand. J. Immunol.* 45 (1997) 276–281.
- [35] C.R. Carvalho, H.L. Lenzi, R. Correa-Oliveira, N.M. Vaz, Indirect effects of oral tolerance to ovalbumin interfere with the immune responses triggered by *Schistosoma mansoni* eggs, *Braz. J. Med. Biol. Res.* 35 (2002) 1195–1199.
- [36] C.M. Rodrigues, O.A. Martins-Filho, N.M. Vaz, C.R. Carvalho, Systemic effects of oral tolerance on inflammation: mobilization of lymphocytes and bone marrow eosinopoiesis, *Immunology* 117 (2006) 517–525.
- [37] R.A. Costa, V. Ruiz-de-Souza, G.M. Azevedo Jr., E. Gava, G.T. Kitten, N.M. Vaz, C.R. Carvalho, Indirect effects of oral tolerance improve wound healing in skin, *Wound. Repair. Regen.* 19 (2011) 487–497.
- [38] S. Yoshino, Antigen-induced arthritis in rats is suppressed by the inducing antigen administered orally before, but not after immunization, *Cell. Immunol.* 163 (1995) 55–58.
- [39] T. Barthlott, G. Kassiotis, B. Stockinger, T cell regulation as a side effect of homeostasis and competition, *J. Exp. Med.* 197 (2003) 451–460.
- [40] J.C. Saldanha, D.L. Gargiulo, S.S. Silva, F.H. Carmo-Pinto, M.C. Andrade, J.I. Alvarez-Leite, M.M. Teixeira, D.C. Cara, A model of chronic IgE-mediated food allergy in ovalbumin-sensitized mice, *Braz. J. Med. Biol. Res.* 37 (2004) 809–816.
- [41] M.L.M. Noviello, N.V. Batista, L.P.A. Dourado, D.C. Cara, Prolonged antigen ingest in by sensitized mice ameliorates airway inflammation, *ISRN Allergy* 2011 (2011) 1–7.