Clinical and Developmental Immunology

Tolerance and Inflammation at the Gut Mucosa

Guest Editors: Ana Maria C. Faria, Daniel Mucida, Donna-Marie McCafferty, Noriko M. Tsuji, and Valerie Verhasselt

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Editorial

Tolerance and Inflammation at the Gut Mucosa

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The gut mucosa is the major site of contact with antigens. It holds the largest mass of lymphoid tissue in the body. Under physiological conditions, microbiota and dietary antigens are the natural sources of stimulation for the gut-associated lymphoid tissue (GALT) and for the immune system as a whole. Most GALT cells are activated, and a variety of proinflammatory mediators are found in this site. Regulatory elements, however, counterbalance local inflammatory events in such a way that a delicate yet robust balance keeps the gut homeostasis in check. Normal antigenic contact through the gut mucosa induces two major noninflammatory immune responses, oral tolerance, and production of secretory IgA. However, under pathological circumstances mucosal homeostasis is disturbed resulting in inflammatory conditions such as food hypersensitivity and inflammatory bowel diseases (IBDs). The number of reported cases of food allergy in children grew 18% in the past decade [1]. At the same time, the incidence and prevalence of chronic inflammatory bowel diseases such as ulcerative colitis and Crohn's disease is increasing yearly in US, Europe, Asia, and Latin America [2]. Although therapies for these diseases have improved, they have many side effects and are still only modestly successful for long-term management. The growing incidence of both types of conditions and the need for therapeutic alternatives demands from the scientific community a better understanding of the mechanisms involved in intestinal homeostasis and the pathological settings that may trigger gut inflammation.

The aim of this special issue is to shed some light on these mechanisms as well as to address alternative therapeutic and preventive approaches for gut inflammatory diseases. It comprises five review articles and ten research articles.

The opening review article by Ramos is a critical appraisal of inflammation as a physiological phenomenon related to the development of multicellular organisms rather than solely a pathological event associated with disease and anti-infectious defense mechanisms. Since the great majority of processes in the gut are a consequence of chronic exposure to large amounts of harmless and often beneficial antigens, the authors discuss how the immune system assimilates these perturbations without generating tissue damage. A. P. da Cunha and H. L. Weiner review the studies on the tolerogenic effects of orally administered anti-CD3 monoclonal antibody in experimental models of autoimmune diseases. Anti-CD3 antibody is biologically active in the gut and may represent a powerful immunologic approach that would be applicable for the treatment of human autoimmune conditions. P. Moingeon and L. Mascarell present a review on the oral lymphoid tissue that comprises various antigen-presenting cells (APCs), resident CD4+ T cells but few mast cells and eosinophils. Interestingly, in the absence of danger signals, the APCs at this site are mostly tolerogenic. These features endow the oral mucosa with properties of a site for immune intervention. Indeed, sublingual vaccines using recombinant allergens are being developed as a novel immunomodulatory strategy to control allergic diseases. The paper by C. T.

Murphy and coworkers reviews the role of leukocyte trafficking to the intestinal mucosa in the pathogenesis of inflammatory bowel diseases (IBDs) and the various strategies employed to target leukocyte migration as a putative therapeutic tool to control these disorders.

In line with these reviewed topics, the original papers in this issue address several aspects of oral tolerance induction, gut inflammatory diseases, and intestinal infection. Two papers present data on the mechanisms involved in oral tolerance to inflammatory reactions occurring in the lung. G. M. Azevedo, Jr., and coworkers showed that intraperitoneal injection of ovalbumin (OVA) in adjuvant minutes before intravenous injection of *Schistosoma mansoni* eggs into OVA tolerant mice blocked the increase of pulmonary granulomas. This indirect effect of oral tolerance to OVA correlates with a reduction in the recruitment of inflammatory cells to the lung suggesting that this is one of the mechanisms by which oral tolerance mediates its indirect effects towards other context-related antigens. The role of regulatory T cells was studied by L. Faustino and coworkers using a model of lung eosinophilic inflammation. Interestingly, at the peak of airway inflammation, the number of regulatory T cells was much higher in allergic mice than in tolerant mice. These regulatory T cells likely play a role in controlling disease progression in allergic mice. The earlier appearance of regulatory T cells in tolerant mice may be the critical parameter that prevents airway inflammation instead of merely limiting it.

Studying regulatory mechanisms induced by oral tolerance, M. Ruberti and coworkers compared effects in the intestinal mucosa of OVA feeding to BALB/c versus OVA TCR transgenic (DO11.10) mice. OVA-fed BALB/c mice had reduced levels of cytokines in the intestinal mucosa, whereas frequencies of intraepithelial lymphocytes (IELs) expressing Foxp3 and CD103 increased. On the other hand, in OVA-fed DO11.10 mice the intestinal mucosa showed signs of inflammation with increased local cytokine production, and reduction in IEL numbers. Having demonstrated previously that DO11.10 mice could not be rendered tolerant to OVA by the oral route, the authors suggest that the altered proportions of mucosal inflammatory/regulatory T cells and IELs in these mice are related to resistance to oral tolerance induction.

Two other papers deal with the controversial practice of delaying the introduction of allergenic foods into the infant diet to prevent food allergy development. Contrary to this wide-spread believe, both studies showed that early antigen exposure by breast feeding is able to prevent sensitization in allergy-prone BN rat pups (A. El-Merhibi and coworkers) and in the offspring of allergy-susceptible BALB/c mice (T. Yamamoto and coworkers). Inhibition of specific IgE production and food allergy by oral tolerance to the breast-fed antigen was followed by increases in regulatory T cells and anti-inflammatory cytokines.

The other reports studied two immunomodulatory strategies for food allergy: a dietary component and a probiotic. O. G. de Matos and coworkers investigated the effect of dietary supplementation with n-3 polyunsaturated fatty acids (PUFA, fish oil source) in an experimental model of food allergy using BALB/c mice. Treatment with n-3 PUFA reduced all inflammatory parameters associated with food allergy including serum levels of antiovalbumin IgE and IgG1, as well as leukocyte recruitment, mucus production, and Paneth cell degranulation in the small intestine. Likewise, administration of *Lactococcus lactis NCC 2287* to BALB/c mice, once sensitized but not before sensitization, reduced allergic manifestations upon allergen challenge. According to A. W. Zuercher and coworkers, the beneficial effect of this strain of *L. lactis* correlates with localized inhibition of Th2 cytokines, particularly IL-13. Therefore, these two strategies characterize promising alternative agents for either prevention or treatment of food allergy.

In addition to hypersensitivity reactions to food, dysregulated gut immune responses are also observed in IBD. Two other papers address the role of IL-10 and autophagia in IBD development. A. C. Gomes-Santos and coworkers report a kinetic study on the changes in cytokines and lymphoid cells in the intestinal mucosa during the course of spontaneous colitis in IL-10-deficient 129 Sv/Ev mice. Although histological signs of colitis only start at 10 weeks of age reaching overt gut inflammation at 16 weeks of age, decrease in the frequency of CD4+CD25+Foxp3+ regulatory T cells and increase in activated T cells and in IL-17 at the gut mucosa can be detected as early as 6 weeks of age. Interestingly, oral tolerance can be induced in diseased 16-week-old mice by a continuous feeding protocol indicating that this could be an alternative therapeutic strategy for established colitis. Autophagy is another process reported to be involved in intestinal immune homeostasis due to its participation in the digestion of intracellular pathogens and in antigen presentation. To investigate the role of autophagy in the development of experimental models of IBD, N. Wittkopf and coworkers generated mice with intestinal epithelial deletion of the autophagy gene Atg7. Knockout mice showed reduced size of granules and decreased levels of lysozyme in Paneth cells. However, this had no effect on susceptibility in mouse models of experimentally induced colitis leaving this association as an open question for future studies.

A. Kantele investigated gut immune responses to persistent intestinal infection by monitoring gut-originating plasmablasts in blood. Even in symptomless patients with *Salmonella, Yersinia,* or *Campylobacter* gastroenteritis and in volunteers receiving an oral typhoid vaccine, these persisting pathogens/antigens keep seeding plasmablasts into the circulation. Assaying these cells might provide a less invasive tool for research on intestinal immune responses in diseases in which persisting microbes have a potential pathogenetic significance.

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Erratum

Erratum to "Phenotypical and Functional Analysis of Intraepithelial Lymphocytes from Small Intestine of Mice in Oral Tolerance"

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In this work, we evaluated the effects of administration of OVA on phenotype and function of intraepithelial lymphocytes (IELs) from small intestine of transgenic (TGN) DO11.10 and wild-type BALB/c mice. While the small intestines from BALB/c presented a well-preserved structure, those from TGN showed an inflamed aspect. The ingestion of OVA induced a reduction in the number of IELs in small intestines of TGN, but it did not change the frequencies of CD8+ and CD4+ T-cell subsets. Administration of OVA via oral + i.p. increased the frequency of CD103+ cells in CD4+ T-cell subset in IELs of both BALB/c and TGN mice and elevated its expression in CD8a+ and CD8β+ T-cell subsets in IELs of BALB/c mice. The frequency of Foxp3+ cells increased in all subsets in IELs of BALB/c treated with OVA; in IELs of TGN, it increased only in CD25+ subset. IELs from BALB/c tolerant mice had lower expression of all cytokines studied, whereas those from TGN showed high expression of inflammatory cytokines, especially of IFN-γ, TGF-β, and TNF-α. Overall, our results suggest that the inability of TGN to become tolerant may be related to disorganization and altered proportions of inflammatory/regulatory T cells in its intestinal mucosa.

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Research Article

Oral Tolerance Induced by Transfer of Food Antigens via Breast Milk of Allergic Mothers Prevents Offspring from Developing Allergic Symptoms in a Mouse Food Allergy Model

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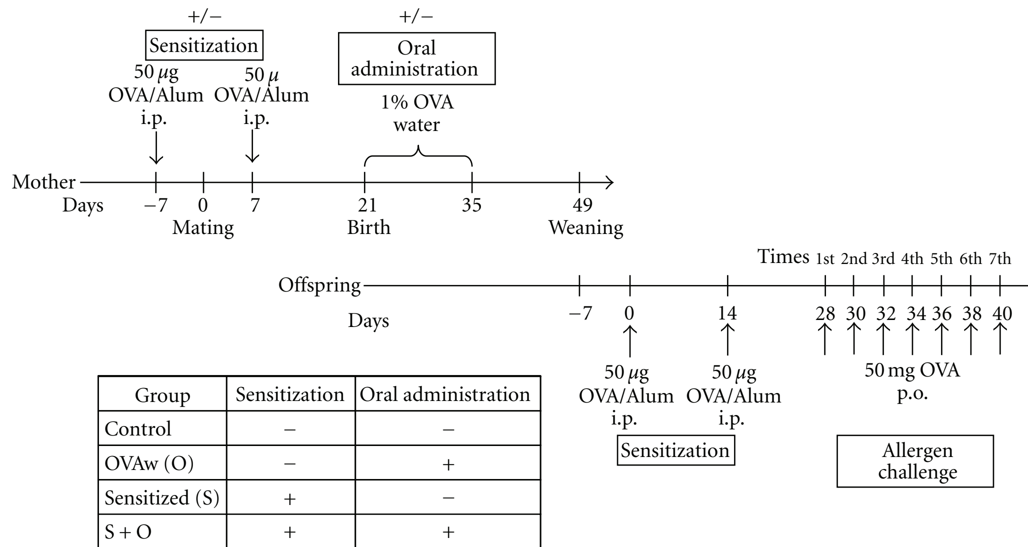
We examined whether maternal exposure to food antigens during lactation and maternal allergic status would affect the development of food allergy in offspring. OVA-sensitized or OVA-nonsensitized BALB/c female mice were exposed or unexposed to OVA during lactation. After weaning, their offspring were systemically sensitized twice with OVA and repeatedly given OVA by oral intubation. While 97.1% of the mice breastfed by OVA-nonsensitized and OVA-unexposed mothers developed allergic diarrhea, 59.7% of the mice breastfed by OVA-exposed nonallergic mothers during lactation and 24.6% of the mice breastfed by OVA-exposed allergic mothers during lactation developed food allergy. Furthermore, OVA was detected in breast-milk from OVA-exposed nonallergic mothers during lactation ($4.6 \pm 0.5\,\mu$g/mL). In addition, OVA-specific IgG1 titers were markedly increased in breast milk from allergic mothers (OVA-sensitized and OVA-unexposed mother: 11.0 ± 0.5, OVA-sensitized and OVA-exposed mother: 12.3 ± 0.3). Our results suggest that oral tolerance induced by breast milk-mediated transfer of dietary antigens along with their specific immunoglobulins to offspring leads to antigen-specific protection from food allergy.

1. Introduction

Over the last few decades, the prevalence of allergic diseases, such as atopic dermatitis, bronchial asthma allergic rhinitis, and food allergy (FA), has dramatically increased in advanced countries. Since FA is relatively common in the early stage of the "Allergy March", in which symptoms are exhibited successively with age, it is very important for infants to outgrow their FA from a viewpoint of primary prevention of various allergy diseases [1, 2]. However, until now, there have not been any effective drug therapies for FA.

The intestinal epithelium is constantly exposed to a multitude of foreign materials that are either harmful or beneficial for humans. Consequently, the intestinal immune system must balance between protective immune responses that are induced by encounters with intestinal pathogens and tolerance against commensal bacteria and food antigens. Despite the large extent of dietary antigenic exposure, the intestinal mucosal immune system has the unique propensity to evoke tolerance against orally administrated antigens and thereby maintains optimal immunological homeostasis in the intestine. It has been assumed that a breakdown in oral tolerance mechanisms or a failure in the induction of oral tolerance results in allergic food hypersensitivity [2–4].

Previous studies have shown that breast milk can influence the incidence of allergic diseases in infants. Epidemiological studies on the protection from allergic diseases caused by breastfeeding have yielded conflicting results. Saarinen and Kajosaari concluded from a prospective study in patients up to 17 years old that the incidence of allergic diseases was lower in the group of patients who were fed by breast milk for a long period [5]. On the contrary, it has been reported that complete breastfeeding for several months postnatally cannot protect children against allergic diseases



Group	Sensitization	Oral administration
Control	−	−
OVAw (O)	−	+
Sensitized (S)	+	−
S + O	+	+

Figure 1: Experimental protocol. Sensitized mice were exposed to 1% OVA in drinking water for 2 weeks immediately after delivery. Offspring were weaned at 4 weeks and 5-week-old offspring were used for the FA model.

[6]. For many years, the American Academy of Pediatricians and the United Kingdom government have recommended maternal food antigen avoidance during pregnancy and lactation to reduce FA. Actually, it is known that there are sufficient concentrations of food antigens for making infants develop FA [7]. However, until now, there have not been any conclusive data that maternal food antigen restriction is protective against FA in infants [8].

Furthermore, it remains controversial whether the mother's allergic status is a significant risk factor for the development of allergy in breastfed infants. In recent prospective studies on a birth cohort of 3,115 children, breast feeding was demonstrated to be associated with a lower asthma risk in children up to 8 years of age without evidence of attenuation of the association and regardless of the child's family history of allergy [9].

It remains unclear whether maternal exposure to food antigens during lactation and maternal allergic status affects the development of allergic diseases in offspring via transfer of factors influencing susceptibility or resistance to allergic diseases. Therefore, we investigated the ability of breast milk from allergic mothers exposed to food antigens during lactation to protect breastfed offspring from FA.

2. Methods

2.1. Animals. BALB/c mice (Japan SLC, Shizuoka, Japan) were used for the experiments and housed in the experimental animal facility at University of Toyama. This study was approved by the Animal Experiment Committee in University of Toyama (Authorization No. is S-2009 INM-9) in accordance with the Guide for the Care and Use of Laboratory Animals in University of Toyama which is accredited by the Ministry of Education, Culture, Sports, Science, and Technology, Japan.

2.2. Experimental Protocols. To study the relationship between breastfeeding and allergic diseases, 9-week-old female BALB/c mice were sensitized twice at a 2-week interval with 50 μg of OVA in the presence of 1.3 mg of aluminum hydroxide gel by intraperitoneal injection. Non-sensitized (vehicle-injected) and sensitized mice were mated with naïve males 7 days after the first intraperitoneal OVA injection. Subsequently, lactating mice were exposed to 1% OVA in drinking water for 2 weeks immediately after delivery (the duration of pregnancy in BALB/c mice is about 21 days). The offspring were weaned at 4 weeks, and 5-week-old offspring were used to test our FA model (Figure 1). Brief, BALB/c mice were sensitized twice at a 2-week interval with 50 μg of OVA in the presence of 1.3 mg of aluminum hydroxide gel (Sigma-Aldrich), which served as an adjuvant, by intraperitoneal injection. Two weeks after systemic priming, the mice were repeatedly given 50 mg of OVA using intragastric feeding needles three times per week. Allergic diarrhea was assessed by visually monitoring mice up to 1 hour following intragastric OVA challenge. Mice with profuse liquid stool were recorded as allergic diarrhea-positive animals. Tissues and plasma were obtained 1hour after the seventh oral OVA challenge.

2.3. OVA-Specific Immunoglobulin Level in Plasma and Breast Milk. OVA-specific IgE, IgA, and IgG1 levels in plasma as well as OVA-specific IgA and IgG1 levels in breast milk were measured by the sandwich ELISA method. The sensitive sandwich ELISAs used to analyze OVA-specific IgE, IgA, and IgG1 levels were developed using biotinylated rat anti-mouse IgE (1 : 1,000; YAMASA, Tokyo, Japan), HRP-conjugated sheep anti-mouse IgA (1 : 2,000; SouthernBiotech, Birmingham, AL, USA), and HRP-conjugated sheep anti-mouse IgG1 (1 : 2,000; SouthernBiotech). Plates were coated with 10 μg/mL OVA (Fraction V; Sigma-Aldrich) at 4°C overnight

and blocked with PBS containing 2% Block Ace (DS Pharma Biomedical, Osaka, Japan). Diluted samples were added to the plates, which were then incubated for 2 hour at 37°C. Each HRP-conjugated anti-immunoglobulin antibody was added and the plates were then incubated for 1 hour at 37°C. Reactions were developed by 3,3′,5,5′-tetramethylbenzidine (Sigma-Aldrich), and color development was terminated by 0.5 N HCl. Endpoint titers of OVA-specific immunoglobulin antibodies were expressed as the reciprocal $\log_2$ of the last dilution that showed a level of >0.1 absorbance over the background levels, which gave an absorbance at 450 nm. Breast milk was collected from the stomachs of 7-day-old neonatal mice and diluted 10 times with RPMI1640 medium. The diluted breast milk was centrifuged, and the supernatant was collected as a breast milk sample.

2.4. Measurement of OVA Concentration in Breast Milk. OVA levels in breast milk were analyzed using ITEA OVA ELISA Kit (Precoated) (ITEA, Tokyo, Japan). Measurements were performed according to the manufacturer's instructions.

2.5. Expression of mRNA in the Intestine. mRNA expression levels in the intestine were examined according to the method previously described [10]. Briefly, 1 hour after the seventh oral OVA challenge, 2 cm of the mouse proximal colon was excised. Total RNA was extracted from the proximal colon using Sepasol Super (Nacalai Tesque, Kyoto, Japan) according to the manufacturer's instruction. Reverse transcription was performed using the ExScript RT reagent Kit (Takara Bio, Shiga, Japan) and random primers, followed by real-time PCR. Real-time PCR amplification of IL-4, IFN-γ, Foxp3, and GAPDH was performed using SYBR Premix Ex Taq (Takara Bio). Target mRNA expression was normalized to GAPDH mRNA expression as an internal control in each sample. The results were expressed as the relative ratio to the naïve group average.

2.6. Immunohistochemistry on Mucosal Mast Cells in the Intestine. Immunohistochemistry was performed according to the method previously described [10]. Briefly, the excised proximal colon was fixed in 4% paraformaldehyde (w/v) in 0.1 M sodium phosphate buffer (PB, pH 7.3) at 4°C for 12–18 hour. Frozen sections (30 μm) were cut at −20°C using a cryostat microtome (Leica Microsystems, Nussloch, Germany). The sections were exposed for 12–18 hour to antiserum against mouse mast cell protease-1 (mMCP-1; a marker of mouse mucosal mast cells; 1 : 5000, Moredun Scientific, Scotland, UK), washed with 0.01 M PBS and incubated for 2 hour with Cy3-conjugated sheep anti-donkey IgG (1 : 200, Jackson Immunoresearch Laboratories, West Grove, PA, USA). The immunostained sections were examined using a fluorescence microscope (IX71 System, Olympus, Tokyo, Japan) with the filter set U-MWIG3 (Olympus) and photographed using an Olympus digital camera (DP70, Olympus). The brightness and contrast of the images were modified with Adobe Photoshop Elements 2.0 (Adobe Systems, San Jose, CA, USA).

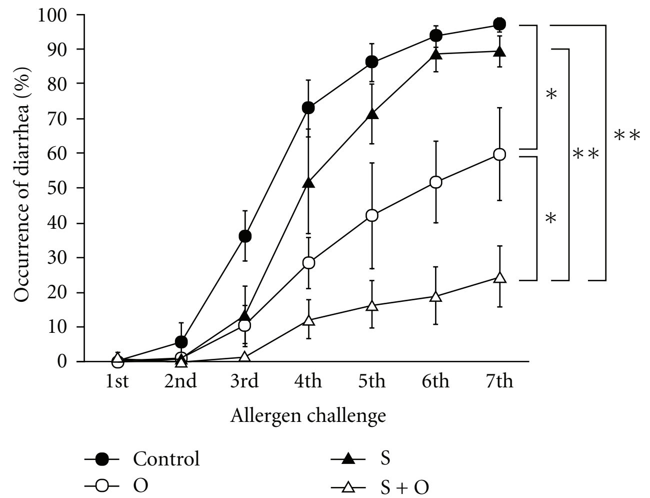


Figure 2: Effects of the maternal exposure to food antigens during lactation and the maternal allergic status on allergic symptoms in FA model. Offspring breastfed by OVA-sensitized and OVA-exposed lactating mothers were more protected from the development of allergic symptoms of FA compared with offspring from OVA-nonsensitized and OVA-exposed lactating mothers. Data are shown as the means ± SE (8 independent experiments, total $n = 62$–181). $^*P < 0.05$, $^{**}P < 0.01$.

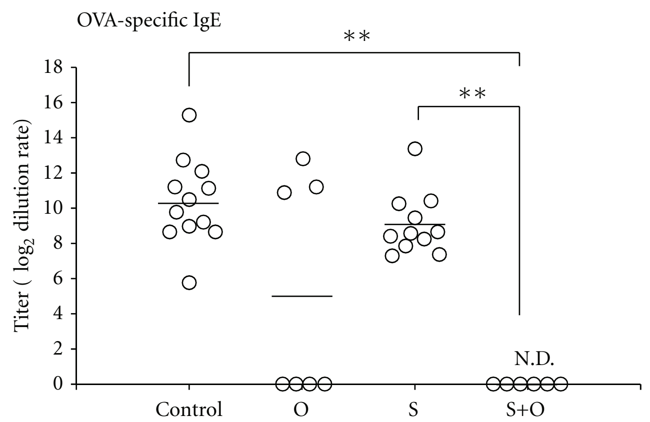


Figure 3: Effects of the maternal exposure to food antigens during lactation and the maternal allergic status on plasma IgE levels of offspring in the mouse FA model. Plasma IgE levels were undetectable in offspring breastfed by OVA-sensitized and OVA-exposed lactating mothers. Data are shown as the means and individual data points. N.D.: not detectable. $^{**}P < 0.01$, $n = 6$–11.

2.7. Data Analyses. The data are expressed as the means ± S.E. or the means and dot plots. Statistical comparisons were made using two-tailed Student's unpaired t-tests or one-way ANOVA followed by Tukey's post-hoc test for multiple comparisons of data with a normal distribution or Games-Howell test for multiple comparisons of data with a nonnormal distribution. P values less than 0.05 were considered to be statistically significant.

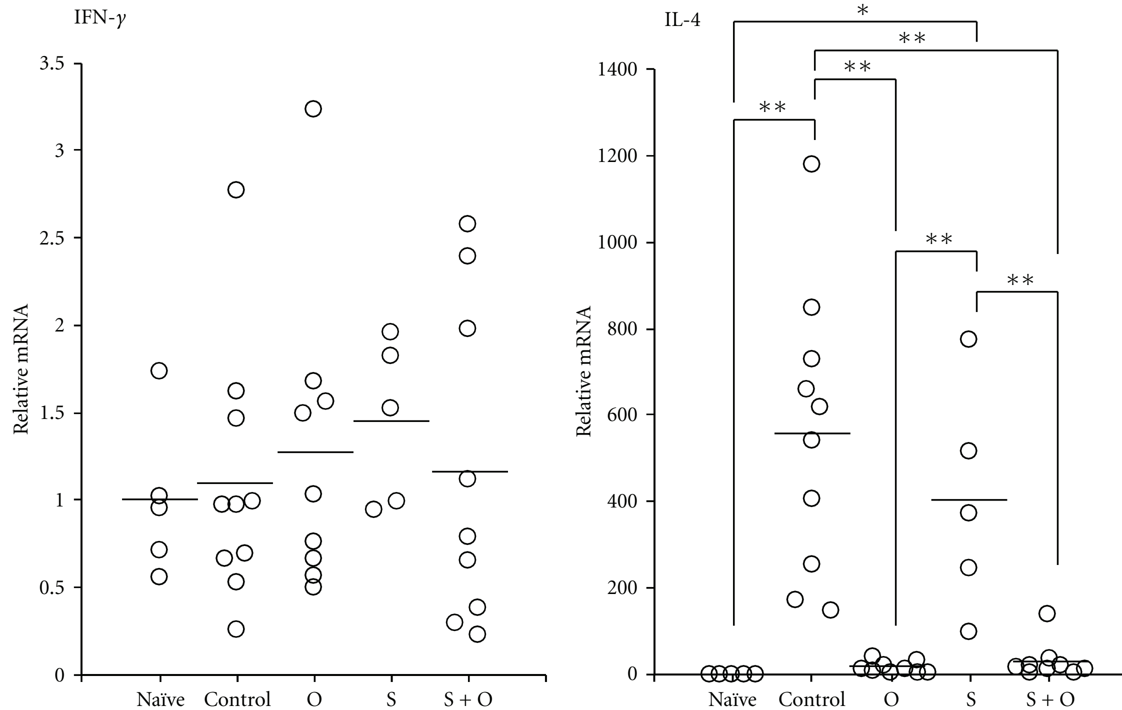


Figure 4: Effects of the maternal exposure to food antigens during lactation and the maternal allergic status on Th1 and Th2 cytokine profiles in the proximal colons of offspring with FA. The mRNA expression levels of IL-4 were completely reduced to the naïve mice range in offspring breastfed by OVA-exposed mothers during lactation, but IFN-γ mRNA expression levels were not altered by the maternal exposure to food antigens and the maternal allergic status. Data are shown as the means and individual data points. $^{*}P < 0.05$, $^{**}P < 0.01$, $n = 5$–10.

3. Results

3.1. Breast Milk from OVA-Exposed Allergic Mothers during Lactation Protects Offspring from Allergic Symptoms in Food Allergy Model. Offspring breastfed by OVA-nonsensitized and OVA-unexposed mothers developed the symptoms of FA (Figure 2: Control group: 97.1 ± 1.9% after the seventh oral OVA challenge). We next determined whether maternal exposure to 1% OVA in drinking water during lactation affects intestinal allergic symptoms in offspring. We found that about half of the offspring breastfed by OVA-nonsensitized and OVA-exposed mothers exhibited allergic reactions after the seventh OVA challenge (Figure 2: O group; 59.7 ± 13.2% after the seventh oral OVA challenge, $P < 0.05$ compared with the control group). Furthermore, when compared with the O group offspring, a reduction in allergic symptoms was evident in offspring nursed by OVA-sensitized and OVA-exposed mothers (Figure 2: S + O group; 24.6 ± 8.8% after the seventh oral OVA challenge, $P < 0.05$ compared with the O group), although breast milk from OVA-sensitized and OVA-unexposed mothers had little effect on the development of FA in offspring (Figure 2: S group: 89.7 ± 4.4% after the seventh oral OVA challenge).

Plasma levels of OVA-specific IgE was very high in the control group offspring (Figure 3). OVA-specific IgE in S + O group offspring were virtually undetectable (Figure 3: $P < 0.01$ compared with the control group), while S group offspring exhibited high OVA-specific IgE levels comparable with the control group offspring (Figure 3). Although OVA-specific IgE levels in O group offspring tended to be lower compared with those in the control group offspring, there were large individual differences among the O group offspring (Figure 3).

3.2. Maternal Exposure to OVA during Lactation Suppresses the Th2-Polarized Cytokine Profile in the Proximal Colons of Offspring with Food Allergy. We examined Th1 (IFN-γ) and Th2 (IL-4) cytokine profiles in the proximal colons of the offspring in each of the four groups. IFN-γ mRNA expression in the proximal colon was not enhanced in the FA model mice and not affected by the maternal exposure to OVA during lactation and/or the maternal allergic status.

Conversely, IL-4 mRNA expression was greatly upregulated in the proximal colons of the control group offspring (Figure 4: $P < 0.01$, 556.4 ± 102.6 compared with naïve mice: 1.0 ± 0.2), and the maternal exposure to OVA during lactation markedly prevented the enhancement of IL-4 mRNA expression in the proximal colons of both the O group and S + O group offspring (Figure 4: $P < 0.01$, 16.4 ± 4.8 and 30.6 ± 14.2, respectively, compared with the control group offspring). The S group offspring exhibited high levels of IL-4 mRNA expression that were comparable to those of the control group offspring (Figure 4: 402.3 ± 116.1).

3.3. Maternal Exposure to OVA during Lactation Prevents Mucosal Mast Cell Infiltration in the Proximal Colons of Offspring with Food Allergy. Mucosal mast cells were dramatically increased in the proximal colons of the control

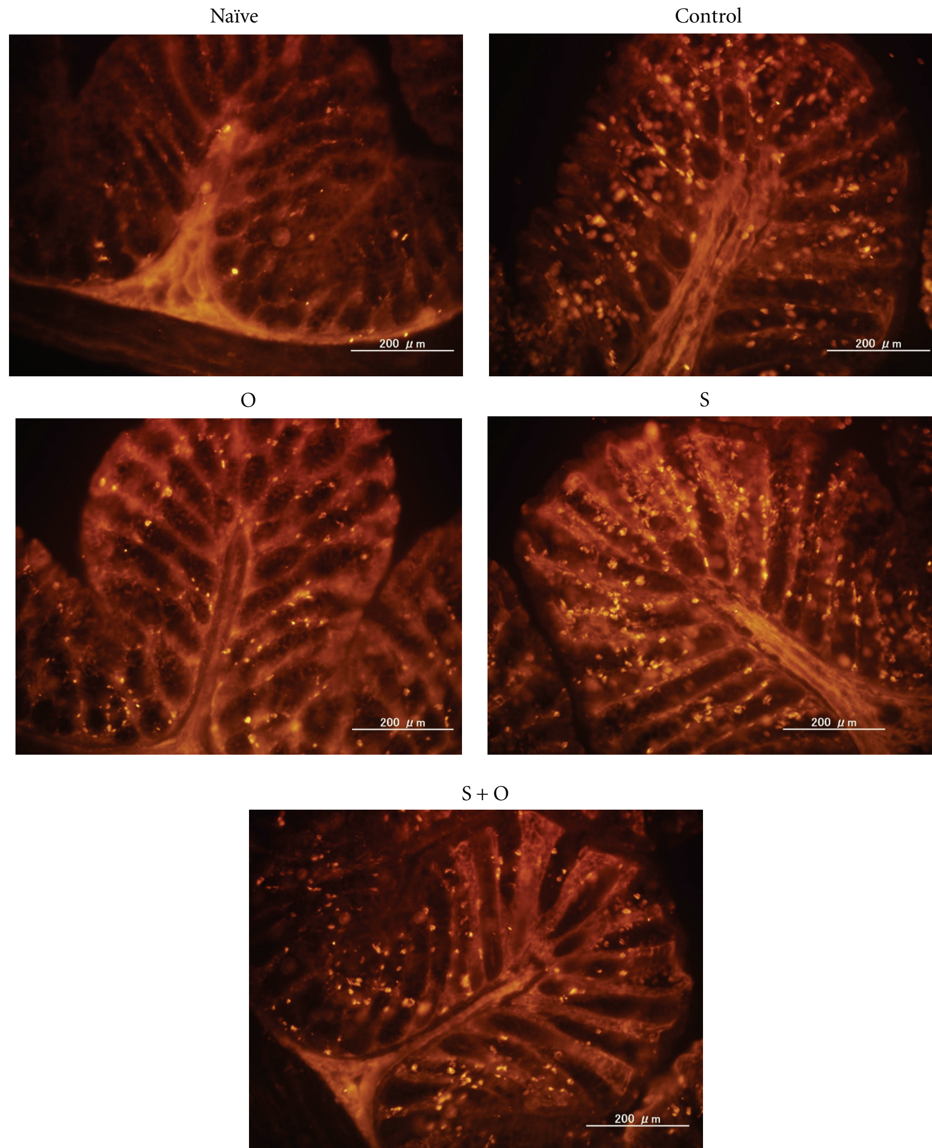


FIGURE 5: Effects of the maternal exposure to food antigens during lactation and the maternal allergic status on mucosal mast cell infiltration in the proximal colons of offspring FA. The infiltration of mucosal mast cells was greatly attenuated in offspring with FA breastfed by OVA-exposed mothers during lactation.

group offspring, while they were markedly decreased in the proximal colons of both the O group and S + O group offspring. Many mucosal mast cells were observed in the proximal colons of the S group offspring, and these levels were comparable to those in the proximal colons of the control group offspring (Figure 5).

3.4. Maternal Transfer of OVA and/or OVA-Specific Antibodies Through Breast Milk. Next, we determined whether the allergic symptoms in offspring were associated with maternal transfer of OVA and/or OVA-specific antibodies (IgG1 and IgA) through breast milk. Among the mothers of the four groups, OVA was only detected in the breast milk of mothers from the O group (4.6 ± 0.5 μg/mL, $n = 5$). Although OVA-specific IgG1 were not detected in the breast milk from the control and O group mothers, the sensitization of mothers to OVA increased the concentration of OVA-specific IgG1 in the breast milk of the S + O group mothers (S group mother: 11.0 ± 0.5, S + O group mother: 12.3 ± 0.3; Figure 6, $P < 0.01$, $n = 5$). In contrast, OVA-specific IgA was detected only in the breast milk from the S + O group mothers (Figure 6, $n = 5$–9).

We further analyzed plasma OVA-specific IgG1 and IgA levels in 7-days-old offspring of the four groups to elucidate the transfer of OVA-specific IgG1 and IgA from mothers to offspring via breast milk. Similarly to OVA-specific IgG1 in the breast milk, plasma OVA-specific IgG1 was detected in both the S group and S + O group offspring but not in the control group and O group offspring (Figure 7). Conversely,

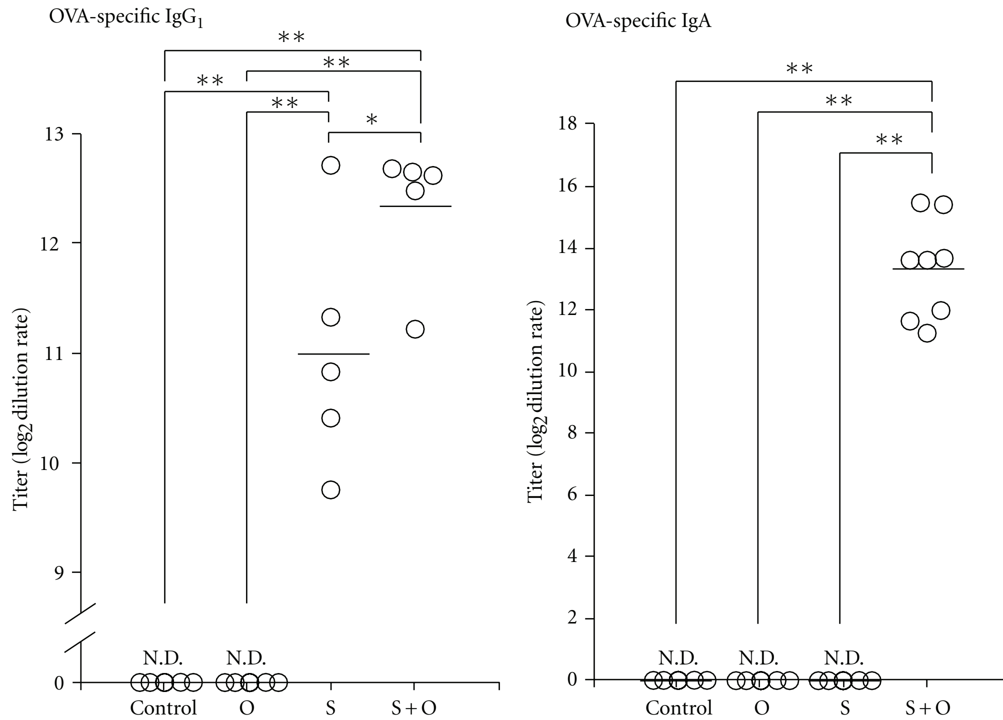


Figure 6: Effects of the maternal exposure to food antigens during lactation and the maternal allergic status on OVA-specific IgG1 and IgA levels in breast milk. We found OVA-specific IgG1 only in the breast milk from OVA-sensitized mothers, which implies that mother mice secrete IgG1 into their breast milk. We found OVA-specific IgA only in the breast milk from OVA-sensitized and OVA-exposed mothers. Data are shown as the means and individual data points. N.D.: not detectable. $^{*}P < 0.05$, $^{**}P < 0.01$, $n = 5$–9.

plasma OVA-specific IgA was undetectable in the offspring of all four groups (Figure 7).

4. Discussion

This study was performed to test our hypothesis that maternal factors transferred through breast milk affect the development of FA in offspring. In the present study, using a mouse FA model, we demonstrated that the induction of oral tolerance can suppress food allergic symptoms and that maternal ingestion of food antigens during lactation, especially in allergic mothers, can induce effective oral tolerance, thereby preventing the onset of FA.

4.1. Induction of Oral Tolerance. Early tolerance induction is an attractive approach for primary prevention of allergic diseases. Recently, the preventive effects of oral tolerance on allergic diseases such as allergic asthma and allergic rhinitis have been reported in experimental models [11–13].

Experimental FA models where repeated oral challenges with OVA in systemically OVA-primed BALB/c mice led to allergic diarrhea require aberrant Th2-type responses, such as an enhanced production of IL-4, IL-5, and IL-13 by the spleen and large intestine and high levels of OVA-specific plasma IgE [14, 15]. We have reported that phosphatidylinositol-3 kinase deficient mice (BALB/c background) selectively lacking gastrointestinal mast cells could not develop allergic diarrhea in our FA model [16], suggesting that mucosal mast cells in the intestine play an important role in the pathogenesis and development of allergic diarrhea.

In 2011, Hadis et al. have demonstrated that the induction of oral tolerance prevents food allergic diarrhea in adult mice [17]. Here we also show that oral tolerance induced by the exposure to OVA prevents the increase in plasma OVA-specific IgE levels and Th2 cytokine mRNA expression in both systemic and mucosal immune system, in addition, the exposure to OVA abrogates the augmentation of the infiltration of mucosal mast cells into the colon (see supplemental materials available at doi:10.1155/2012/721085, Supplemental Figures 1–5).

4.2. Influence of the Maternal Exposure to Food Antigens during Lactation on Allergic Symptoms. Interestingly, Verhasselt et al. demonstrated that breast-milk-mediated transfer of antigens to the neonate results in oral tolerance induction leading to antigen-specific protection from allergic airway disease in a mouse experimental model [18]. However, it remains controversial in humans whether food antigen ingestion by the lactating mother affects the development of FA in neonates. Thus, in the current study, we addressed this issue with our mouse FA model. We found that breast-feeding by OVA-nonsensitized and OVA-exposed mothers moderately reduced the incidence of allergic symptoms in neonates, and food antigen OVA was detected in breast milk only from OVA-nonsensitized and OVA-exposed mothers during lactation, indicating that the prior neonatal exposure to the low levels of food antigens within the breast milk

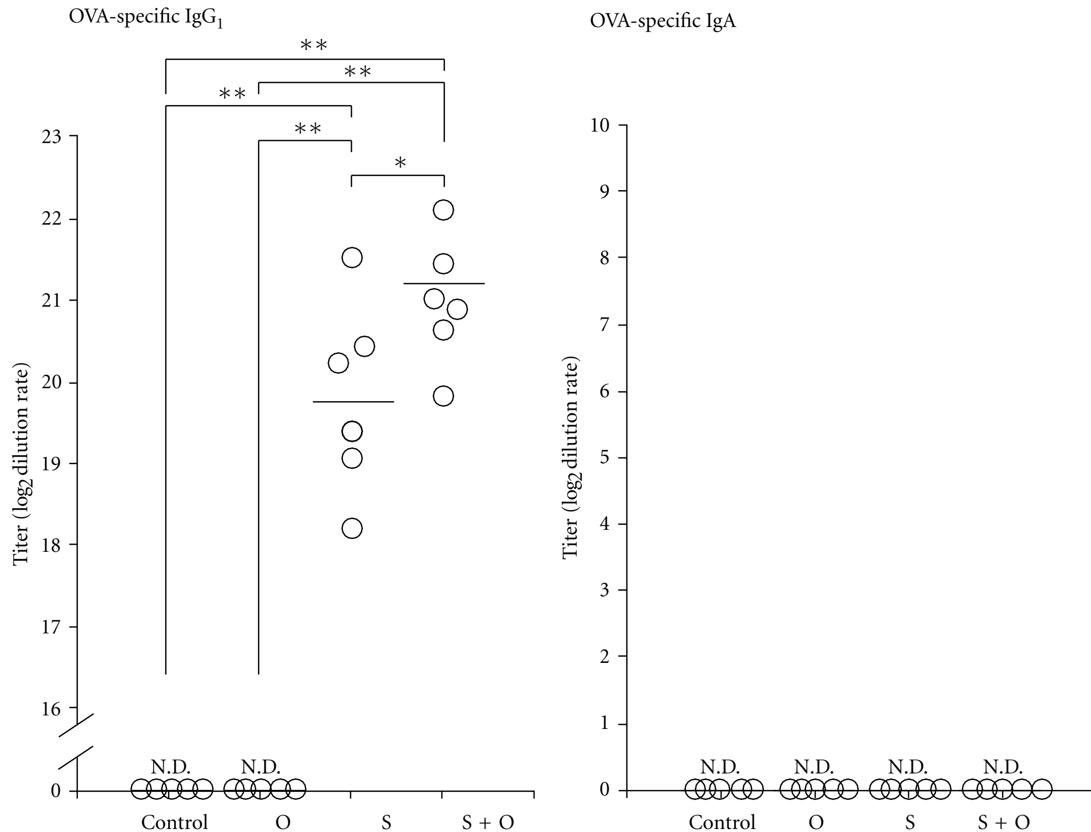


Figure 7: Effects of the maternal exposure to food antigens during lactation and the maternal allergic status on plasma OVA-specific IgG1 and IgA levels in offspring. We found OVA-specific IgG1 only in the plasma of naïve offspring breastfed by OVA-sensitized mothers, which implies that OVA-specific IgG1 transferred from mothers circulate in their offspring. Plasma OVA-specific IgA levels were undetectable in the four groups of offspring. Data are shown as the means and individual data points. N.D.: not detectable. $^{*}P < 0.05$, $^{**}P < 0.01$, $n = 5$–7.

from these mothers for 2 weeks moderately alleviates the susceptibility of their neonates to FA. Furthermore, our results indicate that OVA transferred through breast milk from OVA-exposed mothers during lactation induces oral tolerance, which suppresses the increases in plasma OVA-specific IgE levels and Th2 cytokine mRNA expression levels in the mucosal immune system and the augmentation of the infiltration of mucosal mast cells into the colon, thereby alleviating the development of FA. Food antigens ingested by mothers during lactation are also secreted into breast milk in humans [7, 19]. Taken together, the present results led us to propose the hypothesis that induction of oral tolerance by the maternal ingestion of food antigens during lactation is a strategy for the prevention of FA in infants. However, breast milk from OVA-nonsensitized and OVA-exposed mothers during lactation protects only about half of the offspring from allergic symptoms and drastic increases in plasma OVA-specific IgE levels, implying that the induction of oral tolerance simply by the maternal exposure to OVA during lactation is not sufficient for the prevention of FA in offspring.

4.3. Influence of the Mother's Allergic Status on the Susceptibility of Offspring to Food Allergy. Mosconi et al. demonstrated that breastfeeding by antigen-exposed sensitized mothers abolishes asthma development in progeny. Compared with the protection elicited by antigen-exposed nonsensitized mothers, protection by sensitized mothers was much more profound [20]. Furthermore, López-Expósito et al. showed that low-dose food antigen exposure during pregnancy and lactation reduces the risk of offspring developing first-exposure food antigen-induced anaphylaxis and specific IgE production to active food antigen sensitization in offspring in a mouse peanut FA model [21]. In the present study, we showed that offspring breastfed by OVA-sensitized and OVA-exposed mothers were dramatically protected from the development of FA. Furthermore, OVA-specific IgA and IgG1 were detected in the breast milk from these mothers. In contrast, breast milk from OVA-sensitized and OVA-unexposed mothers had little effect on the development of FA in offspring.

It is known that antigen-specific IgA and IgG form immune complexes with antigens in breast milk [20, 22, 23]. Therefore, it is assumed that OVA that has been ingested by allergic mothers during lactation and then secreted into their breast milk immediately forms immunity complexes with maternal OVA-specific immunoglobulins, thereby masking epitopes of OVA detected by OVA-specific antibodies of OVA ELISA Kit. Thus, we could not measure OVA in the breast

milk from OVA-sensitized and OVA-exposed mothers using OVA ELISA kit, while we could detect OVA in breast milk from OVA-nonsensitized and OVA-exposed mothers. It has been reported that an antigen-IgA immune complexes are efficiently taken up by the body by transcytosis as an immune complex through specific receptors on M cells [24–26].

Moreover, the neonatal Fc receptor for IgG (FcRn), which is the IgG receptor that is expressed on intestinal epithelial cells, is known to contribute to the absorption of IgG and antigen-IgG immune complexes within breast milk [27–29]. Mosconi et al. demonstrated that milk-borne OVA-IgG complexes are actively transferred from mothers to offspring by the FcRn. Furthermore, FcRn-mediated transfer of OVA-IgG complexes resulted in the induction of $FoxP3^+$ regulatory T cells in mesenteric lymph node and that FcRn-deficient mice breastfed by OVA-exposed allergic mice were not protected from allergic airway disease [20]. In this study, we showed that OVA-specific IgG1 was detected in breast milk from allergic mothers, and OVA-specific IgG1 was also detected in the plasma of their offspring, while OVA-specific IgA was not detected in plasma of any offspring. Taken together, these data imply that OVA is effectively transferred into offspring via immune complexes of OVA and OVA specific IgG1 in breast milk through FcRn in comparison to OVA alone in breast milk and thereby effectively induces oral tolerance to protect neonates from FA.

5. Conclusions

Our results indicate that oral tolerance induced by breast-milk-mediated transfer of food antigens by specific immunoglobulins to offspring leads to antigen-specific protection from FA. Our findings may pave the way for the development of novel approaches to primary prevention of allergic diseases such as FA. Furthermore, pioneering researches should be undertaken to target the treatment with immune complexes of food antigen and food antigen-specific IgG1 for the effective prevention strategies against FA.

Authors' Contribution

T. Yamamoto, Y. Tsubota, and T. Kodama contributed equally to this study.

Acknowledgments

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Research Article

Phenotypical and Functional Analysis of Intraepithelial Lymphocytes from Small Intestine of Mice in Oral Tolerance

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In this work, we evaluated the effects of administration of OVA on phenotype and function of intraepithelial lymphocytes (IELs) from small intestine of transgenic (TGN) DO11.10 and wild-type BALB/c mice. While the small intestines from BALB/c presented a well preserved structure, those from TGN showed an inflamed aspect. The ingestion of OVA induced a reduction in the number of IELs in small intestines of TGN, but it did not change the frequencies of $CD8^+$ and $CD4^+$ T-cell subsets. Administration of OVA via oral + ip increased the frequency of $CD103^+$ cells in $CD4^+$ T-cell subset in IELs of both BALB/c and TGN mice and elevated its expression in $CD8\beta^+$ T-cell subset in IELs of TGN. The frequency of $Foxp3^+$ cells increased in all subsets in IELs of BALB/c treated with OVA; in IELs of TGN, it increased only in $CD25^+$ subset. IELs from BALB/c tolerant mice had lower expression of all cytokines studied, whereas those from TGN showed high expression of inflammatory cytokines, especially of IFN-γ, TGF-β, and TNF-α. Overall, our results suggest that the inability of TGN to become tolerant may be related to disorganization and altered proportions of inflammatory/regulatory T cells in its intestinal mucosa.

1. Introduction

Oral tolerance has long been recognized as a physiological mechanism of immune unresponsiveness to dietary antigens (Ags) and indigenous bacterial Ags that maintains tissue integrity by preventing harmful delayed type hypersensitivity responses in the intestine [1]. Three mechanisms were postulated to mediate oral tolerance: clonal deletion, anergy, and active immune suppression. Lower doses of Ag have favored active suppression, and this mechanism is mediated through regulatory cytokines such as TGF-β, IL-10, and IL-4 [2]. T cells producing these regulatory cytokines may downregulate autoreactive T cells in an antigen nonspecific way [2, 3], whereas higher doses favor anergy and T-cell depletion [3]. More recent results show that an activation of T cells is necessary before establishing oral tolerance [4], while enhanced antibacterial immunity can be achieved with concomitant generation of oral tolerance followed by oral administration of soluble antigen such as ovalbumin (OVA) [5].

The immunological consequences of oral administration of antigen ultimately depend on how antigen is taken up and presented to T cells by dendritic cells [6]. Despite of the initiation of oral tolerance remains to be cleared, it seems to involve the active participation of the all gut-associated lymphoid tissue (GALT) [7]. Intraepithelial lymphocytes (IELs) play an important role in the maintenance of mucosal homeostasis by regulating mucosal innate and acquired immunity [8]. The populations of IELs exhibit unique characteristics, perform functions not fully elucidated, and differ widely from their systemic counterparts [9]. IELs in mice and humans include large numbers of cells expressing T-cells receptor (TCRs) $\alpha\beta$ and $\gamma\delta$ [9]. The majority of IELs are $CD8^+$ cells that express a $CD8\alpha\alpha$ homodimer. Among

IELs subsets, it can still be found some $CD4^-CD8^-$double negative cells, $CD4^+CD8^+$ double positive cells and a few $CD4^+CD8\alpha\alpha^+$ cells [8, 10]. Nearly all $CD8^+$ IELs expressed CD69 and had lytic activity [11].

Preliminary results of our group have shown that DO11.10 mice, that bear transgenic anti-OVA TCR, are not susceptible to oral tolerance with OVA [12]. In the present work, we investigated the immune response that takes place in intestinal mucosa during the consumption of OVA in both BALB/c and DO11.10 mice. Since the majority of studies with oral tolerance induction and mucosal immune response have only evaluated the role of $CD4^+\alpha\beta$ T cells [13–15], we analyzed all subsets of IELs of these mice in the context of oral tolerance and immune response to OVA.

2. Material and Methods

2.1. Animals. Breeder pairs of TCR OVA-specific transgenic mice (clone DO11.10) [16] and BALB/c mice were supplied by CEMIB (Centro Multinstitucional de Investigacões Biológicas), UNICAMP. Mice were maintained under specific pathogen-free condition and were provided with autoclaved food and water. The study was approved by the Ethics Commitee for Animal Experimentation of University of Campinas (Protocol no. 736-2).

2.2. Tolerance Induction and Immunizations. Oral tolerance to OVA was induced in 8 weeks old mice as described elsewhere [12]. Briefly, Mice were fed with 4 mg/mL OVA solution (Rhoster Indústria e Comércio, Ltda., Vargem Grande Paulista, SP, Brazil) for seven consecutive days. The mice in the control group received protein-free water. Seven days after the interruption of oral treatment, half of this group of mice was challenged with of 10 μg OVA (Sigma Chemical Co., St. Louis,MO, USA) plus 1 mg $Al(OH)_3$ by intraperitoneal (ip) route. After 14 days, mice were boosted with 10 μg OVA in saline solution via ip. In control group, half of the animals were also challenged with the ip doses of OVA. Seven days after the last ip dose, all mice were bled for serum separation, and then euthanized in a CO_2 chamber.

2.3. Cell Isolation and Purification. Intraepithelial lymphocytes were isolated from the small intestine of BALB/c and DO11.10 mice, according to Montufar-Solis and Klein [17]. Briefly, small intestine tissues were removed, and Peyer's patches were dissected out. Tissues were flushed of fecal material, opened longitudinally, and cut into 3 to 4 mm pieces in RPMI 1640 (Sigma-Aldrich) supplemented with FCS (10% v/v) (Nutricell, Campinas, SP, Brazil), gentamicin 20 μg/mL (GE Healthcare Biosciences, Pittsburgh, PA, USA). Tissue fragments were rinsed several times in $Ca2^-/Mg2^-$ free PBS, transferred to $Ca2^-/Mg2^-$ free PBS containing 5 mM EDTA (Sigma-Aldrich) and 2 mM DTT (Calbiochem; Cleveland's reagent; Merck KGaA, Darmstadt, Germany) and shaken (bath Dubnoff-Marconi, Piracicaba, SP, Brazil) at 37°C for 30 min. Cell suspensions were filtered through 20-mL syringe barrels containing wetted nylon wool, centrifuged, suspended in 3 mL of 40% isotonic Percoll (Amersham and Sigma-Aldrich), layered on top of 70% isotonic Percoll (4 mL), and centrifuged for 30 min at 400 xg. IELs were recovered from the Percoll interface and washed by centrifugation in supplemented RPMI-1640, viability and cell numbers were measured by exclusion of Trypan blue dye and counting in a hemocytometer. IELs from BALB/c mice were further purified by immunomagnetic separation, using beads conjugated with mAb anti-CD90 (Thy-1.2) on MS columns, as recommended by the manufacturer (Midi Macs, Miltenyi Biotec, Bergisch-GladBach, Germany).

2.4. Phenotypic Analysis by Flow Cytometry. Single-cell suspensions from small intestine were suspending in PBS/0,01% BSA (Sigma) w/v supplemented with 0,1% sodium azide. Cells were first incubated with anti-CD16/32 (culture supernatants of clone 2.4G2) for 45 min to block Fc-mediated antibody binding. Then, cells were incubated with relevant mAb for 30 min at 4°C, washed with PBS/2% FCS, and fixed 9 with PBS/1% formaldehyde (Merck Darmstadt, Germany). Three- or four-color flow cytometry acquisition was performed on FACSAria (BD Bioscience, San Jose, CA, USA). A total of 30.000 events were acquired in each analysis. The following antibodies purchased from BDPharMingen were used for flow cytometry: anti-CD3 (clone 145-2C11)-PerCP Cy5.5 or FITC; anti-CD4 (clone GK1.5)-FITC, PE or PE-Cy7; anti-CD4 (clone RM4-5)-PE-Cy7; anti-CD8α (clone 53-6.7)-FITC or PE; anti-CD8β (clone 53-5.8)-FITC; anti-TCRβ (clone H57-597)-PE; anti-TCR$\gamma\delta$ (clone GL3)-FITC or PE; anti-CD152 (CTLA-4) (clone UC10-4F10-11)-PE; anti-CD25 (clone 7D4)-FITC or PE; anti-CD103 (α IEL) (clone M290)-PE; anti-OVA TCR (clone KJ1-26)-PE. Anti-Foxp 3 (clone FJK-16 s)-PE or FITC were purchased from eBioscience (San Diego, CA, USA). Data were analyzed by the software FCS express V3. Respective isotype controls were included for each cell surface stain to exclude nonspecific binding and to determine the optimal setting fluorescence quadrants (BD Bioscience, San Jose, CA, USA). Data were analyzed by the software FCS express V3 (De Novo Software, Los Angeles, CA, USA).

2.5. Histological and Immunohistochemical Staining. For histological analysis, pieces from small intestine were fixed in 4% paraformaldehyde buffered solution (Sigma) and washed with PBS/1% Glycine (J.T. Baker, Mallinckrodt Baker, Phillipsburg, NJ, USA), and 5 μm paraffin-embedded sections were stained with hematoxylin and eosin (Sigma). Slides were observed in an optical microscope (Eclipse E-800 Microscope, NIKON; Tokyo, Japan) and analyzed using the software Proplus image. For immunofluorescence analysis, sections of small intestine were dehydrated, frozen in OCT-embedding compound (Leica) on dry ice, and stored at −70°C. Tissue sections (5 μm) were cut with a cryostat (Microm HM 505 E) and transferred to silane-coated microscope slides. Cryosections were brought to room temperature, fixed with acetone (Merck) for 10 min at 4°C, and blocked with PBS containing 1% of BSA (type V, INLAB, SP, Brazil) for 30 min. After washing with PBS, they were incubated with anti-CD3 FITC-labelled (clone

Table 1: Number of cells recovered from 40/70%-Percoll interface

	Cells from[a,b]			
	BALB/C		DO11.10	
Treatments				
Control	7.07	±2.05	4.27	±1.51
Oral ova	6.50	±1.91	1.57*	±0.72
Oral + IP ova	11.62	±4.97	1.00*	±0.29
IP ova	12.75	±3.41	0.55**	±0.08

[a] Number of cells represents the mean ± SEM × 10^7 cells; [b] Data were obtained from 3-4 independent experiments; * $P < 0.05$; ** $P < 0.01$.

145-2C11 homemade) for 3 h, washed, and incubated with TRITC-phalloidin (Sigma) for 30 min. All incubations were made at room temperature. Vectashield-mounted slides (Vector Laboratories) were visualized by optical microscopy.

2.6. Quantitative Real-Time Polymerase Chain Reaction (PCR). Total RNA was extracted from mouse IELs using PureLink Micro-to-Midi Total RNA Purification System (Invitrogen, SP, Brazil,) according to the manufacturer's instructions. The cDNA was made using SuperScript III First-Strand Synthesis Supermix (Invitrogen) with random primers (Invitrogen) and analyzed for IL-2, IL-4, IL-10, IL-6, IL-17, IFN-γ, TGF-β, and TNF-α gene expression by real-time PCR assay using an 7500 Fast Real-Time PCR (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions; 18S ribosomal RNA (rRNA) was used as an internal control. All mouse primer and probe sets used were predesigned TaqMan Gene Expression Assays (Applied Biosystems). PCRs were performed in four replicates with a 2x TaqMan Mastermix (Applied Biosystems). Relative expression of mRNA species was calculated using the comparative 2 threshold cycle (ΔCT) method [18].

2.7. Statical Analysis. The statistical analysis was performed using GraphPad Prism 4 (GraphPad Software, CA, USA). The statistical significance of differences between control and experimental groups were determined by one-way and two-way ANOVA, followed by multiple comparison Bonferroni's test. The results were expressed as mean ± SEM. Values were considered significant at $P < 0.05$. Supplemental data include two figures (see supplementary material available online at doi:10.1155/2012/208054).

3. Results

3.1. Histological Analysis and Distribution of IELs in Small Intestines of Mice DO11.10 and BALB/c. As depicted in Figure 1(a), the small intestine histoarchitecture of both naïve DO11.10 and BALB/c strains were preserved; however, it was found reduced tunica muscular thickness of DO11.10 when compared with BALB/c mice. Discrete but well-defined histological changes were observed in the lamina propria (LP) of intestinal villi of the transgenic mice after feeding with OVA, mainly in those challenged with OVA by ip route, with a loose connective tissue rupture and mild edema of lamina propria of villous projections in DO11.10 mice. BALB/c mice treated with OVA did not present any of those alterations. The total number of IELs isolated from the small intestine of DO11.10 mice of all experimental groups was always lower than those from BALB/c and markedly dropped upon OVA treatments (Table 1). As illustrated in Figure 1(b), the incidence of CD3 positive cells decreased substantially in the villi of TGN mice but not in the BALB/c. Cytometry analyses of IELs isolated from TGN showed that the clonotype anti-OVA TCR cells (KJ1-26 positive cells) decreased significantly from 65% to less than 20% after oral and ip administration of OVA (Figures 1(c) and 1(d)).

3.2. Analysis of Subsets of IELs after the Induction of Tolerance or Immunization. IELs from BALB/c and D011.10 mice treated with OVA by oral and/or ip route were stained with anti-CD3, anti-CD8α, anti-CD8β, anti-CD4 and analyzed by three-color flow cytometry (Figure 2). As shown in Figure 2(a), the frequency of $CD3^+$ cells in the small intestine of BALB/c and DO11.10 mice was not changed by different treatments with OVA. No significant alteration was observed in the frequency of IEL subsets (CD8$\alpha\alpha$, CD8$\alpha\beta$, CD4/CD8α, CD4) upon treatments with OVA (Figure 2(c)).

The effects of administration of OVA on the distribution of phenotypic markers CD103 and CD25 were assessed in subsets CD4, CD8α, and CD8β of IELs isolated from BALB/c(Supplemental Figure 1, Panel A-D) and DO11.10 mice(Supplemental Figure 1, Panel (E-H)), as well as the frequencies of $\alpha\beta^+$ and $\gamma\delta^+$ T cells in these subsets (Supplemental Figure 2). Frequency and expression of $CD103^+$ cells in subsets of IELs of BALB/c and DO11.10 mice are illustrated in histograms of (Figures 3(a) and 3(b), resp.). Significant increase of $CD4^+CD103^+$ subset of IELs was observed following treatments with OVA by oral + ip routes, in both BALB/c and DO11.10 mice, whereas no antigen-dependent alteration was observed in the frequency of CD8$\alpha\alpha$ and CD8$\alpha\beta$ IELs expressing CD103 (Figure 3(c)). However, the expression of CD103 was significantly augmented in subpopulation of CD8$\alpha\alpha^+$ and CD8$\alpha\beta^+$ IELs when BALB/c mice were fed with OVA, as well as in CD8$\alpha\alpha^+$ cells in ip immunized mice. Conversely, in DO11.10 mice, this marker was significantly reduced in CD8$\alpha\alpha^+$ cells upon immunization with OVA by ip route (Figure 3(c)).

Frequency and expression of $CD25^+$ cells in subsets of IELs of mice BALB/c and DO11.10 are illustrated in histograms of (Figures 4 (a) and 4(b) resp.). No antigen-dependent alteration was observed in the frequency of IELs

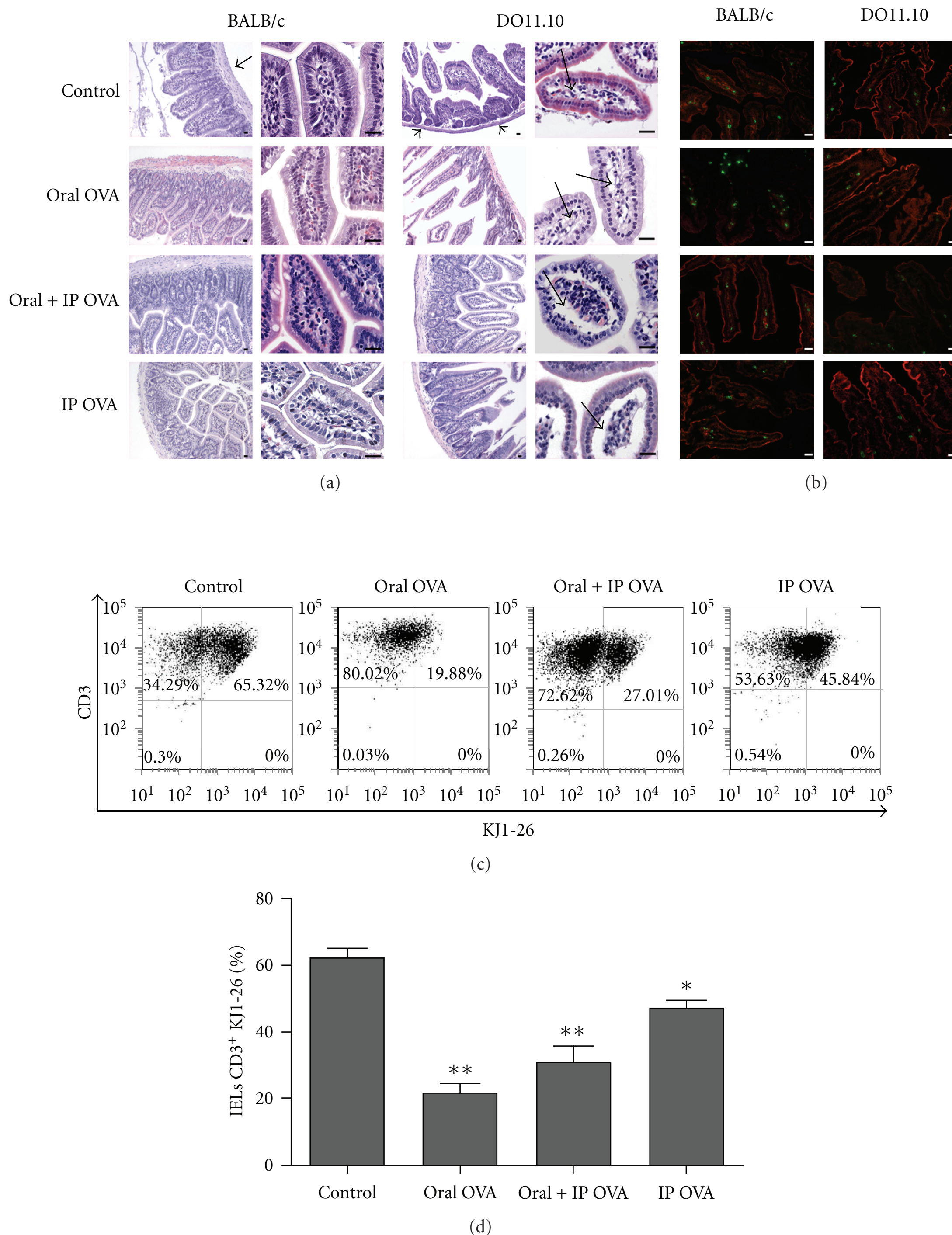
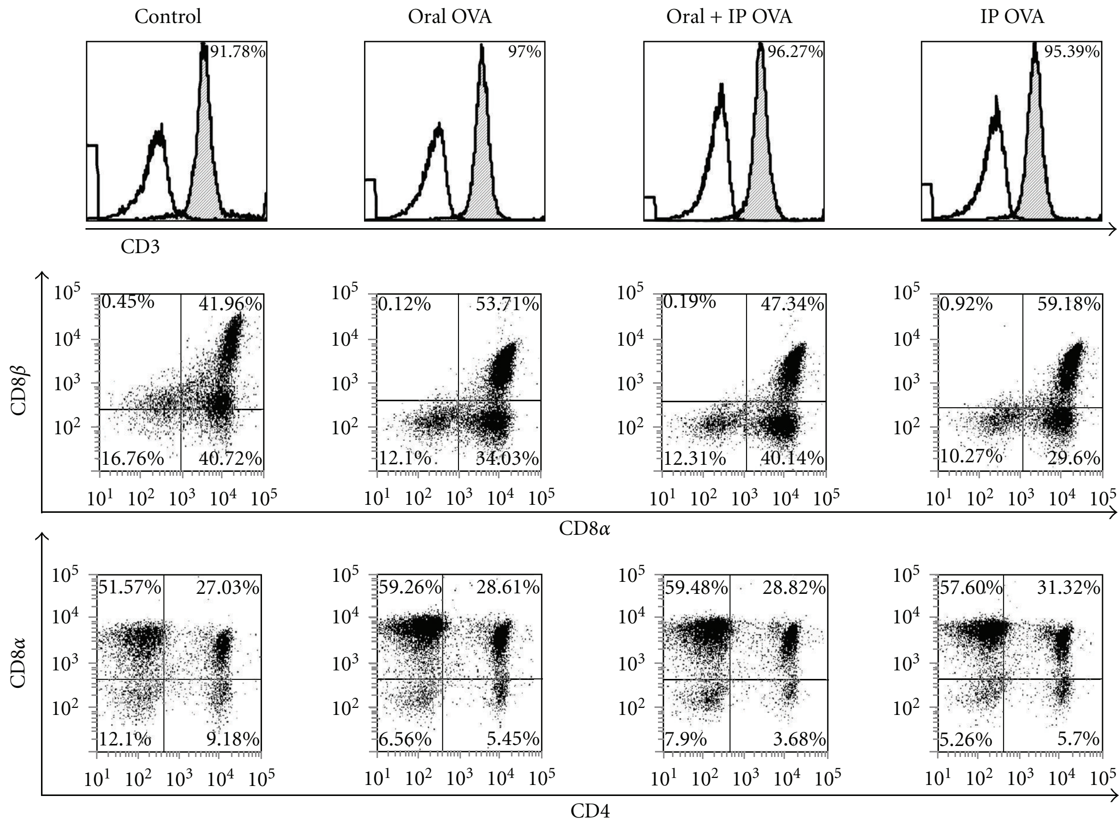


Figure 1: Histological analysis and incidence of T cells in small intestines (jejunum) of BALB/c and DO11.10 mice after treatments with OVA. Mice were fed with OVA solution for 7 days (oral OVA), fed with OVA and challenged by ip route (oral + ip OVA), immunized with OVA only by ip route (ip OVA), or non-treated (control). (a) Hematoxylin/Eosin-stained sections of small intestines in low and high magnification showing details of mucosa villi. Note the reduced thickness of the tunica muscular (arrow heads) in DO11.10 when compared with BALB/c, and loss of connective tissue and mild edema in the lamina propria (thin arrows) in Oral + ip OVA and ip OVA groups of DO11.10 mice. Bars = 50 μm. (b) Immunofluorescence of frozen sections of jejunum counterstained with TRITC-phalloidin (red epithelial cells) showing reduced incidence of CD3 positive cells (green) in the mucosa of DO11.10 in comparison to BALB/c mice. Bars = 25 μm; (c, d) Frequency of KJ1-26 positive cells amongst the intraepithelial lymphocytes freshly isolated from DO11.10 mice treated with OVA. The clonotype anti-OVA TCR cells (KJ1-26$^+$ cells) decreased from 65% to less than 20% after oral and ip administration of OVA. Data represent mean ± SEM ($N = 5$) of three independent experiments.



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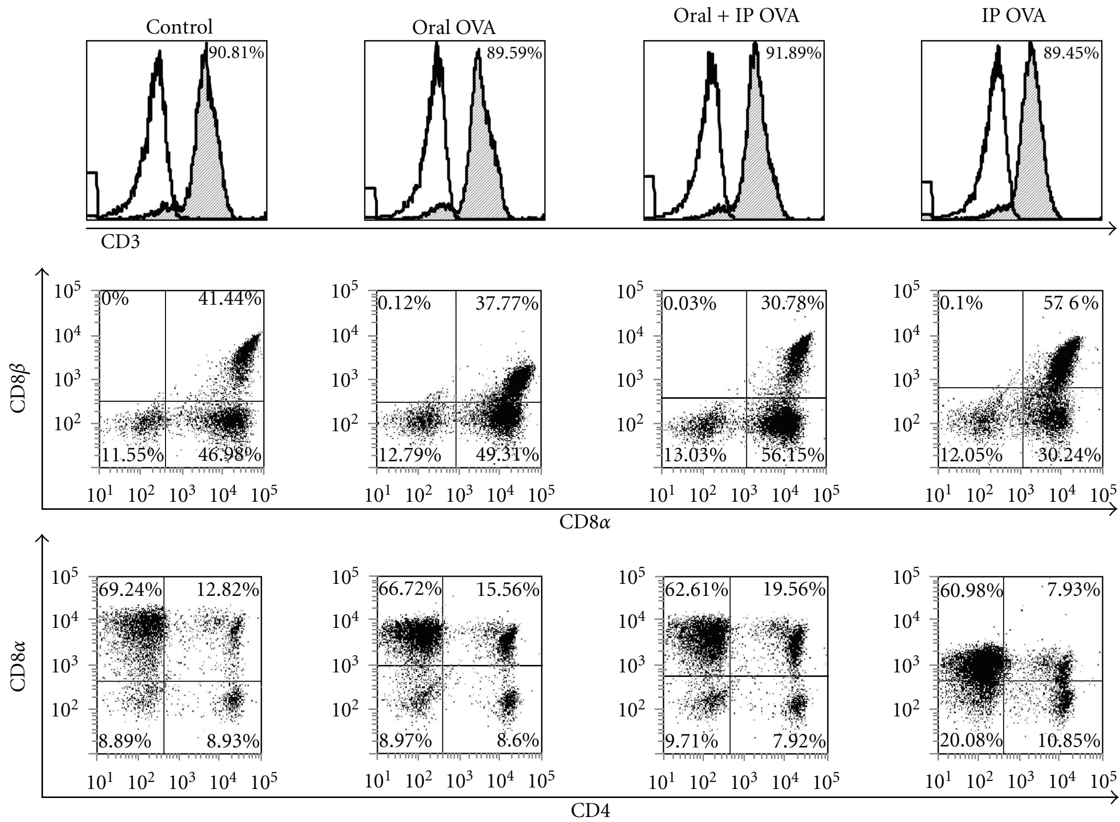

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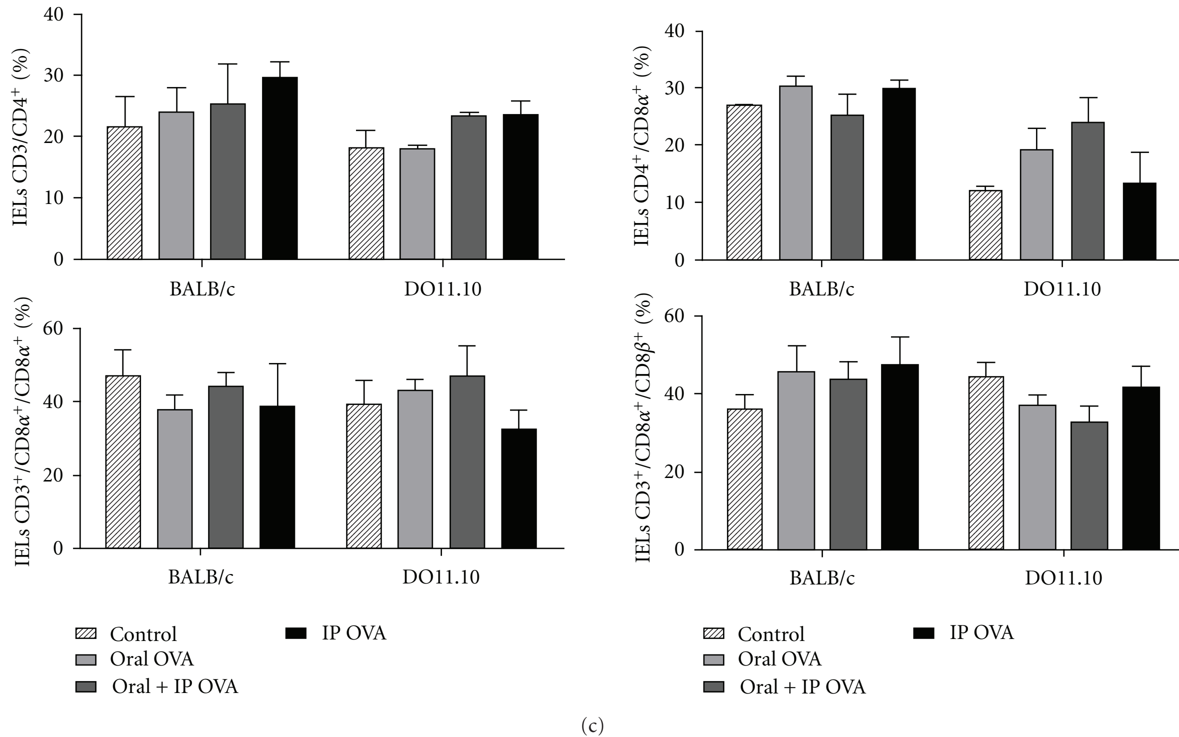


Figure 2: Effects of the treatments with OVA on CD4 and CD8 subsets of IELs. Freshly isolated IELs from BALB/c (a) and DO11.10 (b) mice were gated for $CD3^+$ cells and analyzed for expression of $CD8\alpha$, $CD8\alpha/CD8\beta$, and $CD4/CD8\alpha$ by flow cytometry. No significant difference was found in the frequency of IELs of any subset in both strains of mice. In (c), data represent mean ± SEM ($N = 5$) in each group in three independent experiments.

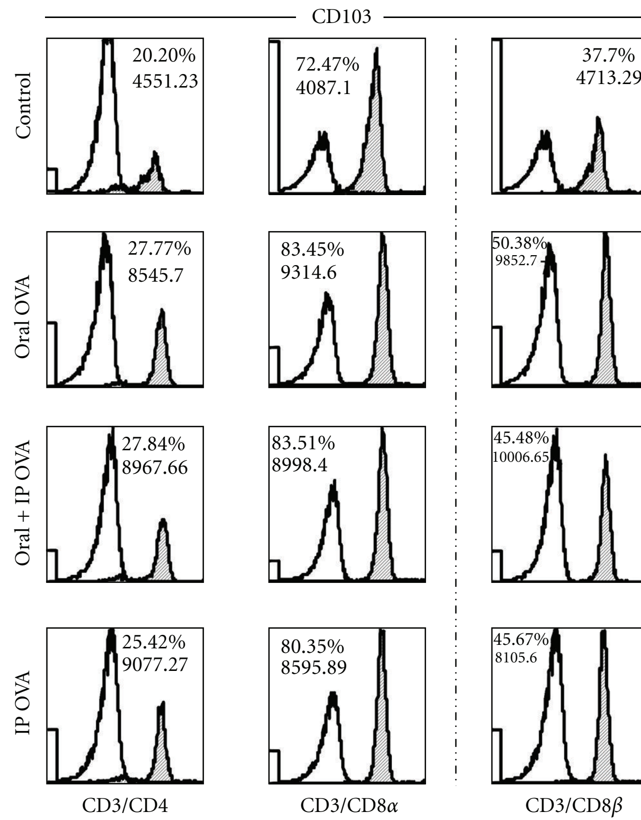
expressing this marker in both DO11.10 and BALB/c mice, except in $CD8\beta^+$ subset of IELs in which this marker was upregulated by treatments with OVA by oral route (Figure 4(c)).

3.3. Evaluation of Foxp3 Expression in IELs after Administration of OVA. To assess possible changes in the frequency of regulatory T cells after oral and/or ip administration of OVA, IELs from BALB/c and DO11.10 mice were stained with anti-Foxp3 and analyzed by flow cytometry in $CD4^+$, $CD8\alpha\alpha^+$, $CD8\alpha\beta^+$, and $CD25^+$ subsets. As shown in Figures 5(a) and 5(c), we observed that oral administration of OVA to BALB/c mice resulted in elevation of the frequency of $Foxp3^+$ cells in $CD4^+$ and $CD8\alpha\beta^+$subsets. Following parenteral administration of OVA, the frequency of cells $Foxp3^+$ in IELs of BALB/c mice was more elevated in the $CD8\alpha\alpha^+$, $CD8\alpha\beta^+$, and $CD25^+$ subsets. On the other hand, only the oral + ip treatment of DO11.10 mice increased the frequency of $Foxp3^+$ cells in the $CD25^+$ subset (Figures 5(b) and 5(c)).

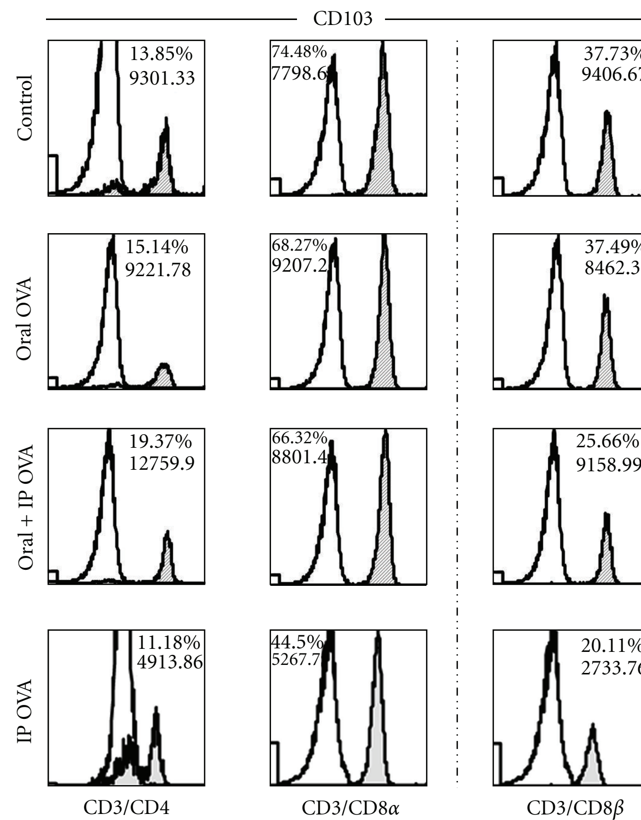
3.4. Effect of Treatments with OVA on Cytokine mRNA Expression in IELs of BALB/c and DO11.10 Mice. In addition to phenotypic analysis, expression of pro-(IL-2, IFN-γ, IL-6, IL-17, and TNF-α) and anti-inflammatory (IL-10, IL-4, and TGF-β) cytokines has been assessed by real-time PCR from extracts of IELs of the small intestine of BALB/c and DO11.10 mice treated with OVA. The results are summarized in Figure 6. It is possible to notice that IELs from OVA-treated mice of both strains present opposite profiles in relation to the gene expression of most cytokines examined. IELs from tolerant BALB/c mice (oral and oral + ip groups) showed a diminished expression of mRNA for cytokines IL-10, IL-2, IFN-γ, TGF-β, and TNF-α in comparison to those from mice immunized by ip route. IELs from DO11.10 mice treated with OVA by oral + ip and ip routes showed an elevated expression of IL-6, IL17, TNF-α, and TGF-β, although differences were not significant in comparison with the oral group. IELs from DO11.10 mice that received OVA by oral + ip, however, showed a mRNA expression for TNF-α, IFN-γ, and TGF-β significantly more elevated than IELs from BALB/c mice of the same group.

4. Discussion

Failure in the induction of oral tolerance seems to be associated with modifications in the gastrointestinal mucosa permeability and, especially, with the immunoregulation that occurs in this environment [19, 20]. Results obtained previously in our laboratory showed that transgenic DO11.10 and BALB/c mice differ in their immune response to oral OVA. While DO11.10 mice develop a specific humoral immune response after the ingestion of native ovalbumin, the BALB/c mice become tolerant to OVA. The transgenic mice fed with ovalbumin produced an immune response that is a mixed



(a)



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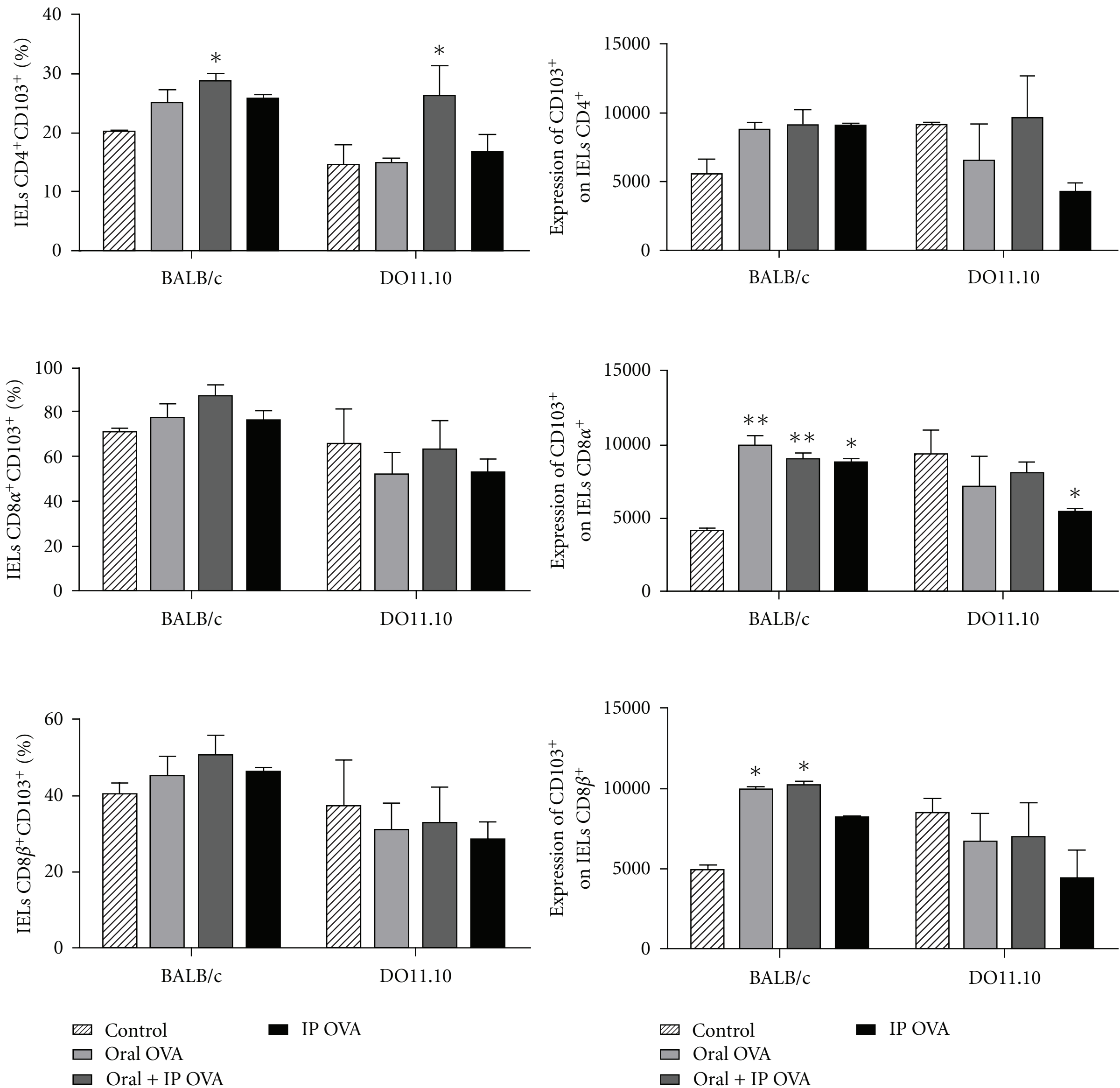
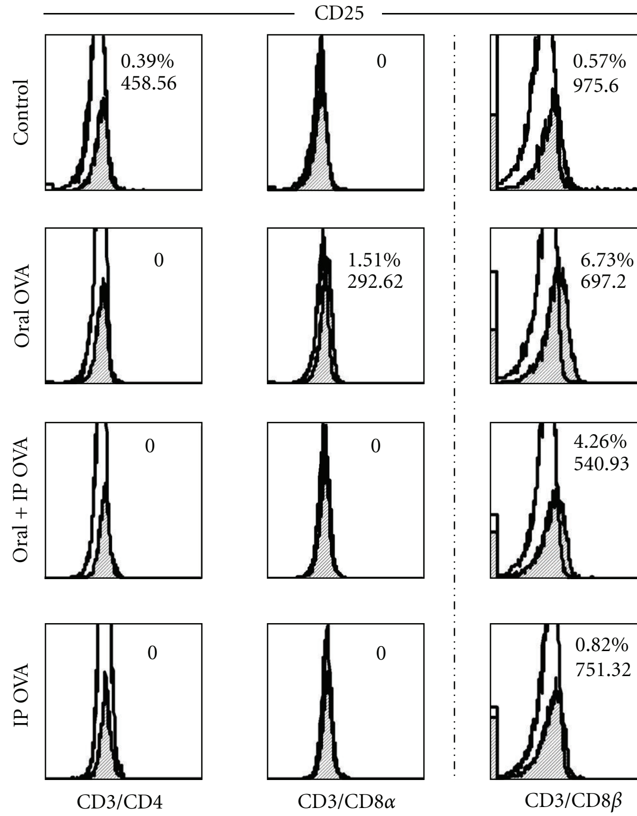


(c)

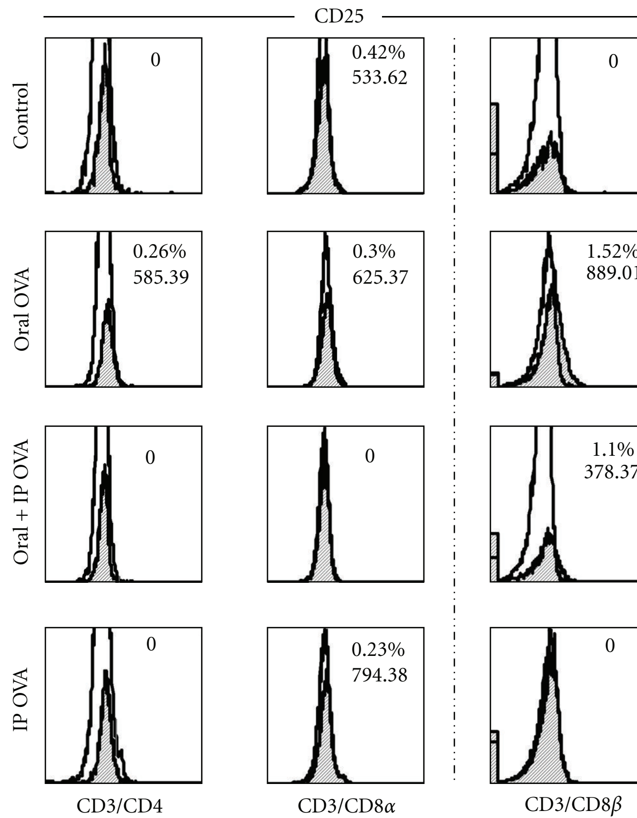
FIGURE 3: Effects of the treatments with OVA on the frequency of CD103+ cells in the small intestine. Freshly isolated IELs from BALB/c (a) and DO11.10 (b) mice were gated for CD3+/CD4+, CD3+/CD8α+, and CD3+/CD8β+ cells and analyzed for expression of CD103 by flow cytometry. Blank histograms indicate isotype control staining. CD3+/CD8β+ subset illustrated at the right column is part of the CD3+/CD8α+ population. (c) Data represent mean ± SEM ($N = 5$) in three independent experiments. Frequency of CD4+ cells were significantly more elevated in IELs isolated from mice BALB/c and DO11.10 treated with OVA by oral + ip routes. The expression of CD103 was markedly augmented in all subpopulations of IELs of OVA-treated BALB/c mice and was reduced in DO11.10 immunized by ip route.

of TH1/TH2, with prevalence of a TH1 pattern [12]. In this work, we observed that even before the oral treatment with OVA, the DO11.10 mice have showed morphological modifications in the intestinal epithelium villi and of the muscular layer in the intestinal tissue. Our results showed a deepening of changes in the epithelium of small intestine in DO11.10 mice treated with OVA, which are consistent to an inflamatory process. These changes have not been observed in the intestinal epithelium of BALB/c mice, which have been presented a good preservation of the villi. A chronic inflammatory process, with lymphocytic infiltration in the lamina propria and increased number of IELs in the epithelium has been shown in double-transgenic mice that express the haemagglutinin from influenza virus A (HA) and TCR HA-specific. The inflammatory reaction, however, is kept under control by the generation of regulatory T cells [14].

Deletion of self-reactive lymphocytes constitutes one of main mechanisms of peripheral tolerance induction and probably of oral tolerance induction [21, 22]. Conversely, intestinal inflammation has been correlated with failure in



(a)



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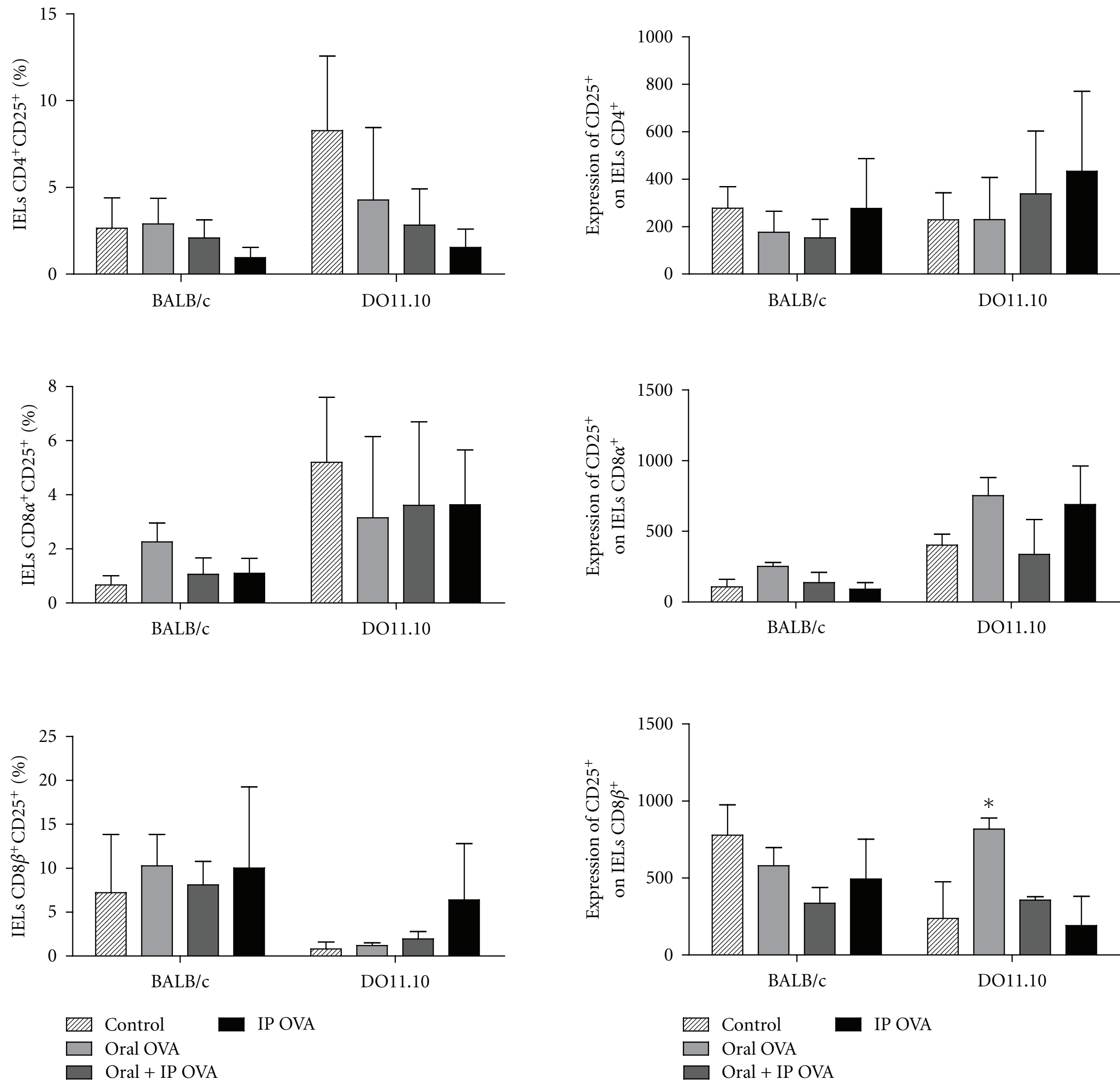
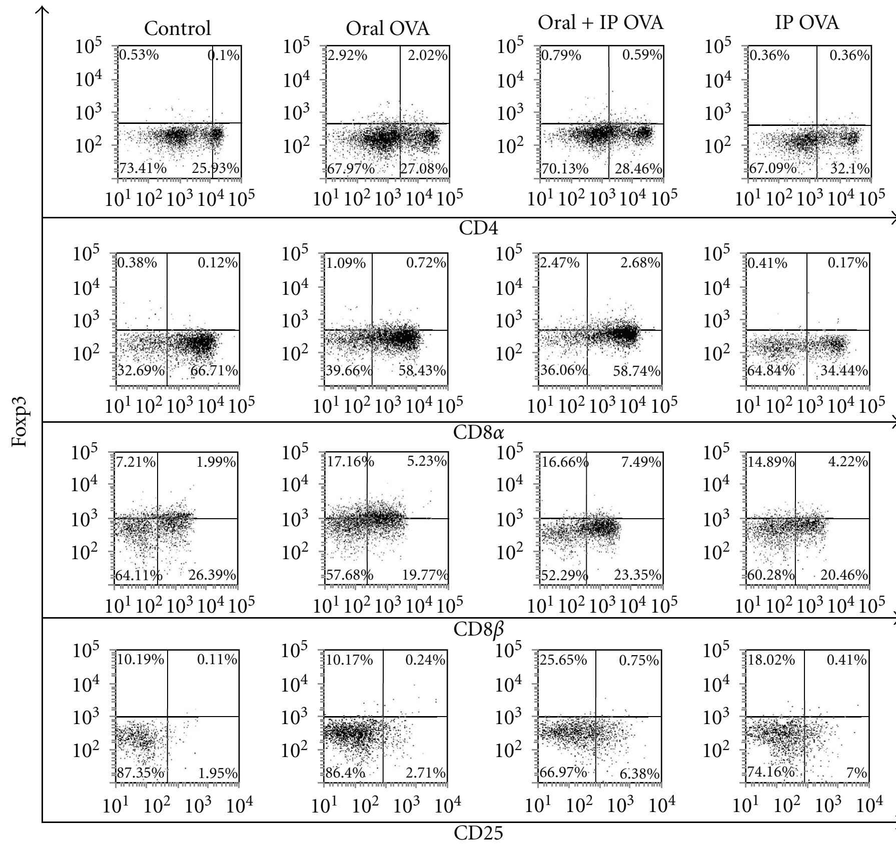


(c)

Figure 4: Effects of the treatments with OVA on the frequency of $CD25^+$ cells in the small intestine. Freshly isolated IELs from BALB/c (a) and DO11.10 (b) mice were gated for $CD3^+/CD4^+$, $CD3^+/CD8\alpha^+$, and $CD3^+/CD8\beta^+$ cells and analyzed for expression of CD25 by flow cytometry. Blank histograms indicate isotype control staining. $CD3^+/CD8\beta^+$ subset illustrated at the right column is part of the $CD3^+/CD8\alpha^+$ population. (c) Data represent mean ± SEM ($N = 5$), in three independent experiments. An increased frequency of $CD25^+$ cells can be observed only in CD8β subset of IELs from DO11.10 mice treated with OVA by oral route.

the induction of apoptosis in lymphocytes present in the mucosa [23]. The initial hypothesis of our study was that resistance to the induction of oral tolerance in DO11.10 might be due to failure in the deletion of OVA-specific lymphocytes in the intestinal mucosa after ingestion of the antigen. Indeed, treatment with OVA by oral and/or ip routes resulted in reduction of IELs in the DO11.10 mice. Besides that, a remarkable reduction in OVA-specific cells (KJ1-26) was observed in small intestine of DO11.10 mice treated with OVA. Previous work has shown that the administration of OVA results in marked reduction of mature lymphocytes KJ1-26^+ in the blood and peripheral lymphoid organs [16]. However, a substantial portion of the IELs and lymphocytes from LP from the DO11.10 mice carries a second nonclonotypical TCR, probably due to the incomplete allelic exclusion of the endogenous TCRα during the rearrangement process in the thymus [21, 24]. Part of the alternative TCR seems to be specific to antigens from the intestinal environment as DO11.10/SCID or DO11.10/RAG$2^{-/-}$ mice do not exhibit reactivity to antigens from the intestinal microbiota [25, 26].



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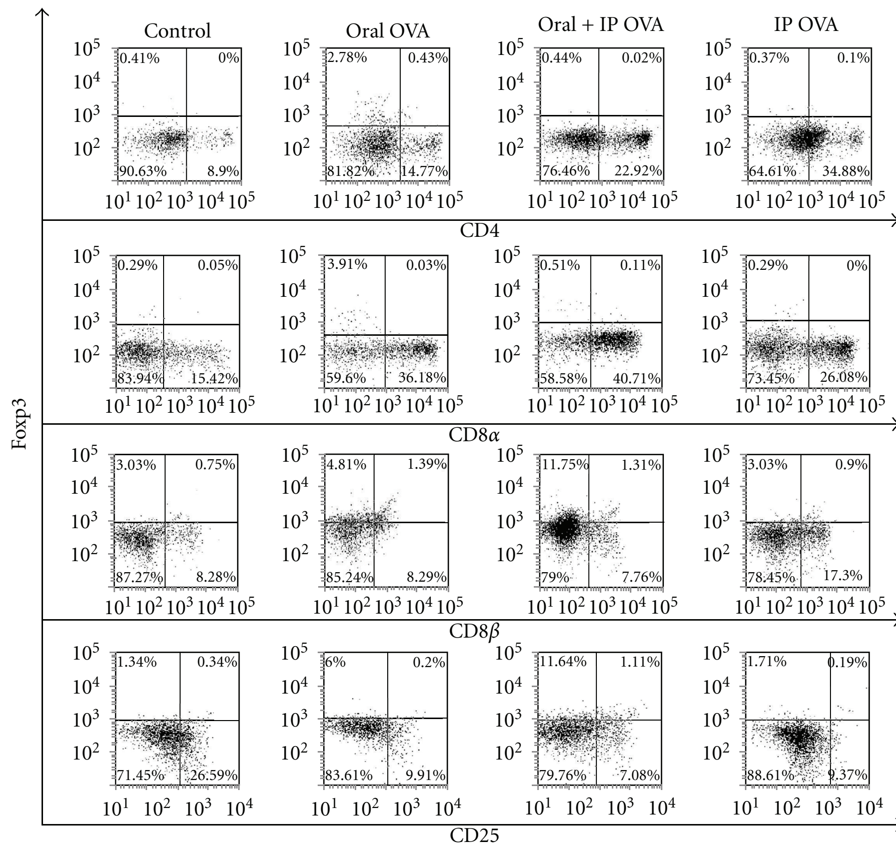

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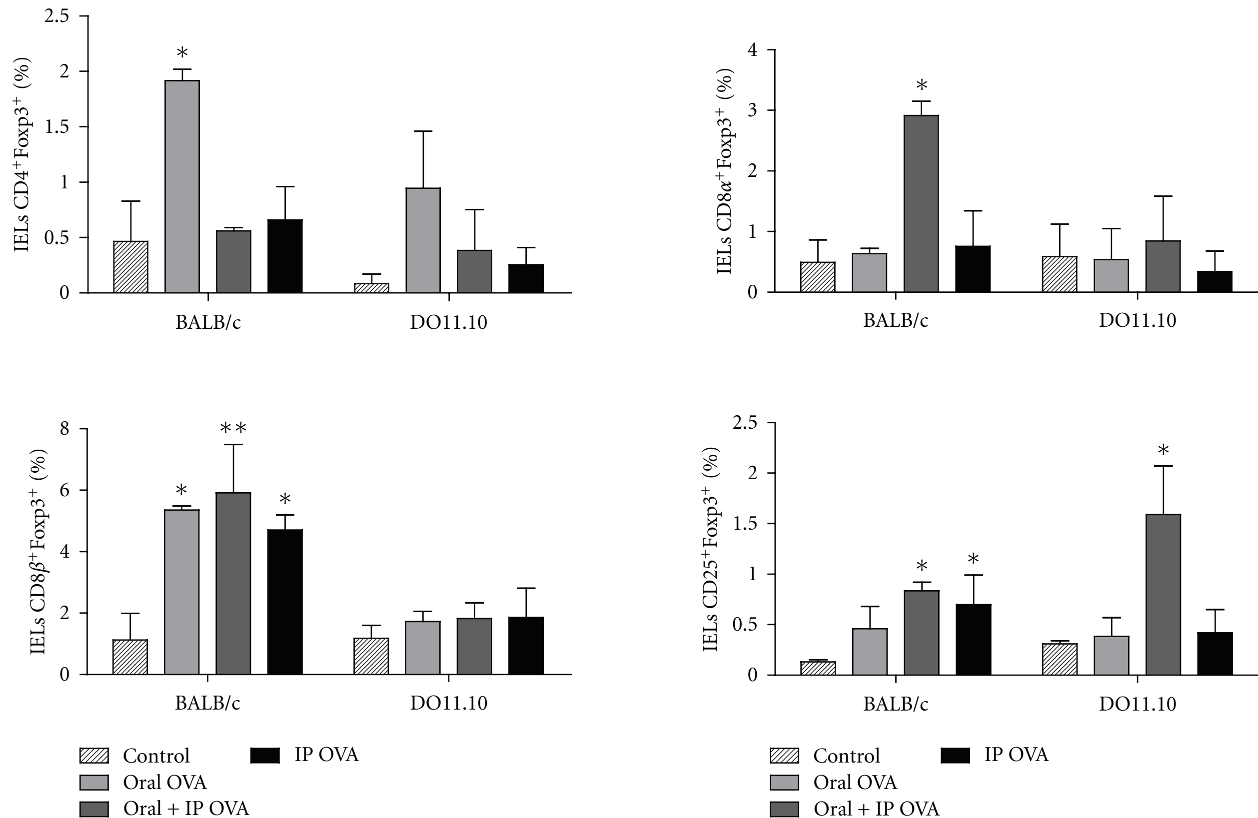

Figure 5: Effects of the treatments with OVA on the frequency of Foxp3$^+$ cells in the small intestine. IELs were isolated from BALB/c (a) and DO11.10 (b) mice, and the frequency of Foxp3$^+$ cells were analyzed on for CD3$^+$/CD4$^+$, CD3$^+$/CD8α^+, and CD3$^+$/CD8β^+ cells. (c) Data represent mean ± SEM ($N = 5$), in three independent experiments. Treatments of BALB/c mice with OVA resulted in increase of the frequency of Foxp3$^+$ cells in all IEL populations. The oral + ip treatment of DO11.10 mice has increased the frequency of Foxp3$^+$cells in IELs CD25$^+$.

Thus, a possible explanation for the escape of DO11.10 from oral tolerance in spite of the occurrence of TCR OVA-specific cells deletion would be the activation of T cells bearing alternative TCR, that would result in local inflammatory response, with activation of nontransgenic IELs carrying TCR to other than OVA epitopes.

The IELs have phenotype similar to either effector or effector memory of cells that are present in other peripheral lymphoid organs [27], presenting a high expression of the CD103 marker ($\alpha_E B_7$), an α-integrin responsible for the retention of IELs in the intestinal epithelium [28]. This molecule has been associated to the immuneregulation in the mucosa as it is expressed by regulatory T lymphocytes [29, 30] as well as by dendritic cells involved in the generation of regulatory T cells [31, 32]. IELs from small intestine of the BALB/c mice treated with OVA orally and/or parenteral have shown marked increase in the expression of CD103, in all populations studied: TCD4, TCD8α, and TCD8β. Furthermore, IELs from orally treated DO11.10 mice have shown no changes in the expression of CD103 in any cell subset. In contrast, TGN immunized intraperitoneally with OVA showed an accentuated reduction in the expression of CD103 in these cells.

Several populations of regulatory cells have been described in oral tolerance, including IL-10 producer cells termed Tr1, TGF-β producing cells called Th3 and TCD4$^+$/CD25$^+$, and its relative importance in the establishment of oral tolerance is still under investigation [1, 20]. Some of these studies have emphasized the role of the CD8$\alpha\alpha$ IELs in the immunoregulation that occurs in the intestinal mucosa [33, 34]. In this work, we did not observe changes in the frequency of this subset of IELs in either BALB/c or DO11.10 mice immunized orally or parenterally with OVA.

There is a consensus in the literature that the oral tolerance is related to the induction of antigen-specific regulatory T cells either by direct or cross-presentation of antigens from the enterocytes [20, 21, 34]. Our results have shown that the ingestion of OVA led to an increase in the frequency of TCD4$^+$/Foxp3$^+$, TCD8α^+/Foxp3$^+$, TCD8β^+/Foxp3$^+$, and TCD25$^+$/Foxp3$^+$ amongst the IELs of BALB/c mice, thus indicating that the establishment of tolerance in wild-type mouse are associated with the increase of regulatory T cells. The frequency of TCD25$^+$/Foxp3$^+$ cells has also increased in the IELs from TGN treated with OVA by oral + ip routes. However, this increase has not been sufficient to inhibit the

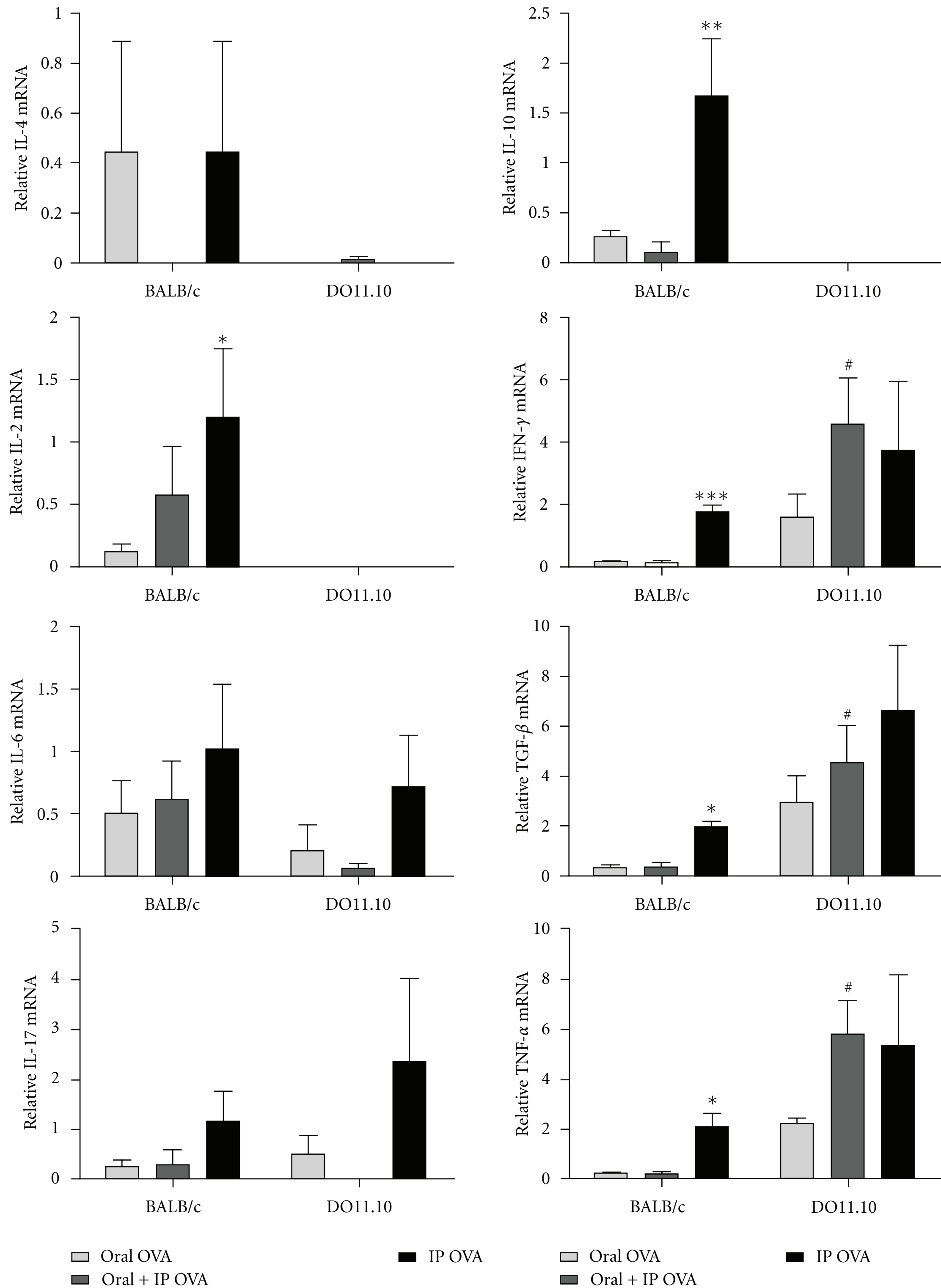

Figure 6: Quantitative mRNA analysis of cytokine expression in intestinal IELs. Total RNA was extracted from freshly isolated IELs of BALB/c and DO11.10 mice ($N = 5$), and cDNA of IL-2, IL-4, IL-6, IL-10, IL-17, IFN-γ, TNF-α, and TGB-β was made using PureLink Micro-to-Midi Total RNA Purification System (Invitrogen, SP, Brazil,). Quantitative PCR analysis was performed with the ABI 7500 Fast Real-Time PCR system (Applied Biosystems), in four replicates, with the TaqMan Mastermix (Applied Biosystems). Samples were normalized to 18S rRNA, and an arbitrary value of 1 was given to control group (naïve mice) for the normalization, and the remaining samples were plotted relative to that value. IELs from BALB/c mice immunized by ip route show an increase in the expression of mRNA of cytokines IL-10, IL-2, IFN-γ, TGF-β, and TNF-α. IELs from DO11.10 mice fed with OVA and challenged by ip route showed increased expression of mRNA for cytokines IFN-γ, TGF-β, and TNF-α. The results are representative of three independent experiments.

inflammation that has settled in the small intestinal mucosa of the TGN after treatment with the protein.

$TCD8^+$ cells also play an important role in the homeostatic maintenance of the intestinal epithelium. In this regard, $TCD8\alpha\beta$ suppressor cells have been correlated with the establishment of antigen specific oral tolerance [30, 35, 36]. Although the ingestion of OVA leads to an increase in the frequency of $Foxp3^+$ cells in all populations of IEL from both BALB/c and DO11.10 mice, our results showed that it is in the $TCD8\beta^+$ population of IELs that occurs the highest frequency of cells carrying this suppression marker.

Due to the exposure of mucosal epithelium to a huge amount of strange antigens, the cytokines IFN-γ and interleukin-(IL-)4 are produced spontaneously under physiological conditions by IELs [37]. In our study, we observed that the expression of IL-2, IL-10, IFN-γ, TGF-β, and TNF-α mRNA was smaller in IELS from BALB/c mice fed OVA and then challenged by ip route than in those that received OVA only by ip route; the levels of IL-17 and IL-6 mRNA were also reduced, but not significantly, in IELs from BALB/c mice. Instead, IELS from transgenic mice of the oral + ip group showed levels of IL-6, IFN-γ, TGF-β, and TNF-α mRNA similar to or higher than the animals immunized only by ip route.

The cytokines IL-10 and TGF-β were always related to anti-inflammatory reactions [1, 20]. More recently, however, these cytokines have been associated with inflammatory reactions by activating the process of differentiation of Th17 cells [38, 39]. $TCD4^+$ cells that secrete IL-17, Th17 cells, are pathogenic in autoimmune diseases, and their development and expansion is driven by the cytokines IL-6, TGF-beta, IL-21, IL-1, and IL-23 [39, 40]. Recent studies have revealed a considerable number of IL-17-producing cells amongst the $TCD4^+$ cells in the intestinal mucosa [41]. Although these cells are important to establish a protective immune response against intestinal bacteria, they can also be responsible for inducing inflammatory response and the development of immunological disorders in the presence of IL-6 and/or IL-23 at mucosal sites [41, 42]. Despite the natural occurrence of higher expression of IL-6, TGF-β, and TNF-α mRNA in the DO11.10 IELS than in those of BALB/c mice, there was no significant change in IL-17 mRNA after administration of OVA orally and/or ip in both BALB/c and DO11.10 mice.

Th1-mediated immune responses are considered to be the primary mediators of most autoimmune and chronic inflammatory diseases. Th17, however, has emerged as a key protagonist in a number of inflammatory diseases. It has been shown that $CD8^+$ Treg cells can suppress both Th1 and Th17 responses, being capable of mediating oral tolerance to OVA independently of their $CD4^+$ counterparts in a normal immune system [43]. In the present work, the increase in the frequency of $CD8\alpha\beta^+Foxp3^+$ cells among IELS of BALB/c mice after ingestion of OVA could explain the absence or reduced expression of IL-6, TGF-β, TNF-α, and IFN-γ.

Recent studies have shown that inflammatory bowel diseases (IBD) such as ulcerative colitis and Crohn's disease may be related to the loss of tolerance to self-antigens or normal flora [44]. Our results indicate that the structural disorder observed in epithelium of intestinal villi of transgenic mice would be a consequence of the preferential expression of pro-inflammatory cytokines by their IELs, in the absence of an efficient immunoregulation. This inflammatory state in intestinal environment may contribute to the impairment of oral tolerance to OVA in DO11.10 mice.

5. Conclusion

Taken together, the results of this study indicate that exposure to OVA orally causes IELs of the small intestine of TGN mice assume inflammatory characteristics, whereas in BALB/c antigen intake leads to the development of IELs with characteristics of regulatory cells. Thus, we speculate that the establishment of oral tolerance in transgenic mice is severely impaired by changes in the amounts and arrangements of T cells during the development of intestinal tissues that compromise the cellular interactions involved in the processes of mucosal immunity.

Abbreviations

GALT: Gut-associated lymphoid tissue
IBD: Inflammatory bowel disease
IELs: Intestinal epithelial cells
IFN-γ: Interferon-γ
LP: Lamina propria
MLN: Mesenteric lymph node
OVA: Ovalbumin
PP: Peyer's patch
TGF-β: Transforming growth factor-β
TCR: T-cell receptor
TGN: Transgenic mice
mAb: Monoclonal antibody.

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Research Article

New Insights into the Immunological Changes in IL-10-Deficient Mice during the Course of Spontaneous Inflammation in the Gut Mucosa

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IL-10 is a regulatory cytokine that plays a major role in the homeostasis of the gut and this is illustrated by the fact that IL-$10^{-/-}$ mice develop spontaneous colitis. In this study, IL-$10^{-/-}$ mice were analyzed for immunological changes during colitis development. We found a reduced frequency of regulatory T cells $CD4^{+}CD25^{+}Foxp3^{+}$ and higher frequency of activated T cells in the colon that precedes the macroscopic signs of the disease. Production of IL-17 and IFN-γ was higher in the colon. Colitis progression culminates with the reduction of $CD4^{+}LAP^{+}$ regulatory T cells in the intestine. Frequency of B1 cells and the secretory IgA production were both elevated. Despite these alterations, 16-week-old IL-$10^{-/-}$ mice could be rendered tolerant by a continuous feeding protocol. Our study provides detailed analysis of changes that precede colitis and it also suggests that oral tolerance could be used to design novel alternative therapies for the disease.

1. Introduction

The intestine is the largest surface of contact between the body and the external environment [1]. Most contacts with foreign antigenic materials occur at the gut mucosa. It has been reported that 130–190 g of protein is absorbed in the small intestine daily [2] and the gastrointestinal tract harbours approximately 10^{14} microorganisms of more than 1000 species mostly in the colon [3]. All these antigenic contacts play an important role in the development of the immune system. Mice reared from weaning up to adulthood on a diet containing exclusively amino acids as nitrogen source have a drastic reduction in the gut-associated lymphoid tissue and in IgG/IgA production with an immunological phenotype that resembles suckling mice [4]. Germ-free mice display similar immunological alterations [5]. Under physiological conditions, the constant exposure to these natural antigens through the gut mucosa leads to local and systemic immunological activities, such as secretory immunoglobulin A (sIgA) production and oral tolerance induction [6].

Oral tolerance has classically been defined as the specific suppression of cellular and/or humoral immune responses to an antigen previously given by the oral route [7]. Several mechanisms have been proposed for the development of oral tolerance, ranging from the deletion of antigen-specific T cells to immune deviation and suppression by regulatory T cells [6]. Studies in both mouse models and human

tissues have suggested that inflammatory bowel disease (IBD) is a consequence of the breakdown of normal mucosal tolerance to luminal antigens. Chronic inflammatory bowel diseases are thought to arise from interacting genetic and environmental factors including altered T cell responses to intestinal antigens [8, 9]. Tolerance to autochthonous microbiota seems to be broken in individuals with IBD providing evidence that inflammatory reactivity to ubiquitous antigens from microbiota is implicated in the initiation and/or perpetuation of IBD [10].

Several murine models of colitis have highlighted the important role that abnormalities of the immune system, particularly those affecting T cells, may play in disease pathogenesis. These models include rats carrying the transgenes for HLA B27 and β2-microglobulin, and mice in which the genes coding IL-2, IL-10, and α or β chains of T cell receptors are absent [8]. In the same line, studies using T cell-restored immunodeficient mice have provided evidence that $CD4^+$ T cells play a key role in the induction and regulation of intestinal inflammation. Cell transfer of $CD45RB^{high}$ $CD4^+$ T cells from normal mice donors into C.B-17 severe combined immunodeficient (SCID) mice led to the development of a severe inflammatory response in the colon [11]. The disease can be prevented by cotransfer of the $CD45RB^{low}$ subset and interleukin IL-10 is an essential mediator produced by this regulatory T cell population [12].

IL-10 is produced by regulatory T cells and a variety of other cell types including epithelia, activated macrophages, dendritic cells, and B1 cells. IL-10 is a key immunosuppressive cytokine that acts directly on antigen-presenting cells (APC) to inhibit IL-12 secretion and down regulate the expression of MHC-II as well as costimulatory molecules such as CD80 and CD86 [13]. This modulatory action on APC indirectly inhibits T cell activation. Some studies have also suggested that IL-10 is a potent costimulant of B-cell differentiation and immunoglobulin secretion [14]. The importance of this cytokine in shaping mucosal immune responses has been demonstrated by the spontaneous onset of gut inflammation in the IL-10-deficient ($IL\text{-}10^{-/-}$) mouse [15].

Under conventional conditions, $IL\text{-}10^{-/-}$ mice develop chronic enterocolitis by 2-3 months of age and there is no evidence of disease in neonates. The disease is characterized by weight loss, splenomegaly, and mild-to-moderate anemia. If maintained under specific pathogen-free (SPF) conditions, mice develop a limited form of colitis [16]. The typical inflammatory lesion found is discontinuous and transmural, affecting usually the lower gastrointestinal tract, with being the small intestine much less affected. Other pathological changes include epithelial hyperplasia, mucin depletion, crypt abscesses, ulcers, and thickening of bowel wall. The inflammatory infiltrate consists of lymphocytes, plasma cells, macrophages, eosinophils, and neutrophils [15]. Development of colitis in $IL\text{-}10^{-/-}$ appears to be mediated by $CD4^+$ T cells and an uncontrolled Th1 response [17]. In addition, the overproduction of numerous inflammatory mediators such as IL-1β, IL-6, tumor necrosis factor (TNF)-α, as found in cultures of mice with colitis [18].

In spite of the fact that enterocolitis in $IL\text{-}10^{-/-}$ mice display features atypical of Crohn's disease including marked crypt hyperplasia, the rare occurrence of granulomas, fibrosis, and the absence of fissures and fistulae, this experimental model resembles Crohn's disease in the transmural and discontinuous inflammation. This inflammation can affect not only the colon but also the small intestine [19]. In fact, Crohn's disease in humans may affect any segment of the digestive tract but most frequently involves the small intestine [20].

This study aimed to characterize morphological and immunological alterations in gut mucosa of $IL\text{-}10^{-/-}$ at 6, 10, or 16 weeks of ages, covering a period since the onset of symptoms until established enterocolitis. We analysed the inflammatory features of each stage of the disease as well as putative changes in immunoregulatory mechanisms that may be involved. Since most of genetically deficient animals used as experimental models for IBD are born without colitis signs [21, 22], they constitute an opportunity to study the immunological changes preceding the disease.

2. Material and Methods

2.1. Animals. Wild-type (WT) and IL-10-deficient ($IL\text{-}10^{-/-}$) mice on a 129Sv/Ev background were obtained from Donna-Marie McCafferty's laboratory (Calgary University, Calgary, Canada). All mice were bred and housed in our facility at Universidade Federal de Minas Gerais, Brazil. Mice were kept in microisolators with autoclaved standard chow and water until weaning. After weaning, all mice were maintained in a conventional facility (open cages). Animals were studied at 6, 10, and 16 weeks of age and age-matched wild-type 129Sv/Ev mice were used as controls. All procedures were approved by the local ethical committee for animal research (Protocol no. 170/2008, CETEA-UFMG, Brazil).

2.2. Macroscopic and Microscopic Assessment of Colitis. The colon was excised and colonic inflammation assessed using a previously defined scoringsystem, which includes features of clinical colitis, such as the presence or absence of adhesions, strictures and diarrhea (diarrhea was defined as loose, watery stool), and the bowel wall thickness (measured in mm). Samples of colon were fixed in formalin and processed for microscopic analysis. Hematoxylin-eosin-stained sections were blindly scored based on a semiquantitative scoring system described previously [23] where the following features were graded: extent of destruction of normal mucosal architecture (0: normal; 1, 2, and 3: mild, moderate, and extensive damage, resp.), presence and degree of cellular infiltration (0: normal; 1, 2, and 3: mild, moderate, and transmural infiltration, resp.), extent of muscle thickening (0: normal; 1, 2 and 3: mild, moderate and extensive thickening, resp.), presence or absence of crypt abscesses (0: absent; 1: present) and the presence or absence of goblet cell depletion (0: absent; 1: present). Scores for each feature were summed up to a maximum possible score of 11.

2.3. Intestinal Tissue Preparation and Cytokine Assay. The intestine was separated into duodenum, proximal jejunum, distal jejunum, ileum, and colon and placed in buffer solution (1 mL/0.1 g). Tissue fragments were homogenized and centrifuged for 10 minutes 600 g at 4°C. Supernatants were collected for cytokine assay. Plates were coated with purified monoclonal antibodies reactive with cytokines IL-17A, IL-6, TGF-β, and IFN-γ (BD-Pharmingen) overnight at 4°C. In the, following day, wells were washed, supernatants were added, and plate was incubated overnight at 4°C. In the third day, biotinylated monoclonal antibodies against cytokines were added and plates were incubated for 2 hours at room temperature. Color reaction was developed at room temperature with 100 μL/well of orthophenylenediamine (1 mg/mL), 0.04% H_2O_2 substrate in sodium citrate buffer. Reaction was interrupted by the addition of 20 μL/well of 2 N H_2SO_4. Absorbance was measured at 492 nm by ELISA reader (BIO-RAD).

2.4. Cell Preparation and Flow Cytometry Analysis. Intraepithelial lymphocytes (IELs) were isolated by a modified version of the method described by Davies and Parrott [24]. Briefly, the entire length of small and large intestine were dissected, opened longitudinally, washed with PBS, and cut into small pieces. Tissue fragments were placed in Petri dishes and washed three times in calcium and magnesium-free HBSS containing 2% fetal bovine serum (FBS). After that, tissue fragments were transferred to culture flasks and incubated at 37°C in HBSS containing 1 mM DL-dithiothreitol (DTT-Sigma) for 30 min, twice. Supernatants were filtered through a 70 μm cell strainer and the IEL fraction kept on ice. For *lamina propria* (LP) cell isolation, tissue fragments were incubated with 100 U/mL of collagenase II (Sigma) for 60 min at 37°C in a shaker. Supernatants were passed through a 70 μm cell strainer and then ressuspended in medium. Cells were suspended in 44% Percoll solution, which was layered on top of 67% Percoll solution and centrifuged at 600 g for 20 min at 4°C. IELs were collected from the interface between the Percoll gradients. Cells from IEL and LP compartments were then labelled with FITC-conjugated anti-mouse CD4, CD5 and CD25, PE-conjugated anti-mouse CD25, Thy1.2, CD44, TCR$\gamma\delta$, TCR$\alpha\beta$ and Foxp3, Cy5-conjugated anti-mouse CD69, CD19, and Biotin anti-mouse LAP (BD Pharmingen). Cells from spleen, Peyer's patches, peritoneum, and bone marrow were labelled with FITC-conjugated anti-mouse CD5 and Cy5-conjugated anti-mouse CD19. Cells were analyzed by a FACSCan (Becton & Dickinson) and data were analyzed by FlowJo (TreeStar). At least 30,000.00 events were counted for each sample.

2.5. Analysis of Ig Isotypes by ELISA. Levels of Ovalbumin-(Ova-) specific and total immunoglobulins were determined by ELISA. Briefly, 96-well plates (Nunc) were coated with 2 μg/well Ova or 0.1 μg goat anti-mouse UNLB antibody, in coating buffer pH 9.8 overnight. Wells were washed and blocked with 200 μL of PBS contain 0.25% casein for 1 h at room temperature. Sera were added to the plate and incubated for 1 h at room temperature, plates were washed, then peroxidase-streptavidin goat anti-mouse or rat anti-goat (Southern Biotechnology) 1 : 15000 was added, and plates were incubated for 1 h at 37°C. Color reaction was developed at room temperature with 100 μL/well of orthophenylenediamine (1 mg/mL) (Sigma), 0.04% H_2O_2 substrate in sodium citrate buffer. Reaction was interrupted by the addition of 20 μL/well of 2 N H_2SO_4. Absorbance was measured at 492 nm by an ELISA microplate reader (Bio-Rad).

2.6. Oral Tolerance Induction. Oral tolerance to Ova was induced by intragastric administration (gavage) of a single dose of 20 mg Ova (Sigma) in 0.2 mL saline (0.15 M NaCl), 7 days before primary immunization. The control group received 0.2 mL of saline. Alternatively, mice received 4 mg/mL solution of Ova in water as their exclusive drinking fluid. The average voluntary intake of a mouse was about 5 mL in 24 h; therefore, the animals were presumed to ingest 20 mg Ova/day. Control groups received filtered tap water. Oral treatment was discontinued 7 days before parenteral immunization. Mice were actively sensitized by an intraperitoneal injection of 0.2 mL of saline containing 10 μg of Ova adsorbed in 1 mg of aluminum hydroxide. Fourteen days later, the animals received the same dose of Ova in PBS.

2.7. Statistical Analysis. Results were expressed as the mean ± standard error of the mean (SEM). The Kolmogorov-Smirnov test, confirmed normal distribution of samples. Significance of differences among groups was determined by Student's *t*-test or analysis of variance (ANOVA) (Tukey's posttest). Means were considered statistically different when $P < 0.05$.

3. Results

3.1. Assessment of Colitis during the Course of Intestinal Inflammation. Macroscopic and histology scores of colonic mucosa were observed in 6-, 10-, or 16-week-old IL-$10^{-/-}$ mice and age-matched wild-type mice. IL-$10^{-/-}$ mice displayed normal colonic histological appearance at 6 weeks of age without macroscopic signs of disease (Figures 1(b), 2(a), and 2(b)). By 10 weeks of age, IL-$10^{-/-}$ mice developed a mild colitis (Figure 1(c)), the severity of which reached a plateau at 16 weeks of age (Figure 1(d)). The macroscopic score did not increase after disease was established since 10- or 16-week-old IL-$10^{-/-}$ mice showed similar scores (Figure 2(a)).

3.2. Morphology of Small Intestine and the IEL Profile in IL-10-Deficient Mice. Regarding the impact of inflammatory changes in small intestine, a previous study has described that IL-$10^{-/-}$ mice can develop enteritis in conventional conditions [15]. Histological analysis of the small intestine revealed signals of inflammation only in the proximal jejunum of 16-week-old IL-$10^{-/-}$ mice (Figure 1(h)). They had altered villus/crypt ratio (Figure 2(c)) due to villus shortening. Noteworthy, 6-weeks-old mice presented villus/crypt ratio similar to older mice (Figure 2(c)) but this

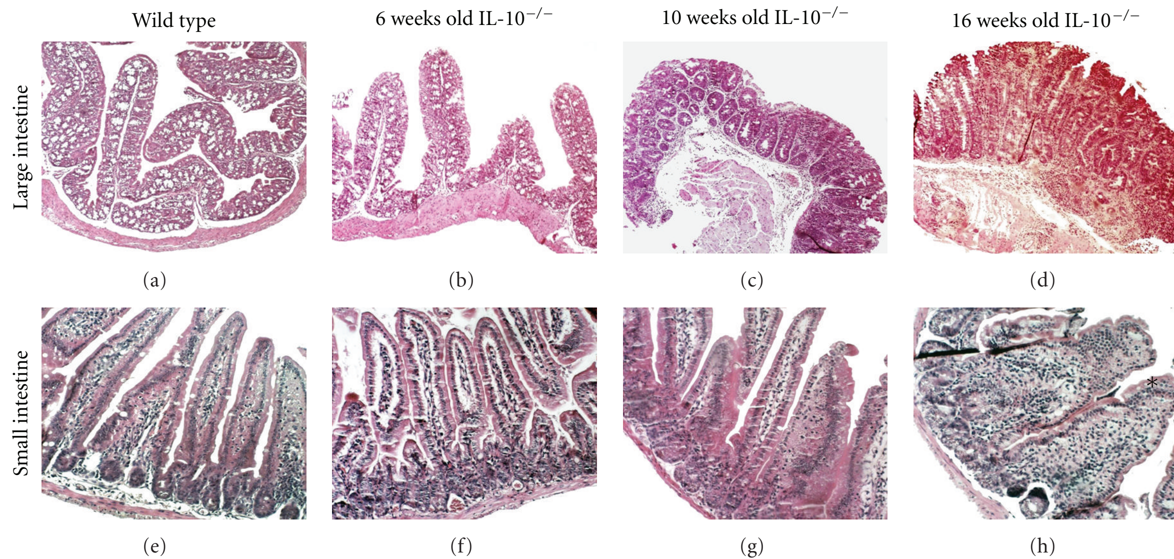

Figure 1: Histopathology of intestinal changes in IL-$10^{-/-}$ mice. (a) Histology of proximal colon representative of wild-type (129Sv/Ev) mice (40x). No cellular infiltration between glands was found. (b) Proximal colon of 6-week-old IL-$10^{-/-}$ mice (40x). Mucosa and submucosa are similar to the ones in wild-type mice. (c) Proximal colon of 10-week-old IL-$10^{-/-}$ mice (40x). Multifocal infiltrations of leucocytes in the mucosa and submucosa. (d) Proximal colon of 16-week-old IL-$10^{-/-}$ mice (40x). Presence of transmural inflammation affecting the muscular layer can be observed. (e) Proximal jejunum representative of wild-type mice (100x). (f) Proximal jejunum of 6-week-old IL-$10^{-/-}$ mice (100x). (g) Proximal jejunum of 10-week-old IL-$10^{-/-}$ mice (100x). (h) Proximal jejunum of 16-week-old IL-$10^{-/-}$ mice showing enteritis and cellular infiltration in the *lamina propria* (asterisks) (100x). Tissues were stained with hematoxylin and eosin.

was due to higher crypt size. Intraephithelial lymphocytes (IELs) are involved in maintenance of intestinal epithelial cell integrity [25]. Frequencies of TCR$\alpha\beta$ and TCR$\gamma\delta$ IEL populations were analyzed in 16-week-old IL-$10^{-/-}$ mice. We used Thy1.2 as a marker to discriminate activated IELs. Despite the similar frequencies of TCR$\alpha\beta$ and TCR$\gamma\delta$ IELs, there was a decrease in the percentage of Thy1.2^+TCR$\gamma\delta^+$ in the IEL population in IL-$10^{-/-}$ mice, whereas no change was found in the frequency of Thy1.2^+TCR$\alpha\beta^+$ IELs (Figure 2(d)).

3.3. IL-10-Deficient Mice Had a Reduced Frequency of Regulatory T Cells in the Small Intestine. In agreement with other studies, we found an increase in the frequency of CD4^+CD44^+ in the colon of IL-10-deficient mice at 6 and 16 weeks of age but a nonaltered frequency in the small intestine (Figure 3(a)). There was no difference in the frequency of T cells expressing the early activation marker CD69^+ in the small and large intestine segments of either 6- or 16-week-old IL-$10^{-/-}$ mice when compared to their wild-type counterparts. Of note, frequency of activated CD69^+ T lymphocyte population increased with age in both wild-type and IL-$10^{-/-}$ mice but only in the large intestine (Figure 3(b)). CD4^+CD25^+Foxp3^+ T cells were reduced in the *lamina propria* of large intestine of IL-$10^{-/-}$ mice regardless of their age (Figure 3(c)). The CD4^+CD25^+LAP$^+$ population of lymphocytes was reduced in the small and large intestines of IL-$10^{-/-}$ at 16 weeks of age. However, the frequency of CD4^+CD25^+LAP$^+$ T cells was enhanced with age in wild-type mice in both small and large intestines (Figure 3(d)).

3.4. Changes in Cytokine Secretion in Small and Large Intestines of IL-10-Deficient Mice. The cytokine profile in IL-$10^{-/-}$ mice was well characterized in colonic extract [18]. However, no information is available about the effect of the lack of IL-10 in the small intestine. IL-17A production enhanced in the colon of 6-week-old IL-10-deficient mice and diminished in the duodenum of 10-week-old IL-$10^{-/-}$ mice (Figure 4(a)). We found alterations in IL-6 in the distal jejunum of IL-$10^{-/-}$ mice with 10 and 16 weeks of age. Levels of IL-6 raised in the colon of 16-week-old IL-$10^{-/-}$ mice (Figure 4(b)). On other hand, production of the anti-inflammatory cytokine TGF-β in IL-$10^{-/-}$ mice increased in the colon at 10 weeks of age and in proximal jejunum at 16 weeks of age (Figure 4(c)). 16-week-old IL-$10^{-/-}$ mice had increased levels of IFN-γ in the colon (Figure 4(d)).

3.5. Frequency of B1 Lymphocytes Increased in Peritoneum and Intestinal lamina propria of IL-10-Deficient Mice with Colitis. Since IL-10 is a potent costimulant of B cell differentiation, immunoglobulin secretion and generation of B1 cells [14], our next step was to investigate the impact of IL-10 deficiency in B lymphocyte populations and immunoglobulin production. There was no significant change in the frequency of B lymphocytes in spleen (SP), peritoneum (PT), Peyer's patches (PP), and gut *lamina propria* (LP) but frequency of these cells was diminished in bone marrow (BM) (Figure 5(a)). Interestingly, frequency of B1 lymphocytes in peritoneum, Peyer's patches, and *lamina propria* of both small and large intestine was augmented in IL-$10^{-/-}$ mice at 16 weeks of age (Figure 5(b)).

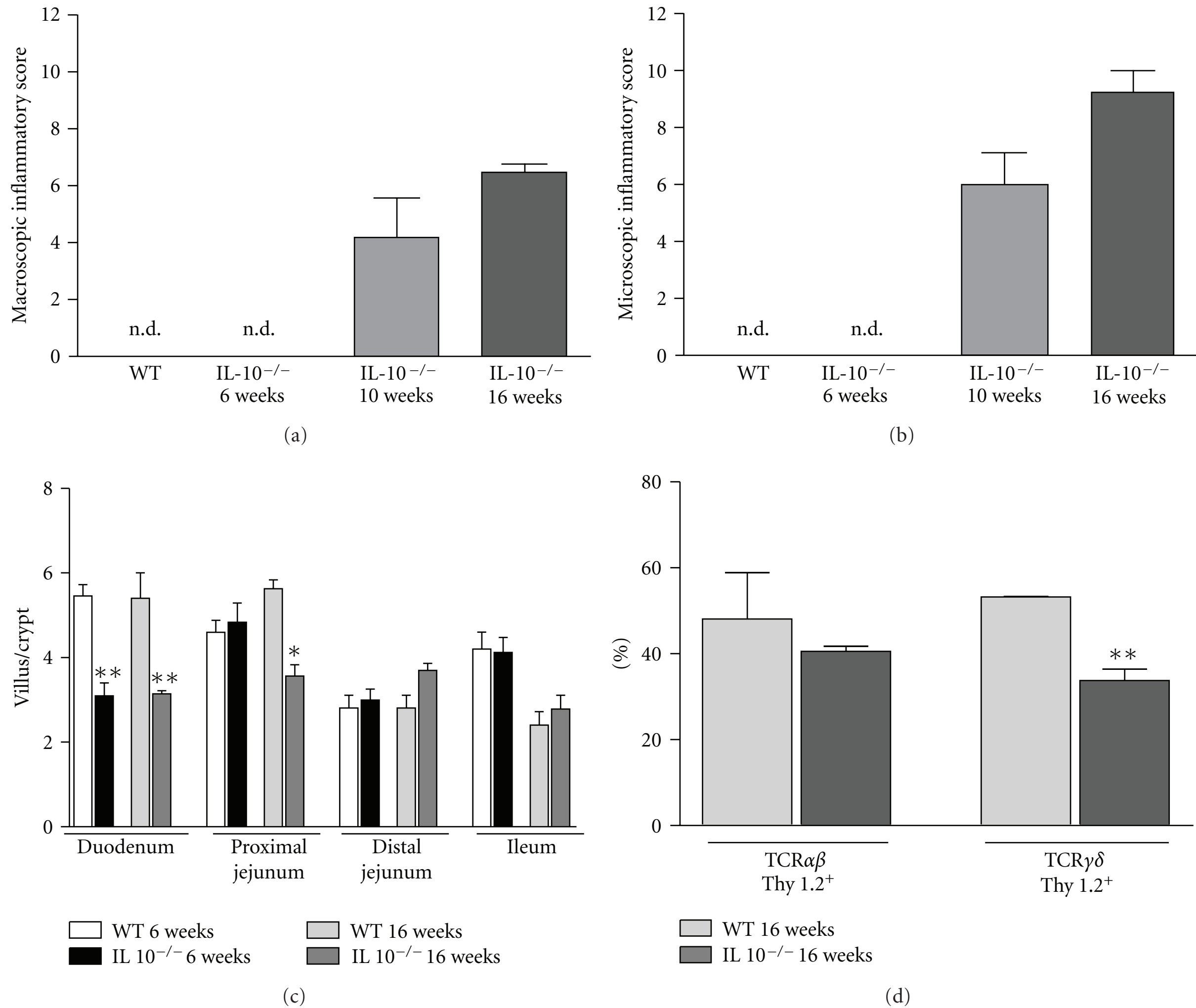

Figure 2: Inflammatory score of IL-$10^{-/-}$ and IEL profile. (a) Macroscopic score of IL-$10^{-/-}$ mice at 6, 10, or 16 weeks of age and control age-matched wild-type mice. (b) Microscopic score of IL-$10^{-/-}$ mice at 6, 10, or 16 weeks of age and control age-matched wild-type mice obtained by histological analysis of the colonic tissues. (c) Villus/crypt ratio of duodenum, proximal jejunum, distal jejunum, and ileum of IL-$10^{-/-}$ mice at 6, or 16 weeks of age. Bars represent the mean ± SEM of 5 mice per group. Asterisks represent differences from age matched WT groups in the same intestinal segment ($^*P < 0.05$ or $^{**}P < 0.01$). (d) Flow cytometry analysis with frequency of IEL isolated from 16-week-old IL-$10^{-/-}$ and control mice stained with fluorescent antibodies to $\alpha\beta$TCR, $\gamma\delta$TCR, and Thy1.2. Cells were analyzed inside the total lymphocytes gate. Bars represent the mean ± SEM of 3 mice per group. Significant difference ($^{**}P < 0.01$) is indicated by the asterisk. n.d means non detected.

3.6. Serum Levels of Immunoglobulins Were Altered during the Course of Disease in IL-10-Deficient Mice. We investigated whether production of the different classes of immunoglobulin was altered in IL-10-deficient mice since weaning and during the course of gut inflammation. There was no difference in the serum levels of IgG, IgM, and IgA in IL-10-deficient mice at 4 weeks of age (Figures 5(c)–5(e)). However, at 6 weeks of age, IL-10-deficient mice had enhanced IgM levels and reduced levels of IgG when compared to levels found in mice at 4 weeks of age. IgA levels were unchanged at this age (Figures 5(c)–5(e)). At 10 weeks of age, IL-$10^{-/-}$ mice had higher concentration of serum IgG. Serum IgA enhanced in IL-$10^{-/-}$ mice from 4 to 10 weeks of age and rose again in 16 week-old mice (Figure 5(e)). Levels of IgE were similar in all ages analysed (Figure 5(f)).

3.7. IL-10-Deficient Mice with Established Gut Inflammation Can Be Rendered Tolerant Only by Continuous Feeding. Finally, we tested the impact of IL-10 deficiency and colitis development in the two major immunological activities that take place at the gut mucosa: secretory IgA (sIgA) production and oral tolerance induction. Concentration of sIgA was measured in the feces. Similarly to what has already been described in the literature, IL-$10^{-/-}$ mice had enhanced levels of sIgA at the time of established inflammation (10 weeks of age) but no difference was found in mice before the onset of colitis at 4 or 6 weeks of age. Interestingly, mice at 16 weeks of age with overt inflammation did not show alterations in sIgA again (Figure 6(a)). To test for oral tolerance induction, mice were either fed 20 mg Ova by intragastric administration (gavage) or given an Ova solution as

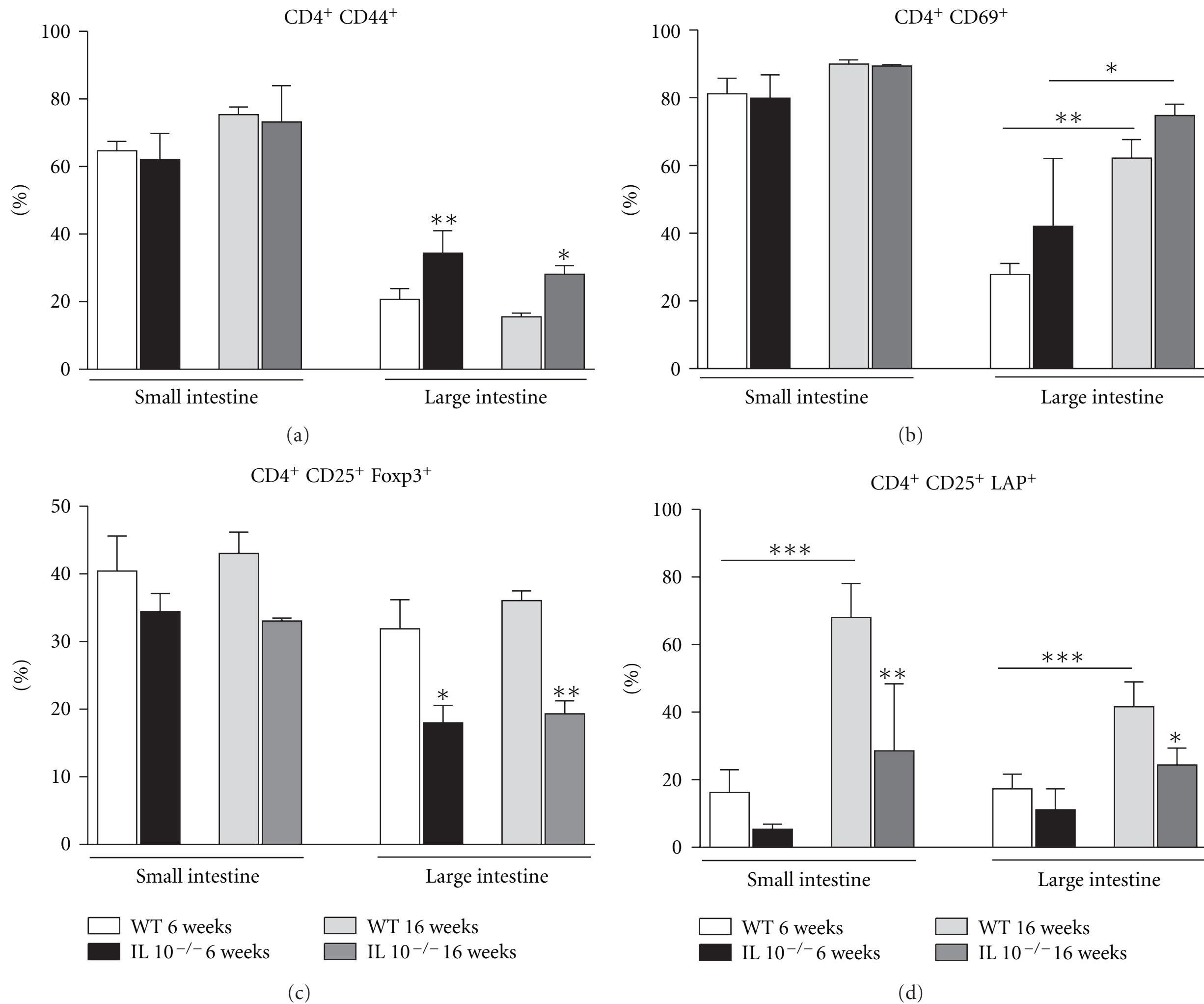

Figure 3: T lymphocyte profile in the *lamina propria* of small and large intestine of IL-$10^{-/-}$ mice during the course of intestinal inflammation. *Lamina propria* T cells from small and large intestine were obtained from either 6- or 16-week-old IL-$10^{-/-}$ mice. Age-matched 129Sv/Ev (WT) mice were used as controls. Frequencies of $CD4^{+}CD44^{+}$ (a), $CD4^{+}CD69^{+}$ (b), $CD4^{+}CD25^{+}Foxp3^{+}$ (c), and $CD4^{+}CD25^{+}LAP^{+}$ (d) T cells gated in $CD4^{+}$ T cells were assessed by flow cytometry. Bars represent the mean ± SEM of 4 mice per group. Significant differences ($^{*}P < 0.05$, $^{**}P < 0.01$ or $^{***}P < 0.001$) are indicated by asterisks.

the only liquid source for one day (continuous feeding). They were intraperitoneally immunized with the same antigen in adjuvant 7 days thereafter. Levels of anti-Ova IgE were reduced in both wild-type (129Sv/Ev) and in IL-$10^{-/-}$ mice that received Ova by either continuous feeding or gavage before immunization when compared to the immunized group (Figure 6(b)). However, only IL-10-deficient mice that were continuously fed Ova could be rendered tolerant for anti-Ova IgG1 production (Figure 6(c)).

4. Discussion

Mouse models of intestinal inflammation have played a key role in understanding the mechanisms that govern the inflammatory response in the intestine, and in designing new therapeutic strategies for human Crohn's disease and ulcerative colitis. Experimental models of IBD usually involve defects in epithelial integrity/permeability or in regulatory elements of the immune system [8]. Several pathological and immunological changes are significantly different between these models. For example, the chemical model induced by dextran sulfate sodium (DSS) display inflammatory features similar to ulcerative colitis, while in IL-$10^{-/-}$ the inflammation resembles Crohn's disease featuring transmural inflammation in colonic mucosa. Indeed, we found that in the IL-$10^{-/-}$ mice there was an overproduction of IFN-γ and IL-17 (Figure 4), whereas in DSS-induced model of colitis occurred a prevalence of IL-6, TNF-α, and INF-γ without alterations in IL-17 production (data not shown). Of note, we observed reduced frequencies of regulatory T cells in mice with DSS-induced colitis (data not shown) similarly to what was found in IL-$10^{-/-}$ mice (Figure 3). However, in DSS-induced colitis, the earliest histological change that predated clinical colitis was the loss of crypt epithelial cells. Inflammation became significant only after the appearance of erosions.

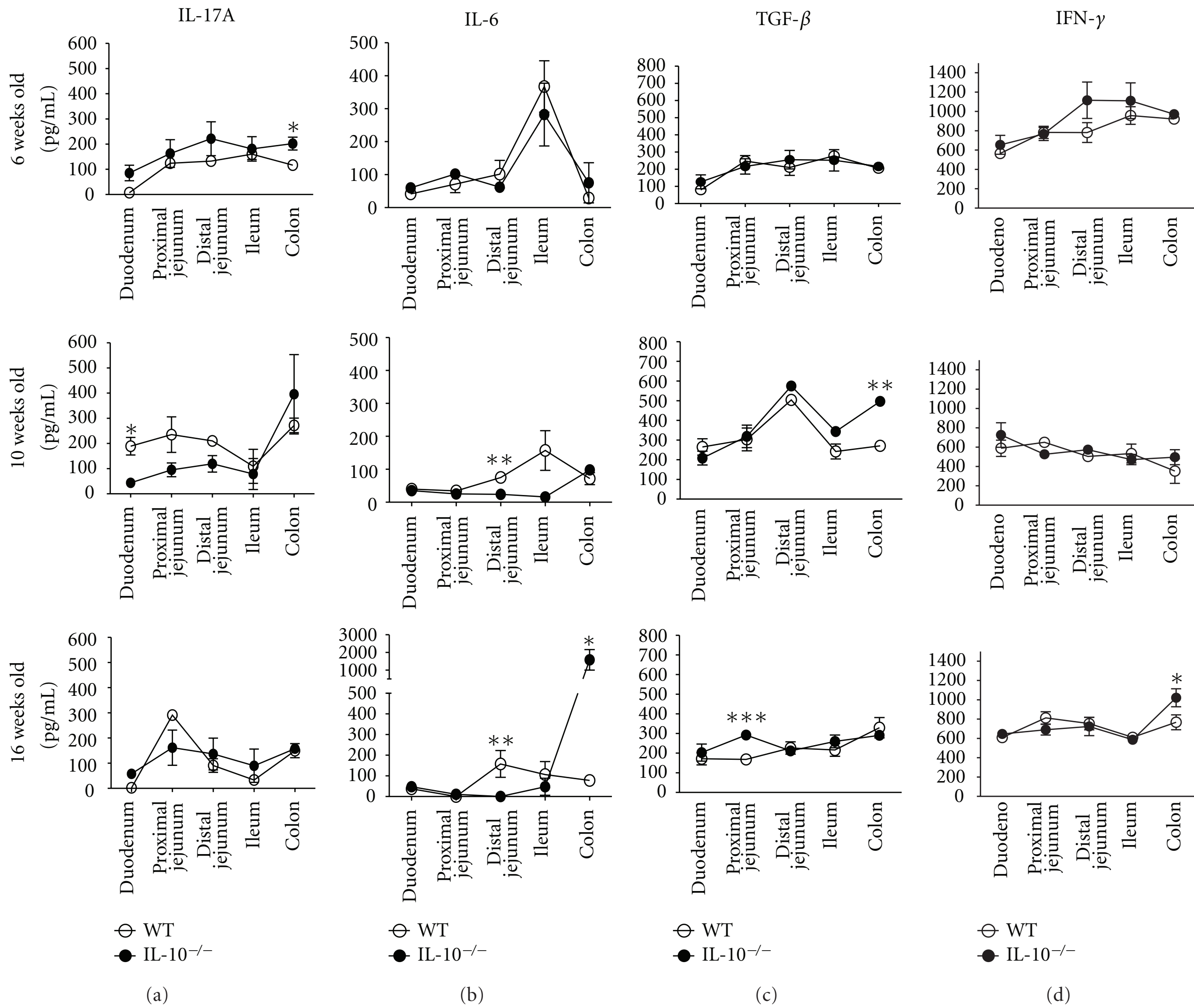

Figure 4: Production of cytokines in the intestinal mucosa during the course of intestinal inflammation. Intestines from IL-$10^{-/-}$ at 6, 10 or 16 weeks of age were removed, separated into duodenum, proximal jejunum, distal jejunum, ileum and homogenized in extract buffer. Age-matched 129Sv/Ev (WT) mice were used as controls. Extract supernatant was collected for cytokine assay. IL-17A (a), IL-6 (b), TGF-β (c), and IFN-γ (d) were measured by ELISA. $n = 5$ mice per group. Asterisks represent differences among groups in the same intestinal segment ($^*P < 0.05$, $^{**}P < 0.01$ or $^{***}P < 0.001$).

On the other hand, in IL-10-deficient mice, inflammation precedes the establishment of clinically detected disease and occurred spontaneously. Therefore, the model of IL-$10^{-/-}$ mouse provides an opportunity to study pathophysiology and immunological changes that precede and accompany the development of the disease.

In this study we used IL-10-deficient mice in the 129Sv/Ev genetic background. Although macroscopic disease can be detected at 3 months of age in IL-10-deficient mice, severity of intestinal lesions vary with the genetic background of the mice. They are most severe in 129Sv/Ev and BALB/c strains, of intermediate severity in 129 x C57BL/6J hybrid mice, and least severe in the C57BL/6J strain. Thus, the 129Sv/Ev strain is considered the one with highest susceptibility to colitis development with homogenous clinical manifestations of the disease among susceptible animals [19]. Since in SPF conditions these mice exhibited only small lesions in the mucosa of proximal colon [16], we chose to undertake our study with mice maintained under conventional conditions, which lead to the full development of enterocolitis.

At 6 weeks of age, just after weaning, no histological sign of inflammation was observed in the large intestine of these mice. On the other hand, IL-10-deficient mice at 10 weeks of age showed already established enterocolitis with infiltration of leucocytes in the gut mucosa and submucosa areas. Severity of the disease progressed as they aged, and 16-week-old mice showed a transmural inflammation in the colon. Parameter such as onset and severity of enterocolitis in IL-$10^{-/-}$ mice varies according to the animal facility that mice are kept. Rederivation of IL-10-deficient mice from conventional SPF environments into germ-free isolators eliminates colitis development, clearly demonstrating the influence of microbiota in the establishment of disease [16].

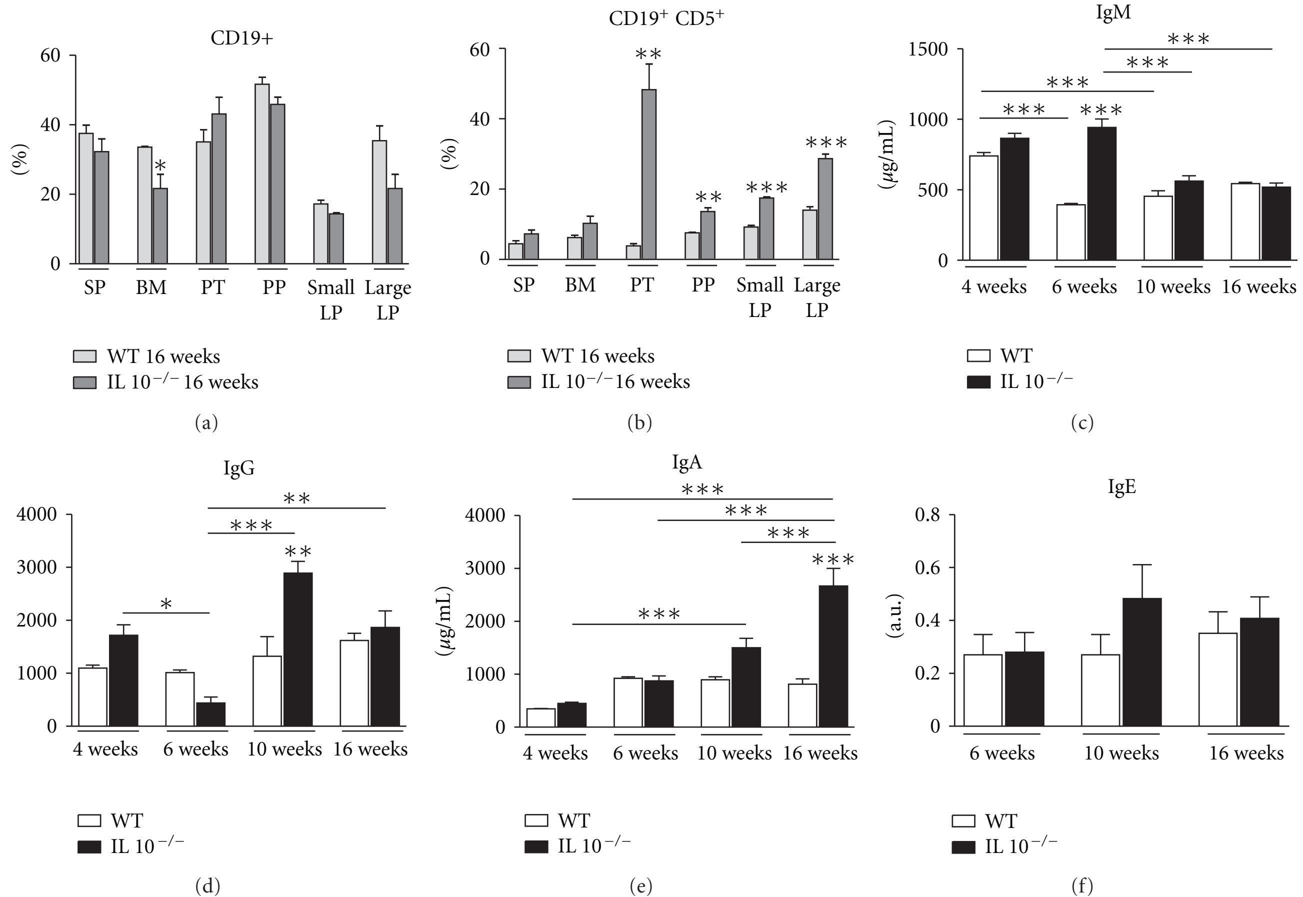

Figure 5: B cells and immunoglobulins isotype during the development of enterocolitis. Cells from spleen (SP), bone marrow (BM), Peritoneum (PT), Peyer patches (PP), or *lamina propria* (LP) from small or large intestine were obtained from 16-week-old IL-$10^{-/-}$ mice. Frequencies of $CD19^+$ B cells (a) or $CD19^+CD5^+$ (b) gated on lymphocytes or $CD19^+$ population, respectively, were assessed by flow cytometry. Bars represent the mean ± SEM of 4 mice per group. Asterisks represent difference between groups ($P < 0.05$). Sera from 4-, 6-, 10-, or 16-week-old IL-$10^{-/-}$ mice were collected and total IgM (c), IgG (d), IgA (e), and IgE (f) were measured by ELISA. A.U.: arbitrary units. Bars represent the mean ± SEM of 5 mice per group. Significant differences ($^*P < 0.05$, $^{**}P < 0.01$ or $^{***}P < 0.001$) are indicated by asterisks.

In addition, IL-10-deficient C3H mice from the same parenteral breeding stocks but maintained in two different facilities had significant differences in histopathological scores at the same age. These differences were attributed to the source of diet and its ingredients and to the water treatment (autoclaved or not) because health monitoring of the two colonies indicates the same SPF status [26]. Berg and coworkers reported small multifocal infiltrates, in *lamina propria* of colon in 3-week-old IL-$10^{-/-}$ mice whereas 12-week-old IL-$10^{-/-}$ mice had multifocal lesions and epithelial hyperplasia in all regions of large intestine [19]. Thus, in mice housed in our animal facility, the onset of enterocolitis was delayed but the progression was faster.

In this study, we evaluated not only inflammatory changes in the large intestine but also in the small intestine of IL-10-deficient mice. Although there was no visible inflammation in the small intestines at 6 weeks of age, the villus/crypt ratio was reduced and this alteration was observed until adulthood (16 weeks of age). Altered villus/crypt morphology has been reported in a number of immune-mediated bowel disorders, including celiac disease, Crohn's disease, and ulcerative colitis. This type of change is due to accelerated epithelial turnover and apoptosis triggered by cytokines released from infiltrating inflammatory cells and from enterocytes themselves [27]. This could contribute to the inflammatory enteritis that we found in 16-week-old IL-$10^{-/-}$ mice.

Small intestinal mucosa harbours a particular population of lymphocytes named intraepithelial lymphocytes (IELs). IELs are in the first line of mucosal surface intertwined with epithelial cells, and the main subpopulations of IELs, $TCR\alpha\beta^+$ and $TCR\gamma\delta^+$, are both described as involved in inhibition of cytotoxic T cells (CTL). The intraepithelial lymphocyte compartment probably provides a first line of defense against infectious pathogens attacking the surfaces of the body and also provides a link between innate and acquired immunity [25, 28]. $TCR\gamma\delta^+$ IELs are also related to immunoregulatory roles such as maintenance of epithelial tissue integrity [25], oral tolerance induction [29], and colitis modulation [30]. In acute colitis induced by

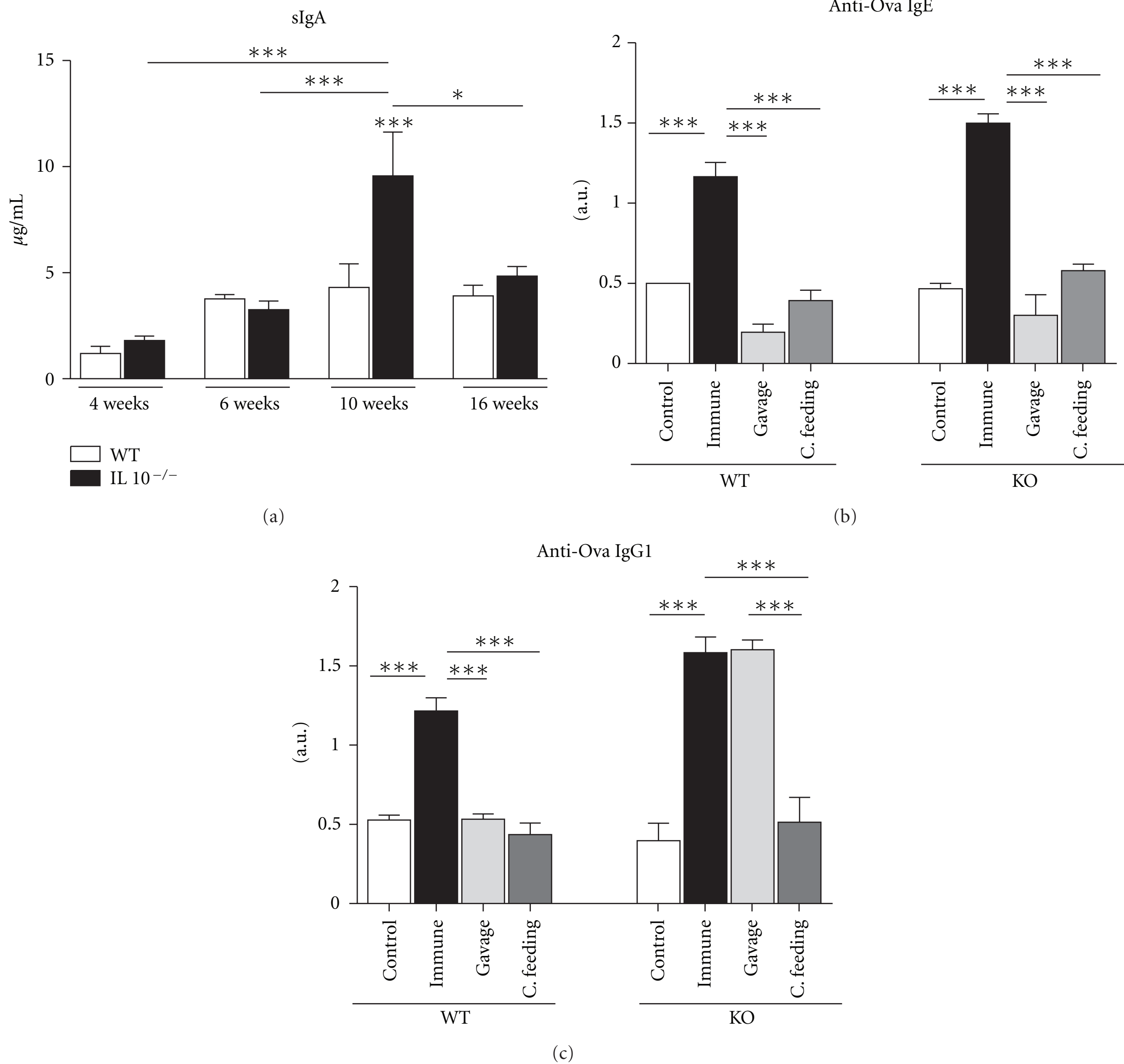

Figure 6: Secretory IgA and oral tolerance. Intestinal feces from 4-, 6-, 10-, or 16-week-old IL-$10^{-/-}$ mice were collected and total sIgA (a) was measured by ELISA. 16-week-old IL-$10^{-/-}$ or wild-type mice received Ova either by gavage or by continuous feeding. Seven days later mice were sensitized by an intraperitoneal (i.p.) injection of 0.2 mL saline (0.9% NaCl) solution containing 10 μg OVA (Sigma, St. Louis, MO) adsorbed in 1 mg aluminum hydroxide. Fourteen days later, animals received the same dose of OVA in PBS. Control animals received 0.2 mL sterile saline. Anti-Ova IgE and IgG1 antibodies were measured by ELISA. A.U.: arbitrary units. Bars represent the mean ± SEM of 5 mice per group. Asterisks represent difference between groups ($^{*}P < 0.05$ or $^{***}P < 0.001$).

administration of either 2,4,6-trinitrobenzene sulfonic acid (TNBS) or DSS, a protective role of $\gamma\delta$ T cells has been demonstrated [30]. Our group has shown recently that the frequency of $\gamma\delta^{+}$ IELs is diminished in aged mice and that this aging-associated change parallels a reduction in susceptibility to oral tolerance induction [31]. In this study, we observed that the frequency of TCR$\alpha\beta^{+}$ and TCR$\gamma\delta^{+}$ T lymphocytes in the IEL compartment did not change (data not shown), but there was a reduction in the frequency of activated TCR$\gamma\delta^{+}$ Thy1.2^{+} cells in IL-10-deficient mice with established inflammation. Since TCR$\gamma\delta^{+}$ IELs are involved in regulatory activities in the gut mucosa, this reduction can represent an instance of immunoregulatory failure associated with disease development in IL-10-deficient mice.

Classical studies reported that the enterocolitis in IL-10-deficient mice is associated with uncontrolled cytokine production by activated macrophages and CD4^{+} Th1-like T cells [19]. The excessive generation of IFN-γ-producing T cells (Th1) driven by IL-12 produced by antigen-presenting cells was described to be responsible for the initiation of disease [32]. However, later studies revealed that IL-23, but not IL-12, drives the intestinal inflammation in IL-$10^{-/-}$ mice. A critical target of IL-23 is memory T cells, which produce the proinflammatory mediators IL-17 and IL-6 [33].

We showed that levels of IFN-γ were elevated in the colon of 16-week-old but not of young IL-$10^{-/-}$ mice. Moreover, 6-week-old IL-$10^{-/-}$ mice had increased levels of IL-17A in their colonic mucosa. These results are consistent with the findings that IL-17A is the cytokine that initiate the intestinal inflammation in IL-$10^{-/-}$ mice followed by an enhanced production of IFN-γ. Th17 cells can convert to Th1 cells through IL-17 induction of IL-12 and IL-23 production by dendritic cells at colonic mucosa [34]. Recently, Mikami and coworkers showed that cotransfer of the mixed Th1/Th17 $CD4^+$ T cells from IL-$10^{-/-}$ with colitis with Th1 $CD4^+$ T cells from $CD4^+CD45^{high}$-induced $RAG^{-/-}$ mice with colitis into $RAG^{-/-}$ mice ameliorate wasting disease. Thus, it seems that Th17 cells compete with Th1 cells, and that predominant secretion of IFN-γ is related to more severe colitis [35]. Moreover, macrophages are usually activated by IFN-γ to produce IL-6 and this could explain the concomitant increase in IFN-γ and IL-6 levels in the colon of 16-week-old IL-$10^{-/-}$ mice [36]. In light of these results, TGF-β production in the colon of 10-week-old IL-$10^{-/-}$ mice may represent an attempt to regulate inflammation by the direct regulatory action of TGF-β. Indeed, production of IL-6 in the colon of 10-week-old IL-$10^{-/-}$ was not high. It is also remarkable that the shift from IL-17A into IFN-γ production in the colonic mucosa of IL-$10^{-/-}$ mice coincided with more severe colitis, spreading of inflammation to the small intestine (Figure 1) and deterioration in the clinical status of the animals.

Cytokine profile was also evaluated in all segments of the small intestine. We found enhanced levels of IL-17A in the duodenum of 10-week-old IL-$10^{-/-}$ mice. On the other hand, there were higher levels of TGF-β in the colon at the same time point. At 16 weeks of age, TGF-β was also produced in the proximal jejunum. Interestingly, the enhanced IL-17A production observed at 10 weeks of age disappeared at 16 weeks of age (Figure 4(b)). The detected upregulation of TGF-β in 10-week-old mice could be an immunoregulatory process triggered in the small intestine to control inflammation. However, it was not a successful modulatory event since histological analysis of the small intestine at that stage showed that inflammation was pronounced in the colon and also in the small intestine. This suggests that although TGF-β can be an important compensatory mechanism in the immune homeostasis of IL-10-deficient mice, these animals show no other inflammatory disorder. Nevertheless, in the gut mucosa, IL-10 seems to be critical and overproduction of TGF-β was not enough to control intestinal inflammation.

Despite the absent intestinal inflammation in young mice, we found enhanced frequency of $CD4^+$ T cells expressing a memory phenotype ($CD44^{high}$) in the large intestinal mucosa before the establishment of colitis (6 weeks of age). Berg and coworkers showed that increased numbers of T-cells were present in IL-10-deficient mice already at 3 weeks of age [19]. In a study using the Gαi2-deficient mouse model of ulcerative colitis, increased numbers of mucosal lymphocytes expressing the $CD44^{high}$ marker were isolated from mice before the onset of colitis [21]. Presumably, the T cell influx into the colonic *lamina propria* is an important factor in the subsequent establishment of colitis. In IL-10-deficient mice with severe disease, the high frequency of $CD44^{high}$ was maintained. We had distinct results for T lymphocytes expressing the early activation marker $CD69^+$. There was an enhanced frequency of $CD69^+$ in both 129Sv/Ev wild-type and IL-$10^{-/-}$ mice from 6 weeks to 16 weeks of age. This indicates that an age-related increase in activated $CD4^+$ lymphocytes that is consistent with other studies [31] and that can be explained by the continuous exposure to antigens under conventional housing. This seemed to be associated with microbiota stimulation since there is no equivalent change in the frequency of activated $CD4^+$ T cells in the small intestine where bacterial colonization is much less intense.

Induced $CD4^+CD25^+Foxp3^+$ T cells have already been shown as important players in the homeostasis of gut mucosa [37]. A reduced frequency of $CD4^+CD25^+Foxp3^+$ regulatory T cells was found in the large intestine of mice as young as 6 weeks old. This could be directly related to the IL-10 deficiency. Moreover, IL-10 secreted by $CD11b^+CD11c^+$ dendritic cells is a fundamental cytokine for the maintenance of Foxp3 expression in induced regulatory T cells in the periphery during colitis development [38].

Our next step was to analyze the frequency of $CD4^+LAP^+$ T cells during colitis development in these mice. $CD4^+LAP^+$ T cells represent a subset of regulatory T cells expressing TGF-β bound to their membranes in its precursor form (associated with the latent associated peptide, LAP). $CD4^+CD25^+LAP^+$ T cells have been shown to participate in the control of intestinal inflammation in experimental models of colitis [39] and $CD4^+LAP^+$ T cells, either expressing CD25 or not, have recently been reported as a distinct subset of T cells [40]. Frequency of $CD4^+CD25^+LAP^+$ was not affected in young IL-10-deficient mice. However $CD4^+CD25^+LAP^+$ T cells were decreased in IL-10-deficient mice at 16 weeks of age both in the colon and in the small intestine. The decrease in frequency of $CD4^+CD25^+LAP^+$ T cells was observed in the small intestine of 16-week-old IL-$10^{-/-}$ in the same time point of TGF-β augment in proximal jejunum. Thus, it is likely that other cell types, including macrophages and the gut epithelial cells, were responsible for the production of TGF-β and not $CD4^+LAP^+$ T cells. We also found enhanced frequencies of $CD4^+LAP^+$ T cells in wild-type mice at 16 weeks of age when compared to the frequencies found in 6-week-old mice. Our group described similar data in a recent study on age-related alterations in the gut mucosa. An increase in $CD4^+CD25^+LAP^+$ cells was observed in mesenteric lymph nodes and Peyer's patches of 6- to 24-month-old mice when compared to young animals (2-month-old), suggesting that these T cell subsets augment after sexual maturity but remain stable afterwards [31].

Not only the cell-mediated but the humoral components of the immune system have been implicated in the pathogenesis of human and mouse models of IBD. Anticolon antibodies have been detected in IL-$2^{-/-}$ mice [41]. Antibody reactive with enteric microbiota such as *Campylobacter jejuni*, have been identified in human patients, particularly those with ulcerative colitis [42]. In this study, we find enhanced levels of serum IgG and IgA in IL-10-deficient mice with established and severe colitis, respectively. Our

findings are in agreement with other studies on IL-10-deficient mice. Kuhn and colleagues were the first authors to demonstrate elevated levels of serum IgG1 and IgA in 8-week-old IL-$10^{-/-}$ mice [15]. Davidson and coworkers also showed the cross-reactivity of IL-$10^{-/-}$ mice serum Ig with colon epithelial and nonepithelial cells in the majority of sera tested. However, the same authors created the B cell-deficient ($B^{-/-}$) strain of lL-$10^{-/-}$ mice and this mice acquired a severe colitis analogous to that of lL-$10^{-/-}$ mice, implying that B cells were not the primary mediator of IBD in this model [17]. Therefore, we cannot say whether that these alterations were a compensatory response to the inflammation or that they were simply a phenomenon related to inflammation of the colon. IL-10-deficient mice at the age of 4–6 weeks had a similar percentage of B cells in thymus and spleen and normal B1 cell subset in the peritoneum [15]. B1 cells were originally identified as $CD5^+$ B cells [43]. Surprisingly, frequency of $CD19^+CD5^+$ cells was increased in the *lamina propria* of small and large intestine as well as in peritoneum and Peyer's patches of 16-week-old IL-$10^{-/-}$ mice. B1 cells from the peritoneum produce predominantly IgM, but class switch to IgA has been shown to occur in the B cell population [44] and *lamina propria* B1 cells are responsible for at least half of the secretory IgA in the gut mucosa [45]. Serum IgA levels enhanced concomitantly with inflammation in IL-$10^{-/-}$ mice. Secretory IgA levels were also higher at 10-week-old mice. Thus, it is reasonable to speculate that B1 cells are the main source of this increment of IgA.

Oral tolerance is a major and common consequence of oral administration of antigen [6]. Although several mechanisms have been proposed to explain the induction, production of anti-inflammatory cytokines such as TGF-β and IL-10 and induction of regulatory T cells such as $CD4^+CD25^+Foxp3^+$ and $CD4^+LAP^+$ T cells seem to be critical for oral tolerance induction [46]. There were previous and controversial reports on the role of IL-10 in this process. Rizzo and coworkers showed that mice genetically engineered to lack IL-4, IL-10 or both cytokines were refractory to oral tolerance [47]. Later, Aroeira and coworkers showed that in vivo depletion of IL-10 with monoclonal antibodies did not affect oral tolerance induction in mice [48]. In the present study, we observed that oral tolerance to IgE could be induced in 16-week-old IL-10-deficient mice by the two regimens of feeding used, gavage, and continuous feeding. However, only continuous feeding of the antigen, but not gavage, could lead to the suppression of anti-Ova IgG1.

Our group has previously described that continuous administration of the antigen is an optimal protocol for oral tolerance induction when compared to antigen administration by gavage [49]. Even aged mice (70-week-old), usually refractory to oral tolerance by gavage, can be rendered tolerant by continuous feeding of the antigen [50]. We have also shown, in a previous study using mice with ethanol-induced colitis, that oral tolerance induction to specific serum IgG1 production was impaired in gavage-fed mice [51]. As already reported, IgG1 is the most resistant immunological parameter to suppression by mucosal administration of proteins. Nasal administration of antigen does not suppress specific anaphylactic serum IgG1, whereas continuous feeding inhibits it very efficiently [52]. In this study, we confirmed these previous data showing that continuous feeding but not gavage was able to render diseased IL-10-deficient mice tolerant to ovalbumin when specific IgG1 antibodies were measured. Therefore, IL-10 deficiency and severe inflammation in the gut mucosa seemed to decrease but not abolish susceptibility to oral tolerance induction. This result represents a key element in designing novel therapeutic approaches for inflammatory bowel diseases. Since these pathological conditions are generated by inflammation, the suppressive modulatory properties of tolerance procedures obtained by oral administration of disease-related proteins would be an alternative. Oral tolerance to target antigens has been successfully tested in many models of other inflammatory illnesses such as autoimmune and allergic diseases [6]. Our present data indicates that even in the presence of severe colitis and enteritis, oral tolerance approaches could be used as an alternative therapeutic tool for inflammatory bowel diseases. Nevertheless, optimal protocols of oral administration, such as continuous feeding, would be a requirement for the use of such strategies.

In conclusion, we demonstrated that IL-10-deficient mice have a progressive and spontaneous inflammation with marked alterations in the immune system. The absence of an important anti-inflammatory cytokine such as IL-10 leads to a dramatic defect of immunoregulation, with reduced regulatory T lymphocytes in the gut mucosa, alterations in immunoglobulins isotypes production and in the frequency of B1 cells. Importantly, we also showed that although several mechanisms of immunoregulation were compromised in IL-10-deficient mice, oral tolerance induction could still be induced. Our study also provides data showing that some immunoregulatory mechanisms were still preserved and oral tolerance could be induced even during overt inflammation.

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Research Article

Persistence of Diarrheal Pathogens Is Associated with Continued Recruitment of Plasmablasts in the Circulation

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Intestinal antigen encounter leads to recirculation of antigen-specific plasmablasts via lymphatics and blood back to the intestine. Investigating these gut-originating cells in blood provides a less invasive tool for studying intestinal immune responses, with the limitation that the cells disappear from the circulation in two weeks. No data exist on situations where pathogens persist in the intestine. Patients with *Salmonella, Yersinia,* or *Campylobacter* gastroenteritis and volunteers receiving an oral typhoid vaccine were assayed for plasmablasts specific to each subject's own pathogen/antigen weekly until the response faded. In vaccinees, plasmablasts disappeared in two weeks. In gastroenteritis, the response faded 2-3 and 3–7 weeks after the last positive *Salmonella* or *Yersinia* stool culture. Even in symptomless patients, pathogens persisting in the intestine keep seeding plasmablasts into the circulation. Assaying these cells might offer a powerful tool for research into diseases in which persisting microbes have a potential pathogenetic significance.

1. Introduction

The intestine represents the largest immunological tissue in the body and carries the majority of all lymphocytes [1, 2]. Pathogens encountered in the intestine activate antigen-specific lymphocytes in Peyer's patches, and these cells migrate to mesenteric lymph nodes and further via lymphatics and blood to the intestinal lamina propria as effector lymphocytes [2–6]. Consistent with this recirculation of activated intestinal lymphocytes, antigen-specific effector lymphocytes have been found in the circulation after intestinal antigen encounter both after oral [7–11] and rectal [10, 12] vaccinations and in intestinal infections [13–15].

The mechanisms underlying this recirculation of activated intestinal lymphocytes have been a subject of extensive research. It has been shown that dendritic cells in the intestine present the antigens to lymphocytes in Peyer's patches and program these cells to express a set of receptors determining their later migratory behavior [2–6]. Next, these activated lymphocytes migrate to mesenteric lymph nodes and return to the mucosal sites with the help of lymphatics and blood. This return once appeared to occur randomly with circulating blood, but, in fact, it exhibits marked tissue selectivity at the final stage of homing from blood through the endothelium into the tissues. This homing is a multistep process requiring an interaction of lymphocyte surface molecules recognizing their ligands distributed in a tissue-specific manner in the body [2–6]. Lymphocytes homing to the intestine express both CCR9 [1–3, 16], a chemokine receptor mediating homing to the small intestine, and $\alpha_4\beta_7$ [1–3, 17], a gut-specific homing receptor (HR) that recognizes Mad-CAM1 on the endothelial venules of the intestine. The majority of gut-originating antigen-specific plasmablasts found in the peripheral blood after oral vaccination [9–11] or in intestinal infections [14] have been shown to express the gut HR, $\alpha_4\beta_7$, implying a preferential homing of these cells to the intestinal lamina propria.

Research into intestinal immune responses in the human gut has been hampered by the instability of antibodies in secretions and, to an even greater extent, by ethical

restrictions. Investigation of gut-originating plasmablasts in the peripheral blood circumvents some of these problems. Plasmablasts (preplasma cells) are close to end-stage cells of the B-cell lineage representing only a minor part of all circulating B cells: recently activated and developed into effector cells, they constitute the part that actively secretes antibodies while being on their way to settling down in the target tissues as plasma cells. Plasmablasts recently activated in the intestine are identified in the circulation as antibody-secreting cells (ASCs) with a spontaneous secretion of antibodies against intestinally encountered antigens [7–15]. Studies with oral vaccines have shown that these cells can be caught from the peripheral blood 3–5 days after the original antigen encounter, they peak in number around day 7 and disappear within two weeks [7, 8, 18], consistent with their homing back to the mucosa. Catching gut-originating effector lymphocytes from the circulation has proved a useful tool in studying mucosal immune responses to both oral vaccines [7–11] and intestinal infections [13–15]. However, a major restriction to this approach is the transient nature of the response: the actual window for catching the cells is no longer than a few days. The kinetics has been explored with oral vaccinations [7, 8, 18] where the exact time of antigen encounter is known and the vaccine antigen persists in the intestine for only a short time. The present study investigates the response in natural infections where the pathogen persists in the intestinal milieu for a longer period of time. This is of interest also in view of diseases in which pathogen persistence plays a role in the pathogenesis of the disease.

2. Methods

Patients with diarrhea and volunteers receiving an oral typhoid vaccine were studied for circulating plasmablasts specific to the pathogen isolated from their own stool sample or to the vaccine antigen, respectively. The kinetics of the response was studied along with determinations of isotypes. In order to confirm the intestinal homing commitment of these migrating cells, the homing profile was determined in a subgroup of volunteers in both groups.

2.1. Patients. 23 patients attending the Central Hospital of Central Finland or the Helsinki University Central Hospital and six healthy volunteers were enrolled in the study. Informed consent was obtained from each patient/volunteer before participation. The study was conducted according to the principles stated in the Helsinki Declaration on human experimentation, and the study protocol was reviewed and approved by the Ethics Committees of the participating hospitals.

22 patients (12 women, 10 men, aged 36–63 years) had bacterial diarrhea, diagnosed on the basis of watery stools and a pathogen isolated from stool samples. One patient had no diarrhea but was examined because of her spouse's positive *Salmonella* culture: her stool culture proved positive for the same pathogen. In her case, the day of the first positive stool sample was defined as day 0, while, for all others, day 0 indicates the day of the first symptoms. None of the patients had a known previous history of diarrhea caused by a bacterial pathogen, nor had they been diagnosed with immune deficiencies or other significant underlying diseases. Six healthy volunteers (all women, aged 24–47), who had not received the typhoid vaccination previously, were each given the oral *Salmonella typhi* Ty21a vaccine (Vivotif, Crucell, Switzerland) in three doses two days apart, according to the manufacturer's instructions. For vaccinees, the day of the first vaccine dose was marked as day 0.

2.2. Collection of Blood Samples, Isolation of PBMC, and Preparation of Antigen. For patients with diarrhea, first blood samples for ASC analyses were drawn 6–33 days after the onset of the symptoms, and, for vaccinees, a series of blood samples was drawn 0, 5, 7, 9, 12, 14, and 16 days after the oral typhoid vaccination and then, in both groups, weekly for as long as the ASCs were detected or the pathogen could be recovered from the stool samples, and the patient was available. Mononuclear cells were isolated from heparinized blood by Ficoll-Paque density-gradient centrifugation as described previously [8] and adjusted to a concentration of 2×10^6 cells/mL. HR expressions were determined in the first blood sample drawn in five patients with diarrhea and on day seven after vaccination in five vaccinees.

Bacterial strains identified in the stool samples were grown on nutrient agar plates and formalin-killed as described previously [13, 14, 19, 20]. The concentration of the bacterial suspension was adjusted to 10^8 bacteria/mL.

2.3. Separation of Receptor-Negative and Receptor-Positive Cell Populations. The separation of the cells into receptor-negative and receptor-positive populations has been described earlier [9, 14, 21]. Briefly, the cells were incubated with the monoclonal antibodies anti-$\alpha_4\beta_7$ (ACT-1) (Millennium Pharmaceuticals, Cambridge, MA), anti-L-selectin (Leu8) (Becton-Dickinson), or anti-CLA (HECA-452) (received from Dr. Sirpa Jalkanen, University of Turku, Finland), washed twice, and incubated with Dynal M-450 magnetic beads coated with sheep anti-mouse IgG (Dynal, Oslo). The beads with the attached cells were separated from the suspension by applying a magnet outside the test tubes and the supernatants with the receptor-negative cells collected. The receptor-positive cells attached to the beads were collected. Both the receptor-positive and receptor-negative cell populations were immediately analyzed with the enzyme-linked immunospot (ELISPOT) assay for numbers of ASC.

The efficiency of the cell separations was checked in pilot experiments as described previously [9, 14].

2.4. Assay of Specific Antibody-Secreting Cells. The total population of PBMC and the receptor-positive and receptor-negative cell populations were each assayed for specific ASC with ELISPOT: the assay of specific ASC has been described previously [8, 13, 14, 19, 20]. In brief, for patients with diarrhea, 96-well microtiter plates (Maxisorp, Nunc.

Denmark) were coated with a whole-cell preparation of formalin-killed pathogen isolated from the stool sample of the same patient (see above) or, for vaccinees, with a preparation formalin-killed *Salmonella* strain SL2404 carrying the O-antigen 9 and 12 similarly to the vaccine strain Ty21a. The cells were incubated in the wells, and antibodies secreted during this time detected with alkaline phosphatase-conjugated anti-human IgA (Sigma-Aldrich, Mo, USA), IgG (Sigma-Aldrich), and IgM (SouthernBiotech, Birmingham, AL, USA) followed by application of substrate (bromo-4-chloro-3-indolyl phosphate p-toluidine salt; Sigma-Aldrich) in melted agarose. Specific ASCs were enumerated by counting the spots in the wells in a light microscope. A response was defined as >2 specific ASC/10^6 PBMC in at least one sample.

2.5. Statistics. The numbers of ASC were calculated as geometric means ± SEM. The proportions of the receptor-positive ASC were calculated as follows: % of receptor-positive cells among ASC = (100 X the number of ASC in receptor-positive population) ÷ (the sum of the number of ASC in receptor-positive and receptor-negative populations). Statistical comparisons were carried out using Student's *t*-test. Results of statistical analyses were considered significant when $P < 0.05$.

3. Results

3.1. Pathogens and Symptoms. In patients with gastroenteritis, *Salmonella enteritidis* was grown as a pathogen in stool culture in 10/23 cases, *Yersinia enterocolitica* in 7/23, *Campylobacter jejuni* in 2/23, and *S. stockholm, S. bradford, S. emek,* and *S. typhimurium* each in one case. In 12/23 patients, only one positive stool sample was obtained, and the second one taken 1-2 weeks later was negative. In 11/23 patients, 2–11 positive stool samples were collected. 12/23 patients had symptoms during the first sampling, and 10/23 of them until 1–5 weeks after it. In 3/23, patients the symptoms had faded away one week, and in 8/23 patients in 2–4 weeks before the first blood sample.

3.2. General Characteristics of the Antigen-Specific Plasmablast Response. All six volunteers vaccinated with Ty21a showed a vaccine-antigen-specific ASC response similar to that in our previous studies [8, 12] (Figure 1).

A response of circulating pathogen-specific plasmablasts was found in 8/10 patients with *Salmonella enteritidis* (Figure 2). In one patient, there was no response despite a positive stool sample one week before (symptoms started 22 days earlier). In one patient, the blood sample was obtained only four weeks after the last positive stool sample and last symptoms. Circulating ASCs were detected in 5/7 patients with *Yersinia enterocolitica* (Figure 3). In one patient with a clear response, no further follow-up samples could be obtained. No ASCs were found in 2/7 patients: in both of these cases, the sample was drawn one week after the symptoms had faded and four weeks after the last positive stool sample.

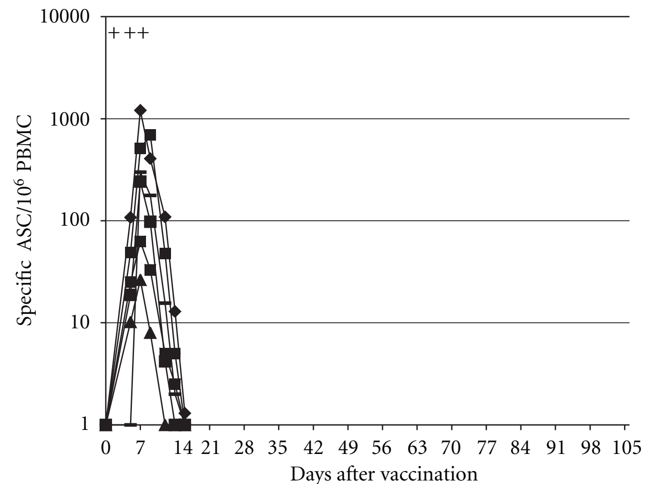

Figure 1: Numbers of vaccine antigen (O9,12)-specific circulating plasmablasts identified as antibody-secreting cells/10^6 PBMC (IgA- + IgG- + IgM) in six volunteers vaccinated with the live oral *Salmonella typhi* Ty21a vaccine. Each volunteer received one vaccine dose on days 0, 2, and 4 (the vaccination days are indicated with "+"). To determine the kinetics of the response, the numbers of plasmablasts were determined on several days for as long as ASCs were found in the samples. The values of each individual are connected with a line.

Patients with *S. bradford, S. emek,* and *S. typhimurium* as pathogens had a response (Figures 4(a)–4(c)). The patient with *Salmonella stockholm* did not show a response even though the samples were drawn one week after positive stool culture and last symptoms. One patient with *Campylobacter jejuni* had a vigorous response (10 000 ASC/10^6 PBMC) (Figure 4(d)), while the other one showed no response; her blood sample was drawn three weeks after the last positive stool sample.

Among the vaccinees, IgA-ASC predominated in three volunteers, IgG in two, and IgM in one. The geometric mean of the peak of the responses (IgA + IgG + IgM-ASC) was 215 ± 184/10^6 PBMC. The isotype distribution is shown in Figure 5.

Out of all patients, the response was dominated by IgA in 15/17 cases and IgM in 2/17 cases (one with *S. typhimurium* and one with *Yersinia enterocolitica*). The geometric mean of the peak of the responses (IgA + IgG + IgM-ASC) was 64 ± 70/10^6 PBMC in patients with *S. enteritidis* and 459±614/10^6 PBMC with *Yersinia enterocolitica*. The isotype distributions are shown in Figure 5.

3.3. Kinetics of the Antigen-Specific Plasmablast Response. In the vaccinees, the response peaked on day 7 and declined thereafter. No samples were drawn after day 16, since the response had faded away/was negligible already on that day in all volunteers (Figure 1).

In patients with gastroenteritis, the numbers of antigen-specific ASC were followed weekly for as long as the response persisted or the stool culture remained positive, and the patient was available. The highest number of ASC was in

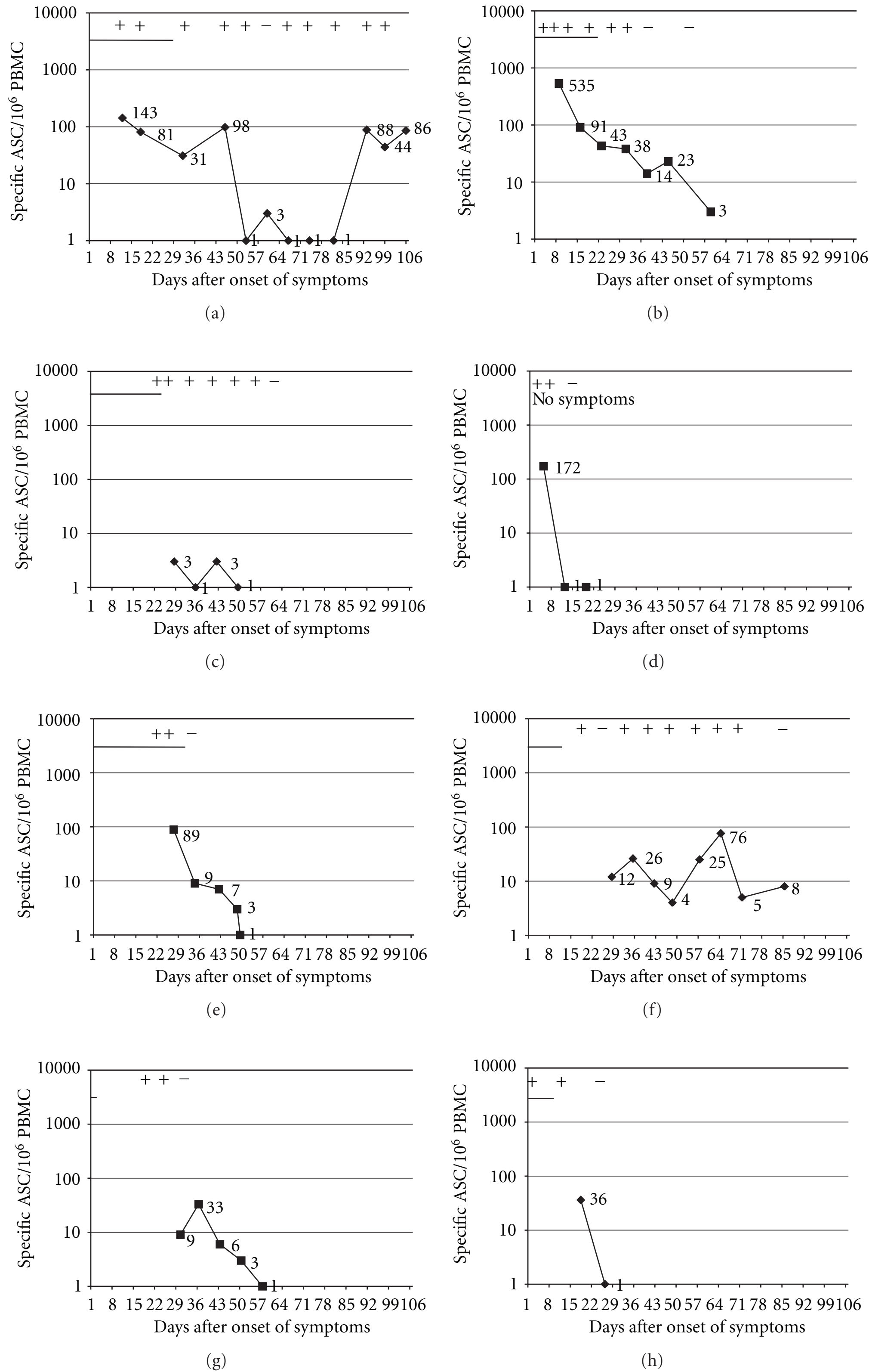

Figure 2: Relation of pathogen-specific circulating plasmablasts (black curve) with symptoms (black horizontal line) and findings in stool samples (+ or −) in eight patients with gastroenteritis caused by *Salmonella enteritidis*. To determine the kinetics of the response, the numbers of plasmablasts were assessed on several occasions as long as they were found in the samples or the pathogen could be isolated from the stool samples, and the patient was available. The plots represent each data from one individual. The day of the onset of the symptoms was marked as day 0.

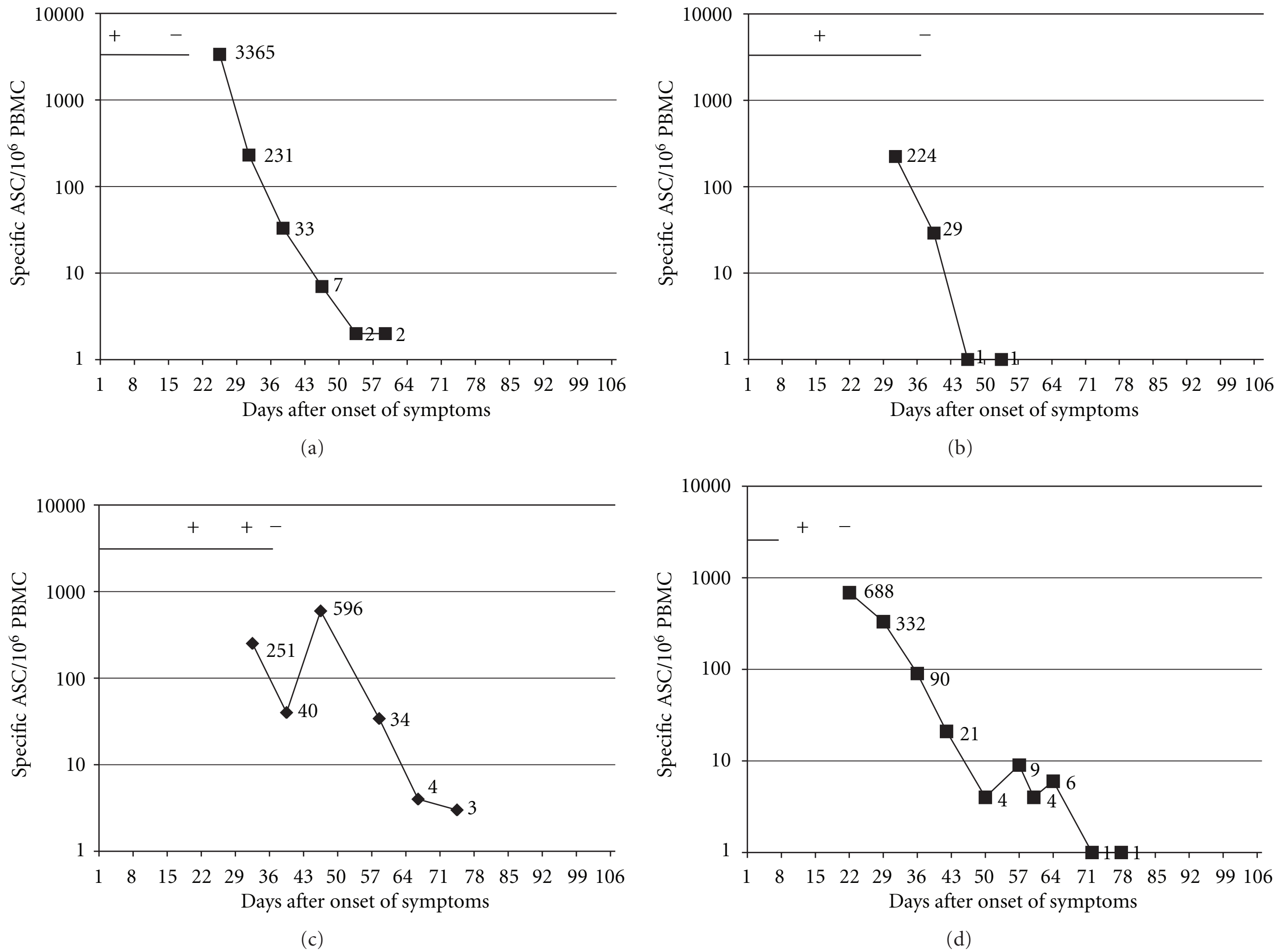

Figure 3: Relation of pathogen-specific circulating plasmablasts (black curve) with symptoms (black horizontal line) and findings in stool samples (+ or −) in four patients with gastroenteritis caused by *Yersinia enterocolitica*. The plots represent each data from one individual. The day of the onset of the symptoms was marked as day 0.

12/16 cases found in the first sample drawn, that is, soon after the beginning of symptoms and after that the magnitude of the response appeared to fluctuate until it faded away (Figures 2–4). The numbers of weekly blood samples drawn varied from one till up to 12 (Figures 2–4). The fading of ASC appeared to be associated with the stool samples turning negative, rather than the subsiding of the symptoms, as the ASC response was prolonged in some volunteers with persisting positive stool cultures even after the symptoms had subsided (Figures 2(a), 2(c), 2(f)). The response appeared to fade away faster after *Salmonella* (2-3 weeks) (Figure 2) than after *Yersinia* infection (3–7 weeks) (Figure 3).

3.4. The Expression of HR on Antigen-Specific Plasmablasts. The expressions of various HR on ASC specific to the diarrheal pathogen or the vaccine antigen were studied in five patients with *Salmonella* diarrhea and in five vaccinees, respectively. In the diarrhea group, the proportion $\alpha_4\ \beta_7$ + ASC among all ASC was $94 \pm 6\%$, L-selectin + ASC $46 \pm 24\%$, and CLA + ASC $4 \pm 4\%$. Among vaccinees, the respective figures were $97 \pm 2\%$, $24 \pm 18\%$, and $1 \pm 2\%$. These data did not reveal any statistical difference in the HR expressions between diarrhea patients and vaccinees but are consistent with the intestinal homing profile of the migrating cells in both groups.

4. Discussion

Investigation of gut-originating plasmablasts from samples of peripheral blood during their recirculation serves as a valuable, less invasive tool for studying intestinal immune response in humans. Instead of examining memory B cells, this approach centers on gut-directed plasmablasts cells spontaneously secreting antibodies against antigens they have encountered recently, that is, the method measures an ongoing intestinal immune response. The transient appearance of these cells in the circulation, which allows only a few days for actual sampling, has posed a severe limitation to this approach. The present study is the first to focus on the kinetics of this response during a natural infection in which the pathogen can persist in the intestine for a longer period of time. It shows that the previously presumed kinetics does

Figure 4: Relation of pathogen-specific circulating plasmablasts (black curve) with symptoms (black horizontal line) and findings in stool samples (+ or −) in four patients with gastroenteritis caused by (a) *Salmonella typhimurium*, (b) *S. emek*, (c) *S. bradburg,* and (d) *Campylobacter jejuni*. The plots represent each data from one individual. The day of the onset of the symptoms was marked as day 0.

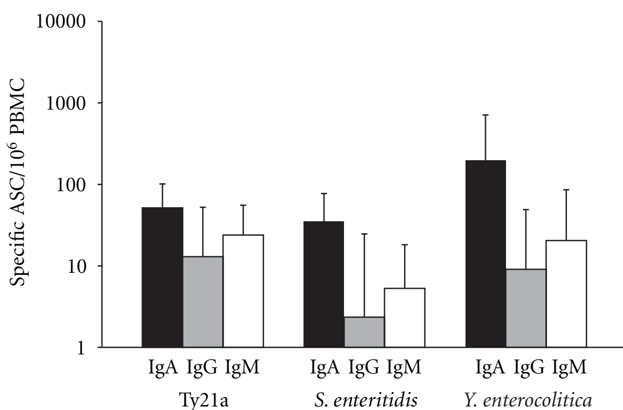

Figure 5: The isotype distribution in the peak of the specific plasmablast response in volunteers vaccinated with the oral live *Salmonella typhi* Ty21a vaccine ($n = 6$) and in patients with gastroenteritis caused by *Salmonella enteritidis* ($n = 8$) or *Yersinia enterocolitica* ($n = 5$). The data are given as geometric means of ASC/10^6 PBMC ± SEM.

not apply to natural infections: if the pathogens persist in the intestine, the actual time frame for sampling can be significantly extended.

4.1. Comparison to Serum Antibody Responses. Assessment of plasmablasts has significant advantages as compared to serum antibody assays. Firstly, plasmablast response measures an immune response to antigens encountered recently, whereas serum antibodies may remain elevated throughout life even if the antigen was encountered decades ago [22]. Secondly, plasmablast assay allows an assessment of the response to each individual's own pathogen. Thirdly, plasmablast response has proved significantly more sensitive than serum antibodies when measuring humoral immune response to intestinal antigen encounter [8]. Our previous studies have shown that shortly after oral vaccination, when antibodies are presumed to exist in intestinal secretions, serum antibodies even fail to rise at all upon intestinal antigen encounter, despite a simultaneous significant plasmablast response [23]. This describes the independent

nature of systemic and mucosal immune systems and further stresses the role of plasmablast instead of serum antibody measurements when assessing intestinal immune responses: plasmablasts represent intestinal immune response, whereas serum antibodies are mainly produced in the bone marrow. Serum antibodies remaining fairly constant for long periods of time do not provide any information as to whether the pathogen persists in the intestine or the pathogen has been cleared. Up until now, it has been assumed that exploring plasmablasts would not provide such information either, as the cells were thought to be found in the circulation for two weeks only. The continued recruitment of plasmablasts in the present study suggests that the use of the assay of specific plasmablasts can be extended and even applied to the evaluation of antigen persistence in the intestine.

4.2. Significance of the Continuous Recruitment of Plasmablasts. Continuous recruitment of plasmablasts appears to be a means of enhancing the immune response to a pathogen the body has not succeeded in expelling. The generally lower numbers of plasmablasts recruited as the time passes and the even negligible numbers seen occasionally in the later course of the response may be a reflection of antibody responses in the intestine already working against the pathogens. However, such fluctuations in the magnitude of the response with occasional negligible numbers of plasmablasts strongly suggest that a single negative sample does not indicate that the pathogen no longer persists, and therefore, multiple sampling may be required. The highest numbers of plasmablasts are found early in the course of the response. Previous to this we have, in fact, shown that if an oral vaccine is given in six instead of three doses, the plasmablast response is prolonged till day 22, and even if no clear peak can be seen, the response is at its highest on day 7 [24]. The early peak in the response means that (possible) comparisons in response magnitude between two groups should be carried out on the basis of samples drawn at early stages of the disease, while, in other samples, a qualitative comparison is mainly feasible. It would be interesting to see if chronic carriers of intestinal pathogens (e.g., Salmonella) still have an ongoing recruitment of plasmablasts, as the pathogen can in such cases be regarded to have become part of their regular intestinal microbiota. As to the disappearance of ASC, it is not possible to draw any definite conclusions. In the present data, however, the response seemed to fade away faster after *Salmonella* (2-3 weeks; Figures 2 and 4) than after *Yersinia* infection (3–7 weeks; Figure 3). As opposed to infections with real multiplying pathogens, the Ty21a vaccine strain is supposed to survive in the intestine only for one or two days. Accordingly, data comparing responses after three versus six doses of Ty21a [24] suggest that in cases with nonreplicating antigens, the plasmablasts disappear from the circulation approximately 8–10 days after the last day of antigen encounter.

Notably, even if the present study focuses on plasmablast response after *intestinal* antigen encounter, a recirculation of antigen-specific plasmablasts also occurs after *nonintestinal* encounter [9, 25–27], for example, in infections at other mucosal sites [10, 19, 20, 28] and after parenteral vaccinations [9, 14, 25–27, 29, 30]. As evidenced by their HR profiles, these plasmablasts are trafficking to nonintestinal sites. Thus, it appears that assaying these cells could be applied to assess persistence of pathogens at nonintestinal sites, too.

5. Conclusion

In conclusion, the recruitment of pathogen-specific plasmablasts in the circulation in gastroenteritis has proved less transient than previously reported. Instead, the recruitment seems to continue as the pathogen persists in the intestine, albeit at a lower or, occasionally, even negligible level, indicating that repeated sampling may be necessary. Continued recruitment of plasmablasts in the circulation not only reflects a continuous stimulation of the immune system but also carries potential for assessing immune response to persisting antigens that are suspected to be of significance in the pathogenesis of a disease.

Abbreviations

ASC: Antibody-secreting cell
CCR: Chemokine receptor
HR: Homing receptor.

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Research Article

Lack of Intestinal Epithelial Atg7 Affects Paneth Cell Granule Formation but Does Not Compromise Immune Homeostasis in the Gut

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Genetic polymorphisms of autophagy-related genes have been associated with an increased risk to develop inflammatory bowel disease (IBD). Autophagy is an elementary process participating in several cellular events such as cellular clearance and nonapoptotic programmed cell death. Furthermore, autophagy may be involved in intestinal immune homeostasis due to its participation in the digestion of intracellular pathogens and in antigen presentation. In the present study, the role of autophagy in the intestinal epithelial layer was investigated. The intestinal epithelium is essential to maintain gut homeostasis, and defects within this barrier have been associated with the pathogenesis of IBD. Therefore, mice with intestinal epithelial deletion of Atg7 were generated and investigated in different mouse models. Knockout mice showed reduced size of granules and decreased levels of lysozyme in Paneth cells. However, this was dispensable for gut immune homeostasis and had no effect on susceptibility in mouse models of experimentally induced colitis.

1. Introduction

Inflammatory bowel disease (IBD) with its two major subtypes Crohn's disease (CD) and ulcerative colitis (UC) is a chronic relapsing inflammatory disorder of the gastrointestinal tract approximately affecting 400 in 100,000 of the adult population in western countries [1]. Patients suffer from varying symptoms including abdominal pain, diarrhoea, rectal bleeding, weight loss, and lethargy. The pathogenesis of IBD is still under investigation but several factors have been shown to play a role in the aetiology of IBD including environmental triggers, genetic factors, bacterial flora, and an overreactive immune response. It is a generally accepted theory that the disease arises due to a dysfunctional interaction between the mucosal immune system and the bacterial microflora in genetic susceptible humans. In healthy individuals, the internal mucosal tissue is separated from the intestinal lumen including food antigens and bacteria by the intestinal epithelial barrier playing a crucial role for gut homeostasis. Disturbance of the epithelial barrier function has been demonstrated to potentially induce intestinal inflammation [2, 3].

Among the identified genetic factors providing an increased risk for the development of IBD, autophagy-related genes such as ATG16L1, IRGM1, and LRRK2 suggest the implication of autophagy dysregulation in the pathogenesis of IBD [4–6]. Autophagy has been demonstrated to be elementary for cellular homeostasis as it is involved in cellular clearance. During the autophagy process, cellular constituents such as aged and damaged organelles or proteins are enveloped by membranes and delivered to lysosomal vesicles for degradation. Furthermore, autophagy is involved in other cellular processes such as development, cellular differentiation, aging, and nonapoptotic programmed cell death [7–9]. It also participates in the clearance of apoptotic bodies of dying cells to prevent tissue inflammation further providing possible implications of autophagy in the pathogenesis of intestinal inflammatory diseases [10].

The association between IBD and autophagy dysregulation is further supported by the role of autophagy in immunity because autophagy participates in the digestion of intracellular pathogens and is involved in antigen presentation [11]. The importance of autophagy has been underlined by the generation of autophagy knockout mice. Mice with a general deletion of the autophagy proteins Atg5, Atg7, or Atg16L1 die within few hours after birth due to the neonatal starvation period [12–14]. Atg7 is an enzyme playing a central role in the elongation of autophagy vesicle membranes. Recent data have shown that liver cell-specific Atg7 deficiency caused hepatomegaly due to accumulation of abnormal organelles and cell swelling [12] while Atg7 deletion in Purkinje cells led to degeneration of axon terminals followed by mouse behavioural deficits [15]. Furthermore, mice with defective Atg16L1 protein in the haematopoietic system were highly susceptible to dextran sodium sulphate- (DSS-) induced colitis, again indicating the crucial role of autophagy in the pathogenesis of IBD [14].

Because of the important role of autophagy in many cellular processes, the association between inflammatory bowel disease and autophagy, the critical involvement of the intestinal epithelial cell (IEC) layer in the pathogenesis of IBD and its special position at the interface between inside and outsides, the role of autophagy in intestinal epithelial cells was investigated. Here, we demonstrated by the analysis of IEC-specific conditional knockout mice that Atg7 deficiency alters the granular morphology of Paneth cells but that Atg7 is generally dispensable for gut homeostasis.

2. Material and Methods

2.1. Mice. Mice carrying a loxP-flanked Atg7 allele ($Atg7^{fl}$) were kindly provided by Komatsu and Tanaka [12]. C57BL/6 mice carrying the sequence for the Cre recombinase under control of the villin promoter (Villin-Cre mice) were described earlier [16]. $Atg7^{fl}$ mice were crossbred with Villin-Cre mice to generate intestinal epithelial-specific Atg7 knockout mice ($Atg7^{IEC\text{-}KO}$). Mice were kept in individually ventilated cages.

2.2. Experimental Model of Intestinal Inflammation. Experimental colitis was induced by challenging mice with dextran sodium sulphate (DSS, MP Biomedicals). 3% DSS were dissolved in sterile drinking water and the solution was continuously applied to the mice as drinking water. DSS solution was exchanged every other day. Development of colitis was monitored by weighing the mice and by regular colonoscopy as previously described [17]. The extent of inflammation was scored as previously performed [17, 18].

2.3. Histological Examination. Freshly isolated tissues were either instantly frozen in liquid nitrogen or fixed in 4% formalin and then paraffin embedded. Sections of paraffin embedded tissues were stained with H & E or combined staining methods with alcian blue, PAS, Elastica, and van Gieson to visualize tissue structures. Immunohistochemical analysis of cryosections was performed using Anti-CD11c antibodies (BD Pharmingen), Anti-lysozyme antibodies (Dianova), and Anti-myeloperoxidase antibodies (Abcam) as primary antibodies, biotinylated secondary antibodies (Dianova) and the TSA Cy3 system (PerkinElmer) as recommended by the manufacturer. Apoptotic cells were detected using In situ Cell Death Detection Kit Fluorescein (Roche) for TdT-mediated dUTP nick end labelling (TUNEL) according to manufacture recommendations. Bacteria were detected by fluorescence in situ hybridization (FISH) of bacterial RNA as previously described [19]. Nuclei were counterstained with Hoechst 3342 (Invitrogen). Immunofluorescent tissue slices were analysed using a fluorescence microscope (Olympus). For analysis of tissues by electron microscopy, tissues were fixed using glutaraldehyde and further embedded in Epon Araldite. Ultrathin sections were analysed using an electron microscope (Zeiss).

2.4. IEC Isolation and Western Blotting. Intestinal epithelial cells were isolated by carefully removing the whole intestine from the mouse corpus, inversion of the intestine, washing in phosphate-buffered saline to clean intestine from feces, and incubating the tissue in prewarmed isolation solution containing HBSS (PAA), 1 mM EGTA (Sigma), 2 mM EDTA (Sigma), and 10% FCS (PAA) for 15 minutes at 37°C. Subsequently, isolated cells were pelleted at 1200 rpm and 4°C for 5 minutes and washed twice with 1x PBS and repeated centrifugation. Proteins were extracted using the mammalian protein extraction reagent (Thermo Scientific) containing protease and phosphatase inhibitor tablets (Complete Mini Protease Inhibitor Cocktail Tablets and PhosStop Phosphatase Inhibitor Cocktail Tablets, Roche). Proteins were separated according to their molecular weight by SDS polyacrylamide gel electrophoresis and subsequent transfer to Protran nitrocellulose transfer membrane (Whatmann). Membranes were blocked in Roti-Block (Roth) and probed with Anti-Atg7-CT antibody (AnaSpec) or Anti-LC3B-antibody (Cell Signaling) over night at 4°C with gentle shaking followed by incubation with secondary HRP-linked Anti-Rabbit antibody (Cell Signaling). Incubating membranes with HRP-linked Anti-Actin antibody (Santa Cruz Biotechnology) for 1 hour at room temperature served as an internal control. For detection of protein bands, Western Lightning Plus-ECL (PerkinElmer) was used according to manufacture recommendations.

2.5. Transcription Analysis. Total RNA was extracted from tissues using an RNA isolation Kit (Nucleo Spin RNA II, Macherey Nagel) and cDNA was generated by reverse transcription using the iScript cDNA Synthesis Kit (Bio-Rad). cDNA samples were mixed with SsoFast EvaGreen (Bio-Rad) and specific QuantiTect Primer assays (Qiagen) and analysed by real-time PCR. *Hprt* was used as an internal control.

2.6. Statistical Analysis. Statistical analysis was performed using Student's t-test. Double asterisks indicate significant differences ($P < 0.01$). n.s. = nonsignificant differences ($P > 0.05$).

3. Results and Discussion

To investigate the role of autophagy in the intestinal epithelial cell layer, we crossbred Villin-Cre mice with mice carrying loxP-flanked Atg7 alleles to generate conditional knockout mice ($Atg7^{IEC-KO}$ mice). $Atg7^{IEC-KO}$ mice showed an IEC-specific deletion of the autophagy protein Atg7 (Figure 1(a)), which is essential for the elongation of autophagy vesicles. During the autophagy process, the cytosolic LC3-I is converted to the lipidated LC3-II by an ubiquitin-like conjugation system involving Atg7. LC3-II is widely ac- cepted as a marker for activated autophagy [20, 21]. In control intestinal epithelial cells, both LC3-I and LC3-II were detected by western blotting. In contrast, only the LC3-I form was observed at an increased level in Atg7-deficient IECs indicating impaired autophagy in these cells (Figure 1(b)). IEC specific Atg7 conditional knockout mice were born healthy and fertile and did not reveal an overt phenotype compared to control littermates. To examine the influence of Atg7 deficiency in IECs on gut homeostasis, the colon of conditional knockout mice and control mice was analysed by colonoscopy. Despite the supposed role of autophagy in the pathogenesis of inflammatory bowel disease in humans, no macroscopic differences were detected indicating that Atg7 deficiency is not associated with spontaneous gut inflammation (Figure 1(c)). Furthermore, chromocolonoscopy using methylene blue demonstrated normal crypt morphology within the colon (Figure 1(c)). H & E staining of colonic cross sections further confirmed the lack of structural alterations in $Atg7^{IEC-KO}$ mice (Figure 1(d)); a finding that was underlined by morphometric analysis, demonstrating comparable general structures of colon and ileum such as length and width of villi and crypts (Figure 1(e)).

Recent studies have demonstrated alterations in Paneth cells of autophagy-deficient mice [22]. Interestingly, Paneth cells of $Atg7^{IEC-KO}$ mice investigated in the current study showed a different morphology compared to Paneth cells of control mice as demonstrated by combined staining of distal small intestine paraffin sections with alcian blue, PAS, Elastica, and van Gieson and by electron microscopy (Figure 2(a)). Accordingly, morphological alterations in Atg7-deficient Paneth cells were indicated by the appearance of more but smaller vesicles compared to Paneth cells in control mice (Figure 2(a), black arrows), suggesting that Atg7 deficiency led to irregularities in granule formation. Paneth cell granules are storage vesicles containing, for example, CD95 ligand, TNF-α, and antibacterial substances such as lysozyme, secretory phospholipase A2, RegIIIγ, and IgA [23]. Importantly, Paneth cell granules are secreted into the gut lumen and participate in innate immune defence. Interestingly, immunofluorescence staining revealed reduction of lysozyme, a marker of Paneth cells, in the small intestine of unchallenged $Atg7^{IEC-KO}$ mice compared to control (Figure 2(b)). A careful statistical analysis of the number of cells at the base of the crypt containing either granules or lysozyme demonstrated that although the number of lysozyme positive cells was decreased, the number of cells containing granules was comparable between $Atg7^{IEC-KO}$ mice and control mice, suggesting that Atg7 deficiency affects the storage and secretion of lysozyme rather than the development or survival of Paneth cells (Figure 2(c)). This was further underlined by quantitative analysis of transcription levels of antimicrobial peptides (AMPs) secreted by Paneth cells demonstrating comparable transcription levels of RegIIIγ, RegIIIβ, Pla2g2a, and Pla2g5 in distal small intestine of unchallenged control and $Atg7^{IEC-KO}$ mice (Figure 2(e)). Furthermore, although diminished levels of lysozyme were detectable in ileal cross sections by immunofluorescence analysis, gene transcription of lysozyme in the distal small intestine of $Atg7^{IEC-KO}$ mice was not significantly different from control mice. Our data are in agreement with data from Cadwell et al., demonstrating altered granular morphology in Atg16L1 and Atg7-deficient Paneth cells [24]. However, while Cadwell et al. reported decreased amounts of granules and diffuse lysozyme staining in Paneth cells of both mice, in contrast, we observed increased numbers and smaller sizes of Paneth cell granules and decreased lysozyme staining. Disturbed lysozyme secretion by Paneth cells suggested decreased antimicrobial defence and alterations in the microbial flora in the intestine of $Atg7^{IEC-KO}$ mice. However, no alterations in the amount of bacteria or the attachment of bacteria to the intestinal epithelial layer were detected (Figure 2(d)). Further studies should investigate whether diminished secretion of the AMP lysozyme by Atg7 deficient Paneth cells influences the composition of the bacterial microflora. In a recent study, Cadwell et al. detected no increased susceptibility of Atg16L1-hypomorphic mice to oral infection with *Listeria monocytogenes*. In agreement with this finding, we found that clearance of orally applied *Citrobacter rodentium*—a commonly used mouse gram negative pathogen mimicking human infectious colitis—was also not affected by IEC Atg7 deficiency (data not shown). Collectively, this implies that Atg7 deficiency and decreased lysozyme in Paneth cells do not affect the attachment of bacteria to IECs and the susceptibility to infections with gram negative bacteria.

Since previous studies had demonstrated the association between autophagy dysregulation and the pathogenesis of IBD [4–6] and also detected decreased secretion of Paneth cell AMPs in the gut of Crohn's disease patients [25, 26], we reasoned that IEC-specific Atg7-deficient mice could be more susceptible to experimentally induced intestinal inflammation. In order to investigate whether deficiency of Atg7 in IECs might modulate intestinal homeostasis under disease conditions, colonic inflammation was induced using dextran sodium sulphate, a commonly used experimental colitis model in mice. $Atg7^{IEC-KO}$ and control mice were continuously treated with 3% DSS in the drinking water. As demonstrated by monitoring mouse body weight changes as an indicator for the general mouse health and by survival analysis, all mice responded comparable to DSS treatment (Figures 3(a) and 3(b)). Development of colitis was followed using colonoscopic video analysis and inflammation scoring. Atg7 deficiency did not affect severity of DSS-induced colitis as demonstrated by comparable signs of inflammation such as granularity of the mucosa, fibrin formation, vascular structure, stool loosening, and thickening of the bowel wall (Figures 3(c) and 3(d)). Extent of inflammation in the colon

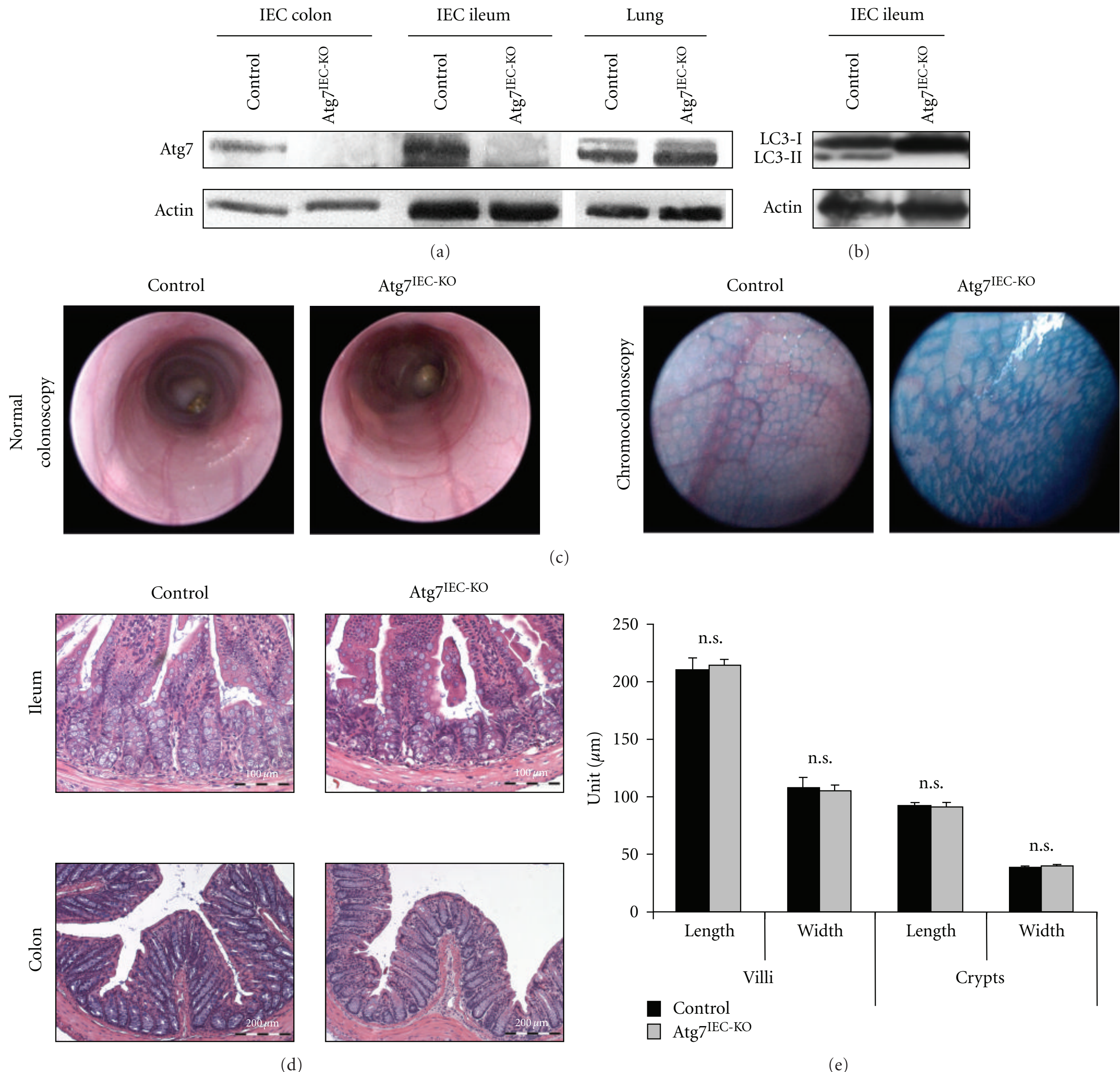
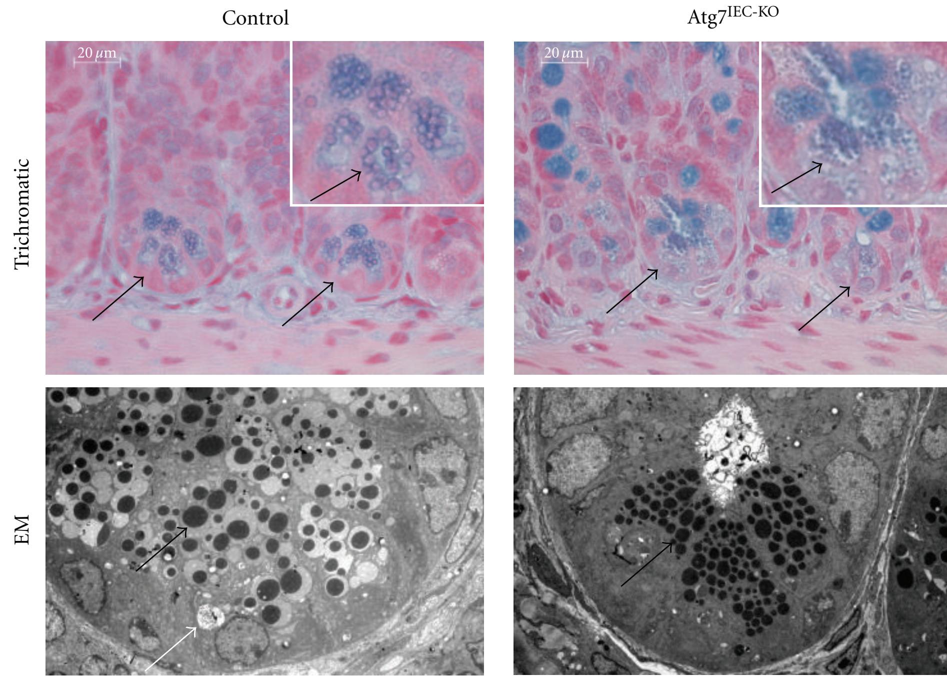


Figure 1: Gut characteristics in unchallenged control and Atg7$^{IEC\text{-}KO}$ mice. (a) Western blot of proteins derived from unchallenged control and Atg7$^{IEC\text{-}KO}$ mice demonstrating lack of Atg7 in isolated intestinal epithelial cells of colon and ileum from Atg7$^{IEC\text{-}KO}$ mice. Other tissues of Atg7$^{IEC\text{-}KO}$ mice display normal Atg7 expression (lung is shown as an example). Actin serves as an internal control. (b) Western blot of proteins extracted from isolated IECs of unchallenged control and Atg7$^{IEC\text{-}KO}$ mice demonstrating deficient autophagy in Atg7-deficient IECs (indicated by lack of the LC3-II form). Actin serves as an internal control. (c) Representative pictures from colonoscopic video analysis using normal colonoscopy (left) and chromocolonoscopy with methylene blue (right) to visualize crypt structures. (d) Representative pictures of paraffin-embedded ileum and colon cross sections stained with H & E. (e) Statistical analysis of distal ileum villi and crypts. Data show mean values of length and width + SEM ($n = 13$ control villi, $n = 24$ Atg7$^{IEC\text{-}KO}$ villi, $n = 24$ control crypts, $n = 27$ Atg7$^{IEC\text{-}KO}$ crypts).

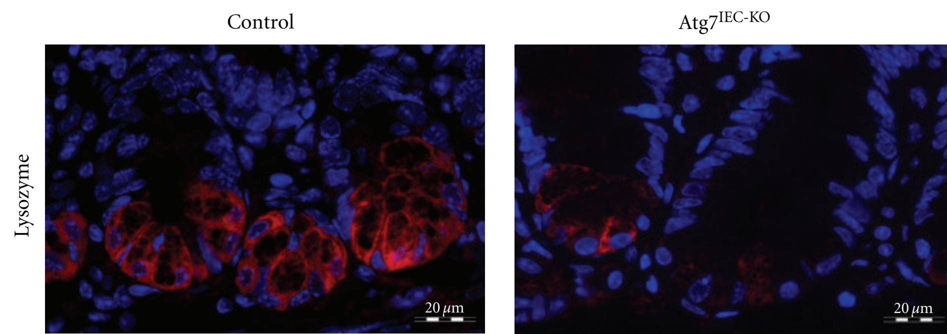
of DSS-treated Atg7$^{IEC\text{-}KO}$ mice was further analysed by TUNEL and H & E staining demonstrating similar tissue destruction and infiltration of immune cells in both mice (Figure 3(e)). We also detected comparable infiltration of immune cells such as dendritic cells and granulocytes into the colonic lamina propria of DSS-treated Atg7$^{IEC\text{-}KO}$ mice and control littermates by immunofluorescence staining (Figure 3(f)). These data demonstrate that Atg7 in intestinal epithelial cells is not essential to manage DSS-induced colitis.

4. Conclusion

In summary, we have analysed intestinal epithelial-specific Atg7-deficient mice and challenged them with mouse models



(a)



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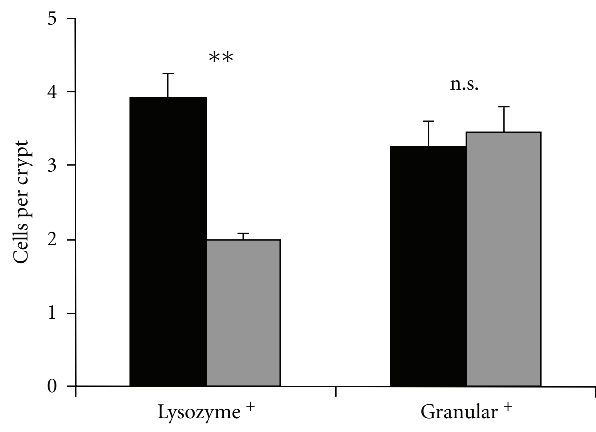

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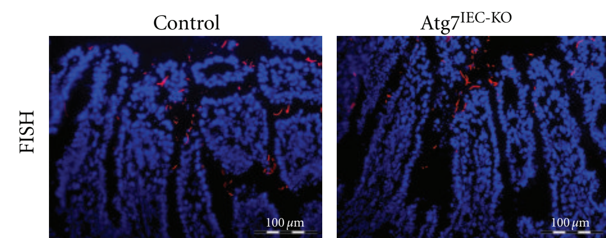
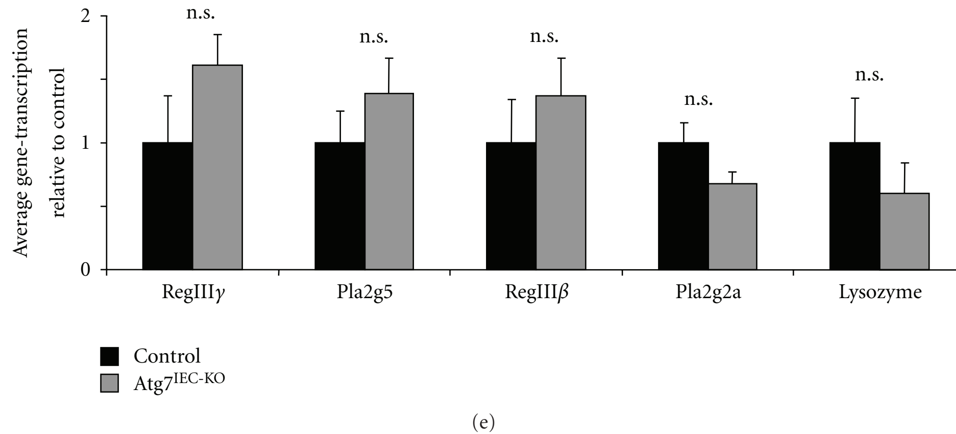


(d)

FIGURE 2: Continued.



(e)

Figure 2: Paneth cell function in unchallenged control and Atg7^{IEC-KO} mice. (a) Histological analysis of Paneth cells by trichromatic staining with alcian blue, PAS, and Elastica van Gieson (top) and by electron microscopy (EM, bottom, 2156x magnification) demonstrates smaller size of granules (black arrows) in Atg7-deficient Paneth cells compared to control Paneth cells. Large autophagosomes (white arrow) were detectable in electron microscopic pictures of IECs derived from control mice but not of IECs derived from Atg7^{IEC-KO} mice. (b) Immunofluorescent staining of distal small intestine for lysozyme (red) reveals decreased levels of lysozyme in Atg7 deficient Paneth cells. Nuclei are shown in blue. (c) Statistical analysis of the number of cells containing granules (granular+) or lysozyme (lysozyme+) at the base of crypts in the distal small intestine of unchallenged control and Atg7^{IEC-KO} mice ($n = 187$ control crypts and $n = 140$ Atg7^{IEC-KO} crypts for analysing granular+ cells, $n = 75$ control crypts and $n = 43$ Atg7^{IEC-KO} crypts for analysing lysozyme+ cells). (d) Detection of bacteria (red) in the distal small intestine of unchallenged control and Atg7^{IEC-KO} mice by fluorescence in situ hybridization (FISH). Nuclei are shown in blue. (e) Quantitative analysis of the transcription of antimicrobial peptide genes in the distal small intestine of unchallenged control and Atg7^{IEC-KO} mice. Data show mean values + SEM ($n = 5$ control mice, $n = 4$ Atg7^{IEC-KO} mice).

of experimentally induced colitis. Our data suggest that deficiency of the autophagy protein Atg7 selectively affects Paneth cell granule formation while no other intestinal epithelial cell lineage seemed to be affected. This finding is surprising, as many other autophagy-related conditional knockout mice have demonstrated severe phenotypes and impaired homeostasis of the respective organs. For example, loss of Atg7 in the liver led to cell swelling due to accumulation of abnormal organelles in hepatic cells and mutant mice developed hepatomegaly [12]. Neural-cell-specific Atg7 knockout mice also developed a severe phenotype as they had a decreased number of Purkinje cells leading to behavioural deficits [15, 27]. Although Atg7 deletion was demonstrated to result in autophagy deficiency in IECs, we did neither observe cell swelling nor increased cell death suggesting that autophagy is dispensable for homeostasis of the intestinal epithelium in healthy individuals. We propose that no other cells than Paneth cells are affected by Atg7 deficiency as these cell lineages (enterocytes, goblet cell, and enteroendocrine cells) have a shorter lifetime (4-5 days) [28]. In these cells, autophagy might be not essential as an intracellular clearance mechanism as damaged organelles and proteins may not accumulate to toxic concentrations, given the short lifetime of these cells. The susceptibility of Paneth cells to Atg7 deficiency may be based on their longer lifespan and the abundant endoplasmic reticulum (ER). Autophagy defects might lead to impaired turnover of ER resulting in increased endoplasmic reticulum stress. For example, mice deficient for Xbp1, a transcription factor required for ER expansion, have reduced numbers of lysozyme-positive Paneth cells and remaining Paneth cells contained compressed ER demonstrating the crucial role of ER homeostasis for Paneth cell biology [29]. Furthermore, membranes derived from the endoplasmic reticulum are the source of many intracellular membranous bodies suggesting that increased ER stress caused by autophagy deficiency results in impaired formation of granules in Paneth cells of Atg7^{IEC-KO} mice.

Interestingly, we could not detect inflammatory alterations in unchallenged Atg7^{IEC-KO} mice suggesting that Atg7 deficiency in the intestinal epithelium is not sufficient to induce a Crohn's disease like phenotype. This was very surprising, as polymorphisms in autophagy genes have been associated with an increased risk to develop inflammatory bowel disease. Furthermore, other studies have demonstrated that rapid clearance of apoptotic bodies is essential to prevent tissue inflammation [30] and that Atg5 general knockout mice showed a decreased removal of apoptotic cells and increased tissue inflammation [10]. However, the absence of tissue inflammation in unchallenged Atg7^{IEC-KO} mice might be reasoned by a minor role of autophagy in short living tissues such as the intestinal epithelial cell layer and the shedding of dead IECs into the gut lumen being unable to cause inflammation. The functional role of Atg7-mediated autophagy in the pathogenesis of IBD was further analysed in experimental models of colitis. Although Atg7^{IEC-KO} mice displayed Paneth cell granule abnormalities similar to those observed in Crohn's disease patients [22], Atg7^{IEC-KO} mice did not show increased susceptibility towards DSS-induced or *Citrobacter rodentium* induced colitis. However, other environmental triggers such as intestinal infections with *Salmonella typhimurium*—an intracellular pathogen requiring autophagy for its clearance—might be a more promising experimental model and should be analysed for its relevance in Atg7^{IEC-KO} mice. In contrast to our data,

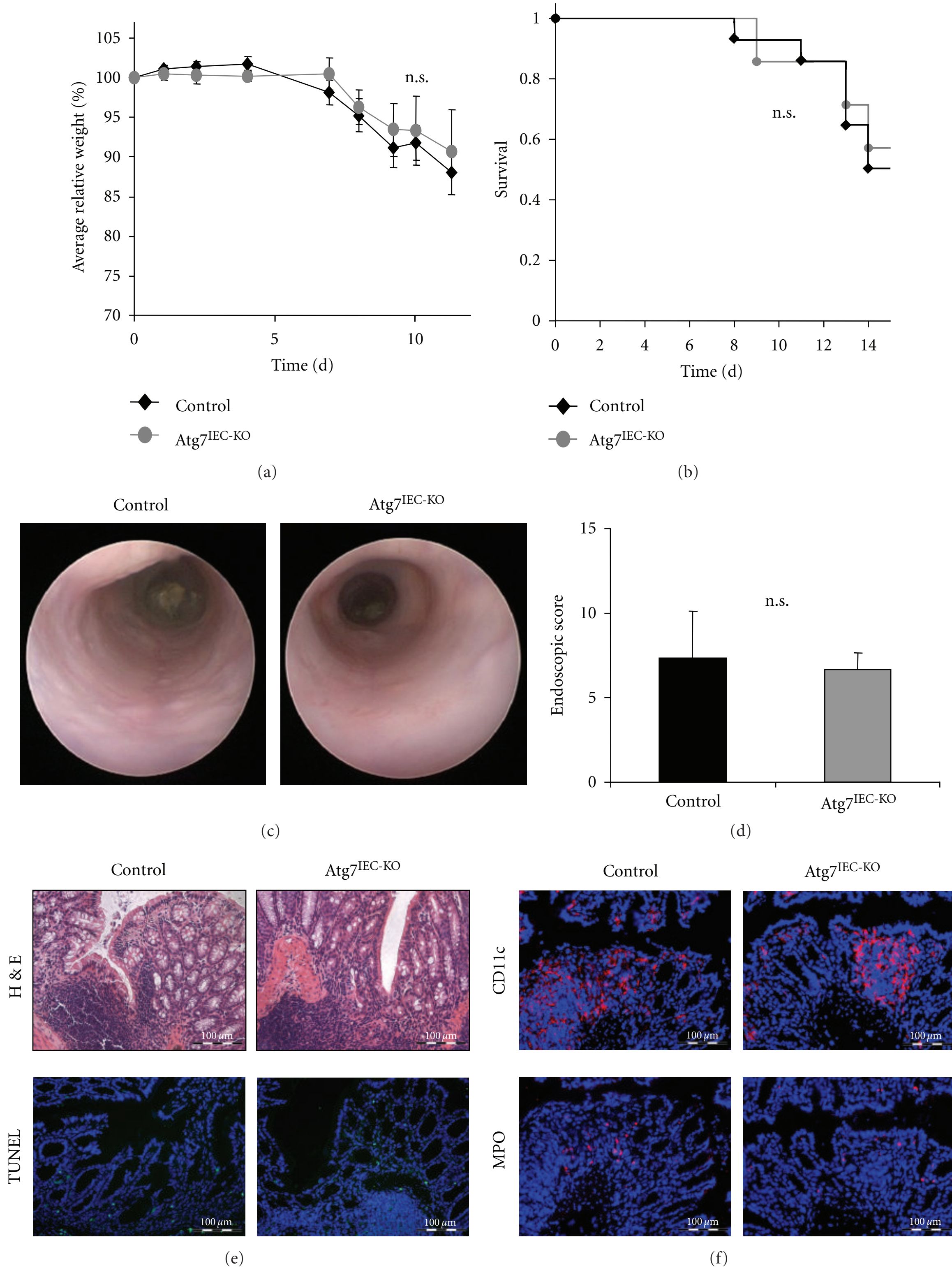


Figure 3: Experimentally induced colitis in control and Atg7$^{\text{IEC-KO}}$ mice. Control and Atg7$^{\text{IEC-KO}}$ mice were continuously challenged with 3% DSS in the drinking water. Illustrated data are representative ($n = 4$ independent experiments). (a) Average weight dynamics were calculated from mouse body weights relative to day 0. Data show mean values ± SEM ($n = 14$ control mice, $n = 7$ Atg7$^{\text{IEC-KO}}$ mice). (b) Survival analysis. (c) Colonoscopic pictures of control and Atg7$^{\text{IEC-KO}}$ mice 14 days after beginning of DSS treatment. (d) Scoring of the extent of colitis. Data show mean values + SEM ($n = 3$ mice in each group). (e) Histological analysis of the distal part of the colon from control and Atg7$^{\text{IEC-KO}}$ mice, treated with DSS for 14 days, by H & E staining (upper row) and TUNEL (bottom row). (f) Infiltration of CD11c+ cells (red, upper row) and MPO+ cells (red, bottom row) into the colon of DSS-treated control and Atg7$^{\text{IEC-KO}}$ mice was detected by immunohistochemical analysis. Nuclei are shown in blue.

Cadwell et al. showed an increased susceptibility of hypomorphic Atg16L1 mice to DSS-induced colitis. However, this effect was dependent on the infection with a certain virus strain [31]. Furthermore, hypomorphic Atg16L1 mice have a defective autophagy in all cell types and therefore, these results are not comparable to our study. Thus, our data show that Atg7 deficiency in the intestinal epithelium alone does not lead to altered responses to DSS treatment indicating that autophagy dysregulation in intestinal immune cells might play a more important role for the pathogenesis of IBD.

In conclusion, these data demonstrate that Atg7 deficiency in IECs affects Paneth cell biology and suggest that Atg7 in intestinal epithelial cells is dispensable for gut home- ostasis. Further studies have to investigate why stem cells, although they are long living, seem to be unaffected by Atg7 deficiency and if other environmental triggers render $Atg7^{IEC-KO}$ mice more susceptible to experimentally induced colitis.

Acknowledgments

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Research Article

Early Oral Ovalbumin Exposure during Maternal Milk Feeding Prevents Spontaneous Allergic Sensitization in Allergy-Prone Rat Pups

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There are conflicting data to support the practice of delaying the introduction of allergenic foods into the infant diet to prevent allergy development. This study investigated immune response development after early oral egg antigen (Ovalbumin; OVA) exposure in a rat pup model. Brown Norway (BN) rat pups were randomly allocated into groups: dam reared (DR), DR pups challenged daily (days 4–13) with oral OVA (DR + OVA*c*), DR pups challenged intermittently (on day 4, 10, 12, and 13) with oral OVA (DR + OVA*i*), formula-fed pups (FF), and FF pups challenged daily with oral OVA (FF + OVA). Immune parameters assessed included OVA-specific serum IgE, IgG1, and IgA. Ileal and splenic messenger ribonucleic acid (mRNA) expression of transforming growth factor-beta (TGF-β1), mothers against decapentaplegic (Smad) 2/4/7, and forkhead box P3 (Foxp3) were determined. Ileum was stained for TGF-β1 and Smad4. *Results*. Feeding OVA daily to DR pups maintained systemic and local gut antibody and immunoregulatory marker mRNA responses. Systemic TGF-β1 was lower in DR + OVA*i* pups compared to DR and DR + OVA*c* pups. Feeding OVA to FF pups resulted in significantly greater OVA-specific IgE and IgG1, and lower IgA and TGF-β1 and Smad expression compared to DR pups. *Conclusions*. Early daily OVA exposure in the presence of maternal milk maintains immune markers associated with a regulated immune response, preventing early allergic sensitization.

1. Introduction

Allergic disease arises due to a complex interaction between genetic predisposition and environmental factors, breast or formula feeding and patterns of early microbial exposure [1–3]. The most common food allergies emerging in young infants are to egg and peanut antigens. Approximately 6–8% of children under three years of age are affected, with the incidence of these allergies increasing [4–7]. Food allergy to milk and eggs typically disappears by age three to five, however there are data to suggest that the natural history of food allergy may be changing and even food allergies, such as egg and milk, which we think of as typically transient are showing greater persistence into teenage and adult years [8, 9].

Antigen (allergen) stimulation of the mucosal immune system is thought to be critical for the development of oral tolerance. In early life, exposure to repeated doses of food antigens may help prime the developing immune response toward induction of oral tolerance [10]. The ability to develop tolerance to allergens also appears to coincide with the establishment of healthy gut colonization by commensal bacteria [11]. Failure to develop oral tolerance is thought to be associated with development of food-allergic disease. However, the mechanism(s) by which the normal intestinal immune system responds to food and its involvement in the development of food allergy remains unresolved. Understanding the mechanisms involved would allow for the potential to develop intervention strategies for the prevention of food allergy and also therapeutic treatments for infants who have already developed food allergy.

Oral tolerance to food antigens can be induced experimentally, but optimization of the dose used for sensitization

is critical [12]. For example, induction of tolerance to peanut requires a significantly higher oral dose than for egg. Animals fed high doses of chicken OVA secrete more interleukin-4 (IL-4; associated with allergy) and less TGF-β (associated with tolerance) than those fed low doses, where more TGF-β and less IL-4 are produced [13]. There are only a few studies in neonates assessing timing of antigen exposure in inducing oral tolerance. In an animal model, Strobel et al. [14] have shown that oral OVA given in the first week of life to mice induces humoral as well as cell-mediated immunity [14]. In contrast, recent studies associate early antigen exposure with development of tolerance [15, 16]. More research is required to determine the optimum intervention strategy to promote oral tolerance.

Maternal milk cytokines, such as TGF-β2 and interleukin (IL-10) have the potential to regulate immune responses to food antigens and promote tolerance [17–23]. Although the relationship between breastfeeding and allergy prevention is controversial [24–26], there has recently been a growing interest in the role of breast milk in regulating immune response development to food antigens as new foods are introduced into the diet [16, 27].

During infancy, T helper 1 (Th1) immune response development is important in preventing persistent T helper 2 (Th2) responses and the subsequent promotion of allergic disease [3]. The maturation of naïve T cells into committed effector and regulator cells depends on complex interactions between antigen, immune cells, and the immediate cytokine environment. TGF-β, which predominantly signals through the Smad family of proteins, plays a major role in the development of T-cell lineage. TGF-β induces development of Foxp3$^+$ T regulatory cells (Tregs) to promote tolerance [28, 29]. IL-4 together with TGF-β inhibits the generation of Foxp3$^+$ Tregs by promoting Th cells that secrete IL-10, but which do not have regulatory function [30]. TGF-β in the local gut environment plays an important role in development of the infant immune response to food antigens as they are introduced into the diet [23, 31].

The interactions between breastfeeding and the timing of food antigen encounter are key factors which influence food allergy development [15, 32]. Currently there is a concern that delayed feeding until after 6 months (traditional weaning age) may program the developing immune response toward sensitization instead of tolerance [33, 34]. In countries where delayed feeding has been recommended, rates of food allergy have escalated, including a greater than 5-fold increase observed in food anaphylaxis in Australian children under 4 years of age [35]. The local intestinal environment plays an important role in regulating immune response development during introduction of food antigens. Since analysis of the local gut immune response during oral antigen introduction is not ethically feasible in infants, we assessed in an atopic rat pup model the developing immune response after daily early oral OVA exposure (continuous), as compared to intermittent (occasional) OVA exposure. In this *in vivo* study we focused on an early weaning time point (day 14). The developing immune response was assessed when OVA was introduced into the diet during a critical time in early life. Formula-fed groups were included as controls, as we have previously shown sensitization after early oral antigen feeding in formula-fed pups [16]. Egg ovalbumin was used as the target antigen to assess antigen-specific responses as it is one of the most common causes of food allergy in infants.

2. Materials and Methods

2.1. Animals. The BN rat has a naturally occurring genetic predisposition toward allergy development [36–39]. BN rats were bred and housed in the Animal Facility of the Child, Youth and Women's Health Services, Adelaide and experimentation was completed with approval from the Child, Youth and Women's Health Services Animal Ethics Committee.

2.2. Cannulation and Maintenance. The details of the artificial rat milk (formula) composition (Wombaroo Food Products, South Australia, Australia; Table 1 of Supplementary Material available doi 10.1155/2012/396232) and the procedure for artificial feeding have been previously described [16, 23]. We have also previously shown that the artificial rat milk (formula) does not contain active TGF-β [18, 40]. Briefly, at day 4 of age, rat pups in the formula fed groups were lightly anesthetized using forthane (Isoflurathane) and surgically implanted with a flexible i.g. cannula. Artificial rat milk was delivered to rat pups through a polyethylene line connected to the cannula using a multisyringe infusion pump (KDS220 multisyringe infusion pump; KD Scientific). We have demonstrated that changes in immune markers are directly attributed to the formula and not the surgical procedure [17].

2.3. Experimental Design. Rat dams were fed a standard non-purified diet which does not contain OVA (Ridley Agriproducts Pty Ltd, Victoria, Australia). Rat pups from 12 BN litters were randomly assigned to groups ($n = 8$/group). Each group (including the dam reared groups) were composed of a mix of pups taken from litters originating from a number of different dams. A daily or an intermittent oral OVA exposure regime was used. There was five feeding groups: dam reared pups (DR), DR pups receiving daily oral gavage (0.1 mL) of 10 mg OVA/day (OVA: Sigma-Aldrich, St.Louis, Mo, USA) from day 4–13 (DR + OVA*c*), DR pups receiving an initial oral gavage of OVA at day 4 followed by subsequent gavage with OVA on day 10, 12, and 13, (0.1 mL) of 10 mg OVA/day (DR + OVA*i*), formula-fed pups (FF), and FF pups receiving a daily oral gavage (0.1 mL) of 10 mg OVA/d (FF + OVA). Rat pups were killed at day 14 (prior to weaning).

Blood was collected by cardiac puncture and sera stored at −80°C. The spleen was removed, snap-frozen in liquid nitrogen, and stored at −80°C. The gastrointestinal tract was excised, and tissue from the ileum was isolated and either weighed and snap-frozen in liquid nitrogen for later RNA and protein analysis or fixed in 4% neutral buffered formaldehyde for 24 hour and transferred to 70% (v/v) ethanol for later processing.

2.4. IgE, IgG1, and IgA Analyses. Serum OVA-specific IgE and OVA-specific IgG1 were quantified by ELISA as previously

Table 1

Gene	Forward primer	Reverse primer
TGF-β1	TGCGCCTGCAGAGATTCAAGTCAA	AAAGACAGCCACTCAGGCGTATCA
Smad2	TGAGCTTGAGAAAGCCATCA	TGTGTCCCACTGATCTACCG
Smad4	GGCATTGGTGTAGACGACCT	GGGGTTTCTTTGATGCTCTG
*i*Smad7	GCAGCAGTTACCCCATCTTC	TGATGGAGAAACCAGGGAAC
FoxP3	CCACACCTCCTCTTCTTCCTT	TGACTAGGGGCACTGTAGGC
Cyclophilin A	GGTTGGATGGCAAGCATGTG	TGCTGGTCTTGCCATTCCTG

described [23] but OVA was used for coating. Sera from the pups were diluted 5-fold for analysis in the ELISA assay. Standards and samples were added in duplicate and detected colorimetrically using 3,3′,5,5′ tetramethylbenzidine (TMB; Sigma-Aldrich Chemical Co., St. Louis, Mo, USA). The limits of detection for the OVA-specific IgE and OVA-specific IgG1 ELISA assays were 1.95 and 0.78 ng/mL, respectively. The plates were read with a Sunrise Magellan plate reader at 450 nm (Tecan Group Ltd, Mannedorf, Switzerland) and data expressed as ng immunoglobulin/mL sera.

IgA was quantified by ELISA using ileal tissue. Ileal protein lysates for use in the IgA ELISA were prepared as described in Tooley et al. [16]. Briefly, ileal protein lysates were prepared by adding a cocktail of protease inhibitors (Sigma-Aldrich Chemical Co., St. Louis, Mo, USA) to intestinal tissue (1 mL/100 mg tissue), which was then homogenized and centrifuged twice. Supernatants were collected, aliquoted, and stored at −80°C until analysed. Samples from pups for IgA analyses were diluted 1/2000 for DR groups and 1/5 for FF groups. The standard, purified rat IgAκ, capture antibody, mouse anti-rat IgA and the secondary, biotin mouse anti-rat IgA were all purchased from BD Biosciences (Franklin Lakes, NJ, USA). Briefly, 96-well plates (Greiner, Frickenhausen, Germany) were coated with 2 μg/mL mouse anti-rat IgA in phosphate-buffered saline (PBS) overnight at 4°C. The wells were washed five times with wash buffer (PBS/0.05% Tween20) and then blocked for 1 hour at room temperature with 1% Polypep protein digest (Sigma-Aldrich Chemical Co., St. Louis, Mo, USA) in PBS. The samples and standards (purified rat IgA; standard range: 125 ng/mL to 1.95 ng/mL) were then added to the plate and incubated for 1 hour at room temperature. After incubation, the plates were washed five times and biotin mouse anti-Rat IgA was added (0.5 μg/mL). Plates were incubated at room temperature for 1 hour and then washed six times. Following the final wash, a solution of ABC reagent (Vector Laboratories, Inc. Burlingame, Calif, USA) was added and the plates incubated for 30 minutes at room temperature. Plates were then washed six times; after washing TMB substrate was added to the wells for 30 minutes after which time the reaction was stopped using 50 μL of 2 N HCl and read at an absorbance of 450 nm. The limit of detection for the IgA was 1.95 ng/mL. Data was expressed as ng immunoglobulin/g of tissue.

2.5. Real-Time PCR. RNA extraction from the spleen and ileum, cDNA synthesis, primer design, real-time PCR, and analysis were performed as previously described [16]. Primers for TGF-β1, Smad2, Smad4, *i*Smad7, and Foxp3 are provided in Table 1.

2.6. Histological Assessment. Immunohistochemical analyses of TGF-β1 and Smad4 were carried out on segments of the ileum. Four-micrometer sections were cut from paraffin-embedded tissue and placed on gelatin-coated slides. Sections were deparaffinized with xylene and rehydrated in graded ethanol in water. Sections were then placed in 10 mM citrate buffer (1.8 mM citric acid; 8.2 mM sodium citrate, pH 6.0) and subjected to heat-induced epitope recovery using microwave irradiation [41]. Sections were then cooled at room temperature for 30 minutes before staining.

For TGF-β1, sections were stained as described in Penttila et al. [40]. For Smad4 staining, tissue sections were first incubated with 5% normal horse serum/1% bovine serum albumin (5% NHS/1% BSA) in Tris buffered saline (TBS) for 30 minutes at room temperature to block nonspecific binding of the secondary antibody. The blocking antibody was then decanted and 100 μL of anti-Smad4 IgG (8 μg/mL; Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA) was added, and the sections were incubated overnight at 4°C. After incubation the sections were then washed three times in TBS containing 0.05% Tween 20 (TTBS; 5 minutes/wash) and then incubated in 3% (v/v) hydrogen peroxide for 15 minutes at room temperature to quench endogenous peroxidase activity. The sections were then washed three times with TBS (5 minutes/wash) after which the secondary antibody (HRP conjugated donkey anti-mouse—3.2 μg/mL; Jackson Immuno Research Laboratories, West Grove, PA. USA) was applied to the sections (100 μL per section) and the sections incubated for 60 minutes at room temperature. The sections were then washed with TTBS (5 minutes/wash) two times followed by two washes with TBS (5 minutes/wash). For both TGF-β1 and Smad4 staining, immunohistochemistry reactions were visualized using a 3,3-diaminobenzidine (DAB) substrate plus enhancer (Invitrogen, Carlsbad, Calif, USA). After substrate development, sections were counterstained, dehydrated with graded ethanol, and mounted.

Control samples for TGF-β1 included sections incubated with normal chicken IgY (R&D Systems Inc, Minneapolis, MN, USA) or with antibody dilution buffer only. Control samples for Smad4 included sections incubated with 5% NHS/1% BSA (R&D Systems Inc, Minneapolis, MN, USA) or with the isotype control, mouse IgG1 (8 μg/mL). Digital images of both TGF-β1 and Smad4 immunohistochemical sections (400x magnification) were taken and analysed using

Image Pro Plus software, version 5.1 (Media Cybernetics, Bethesda, Md, USA).

2.7. Statistical Analyses. All data were expressed as the mean + standard error of the mean (SEM). Data was assessed for Normality before analysis. OVA-specific IgE and IgG1 and TGF-β1, Foxp3, Smad2, Smad4, and *i*Smad7 mRNA expression data were evaluated utilizing a nonparametric one-way ANOVA (Kruskal-Wallis) followed by a Dunn's Multiple Comparisons post hoc test. Differences were considered significant at $P < 0.05$. All statistical analyses and comparisons were made using GraphPad Prism software, version 3 (GraphPad Software Inc, San Diego, Calif, USA).

3. Results

3.1. Bodyweight Change. Feeding OVA to either DR or FF pups did not affect body weight gain at day 14 (data not shown).

3.2. OVA-Specific IgE, OVA-Specific IgG1 and IgA. OVA given during formula feeding resulted in a significantly increased OVA-specific IgE titer compared with the DR and DR + OVA*c* groups ($P < 0.05$; Figure 1(a)). Serum OVA-specific IgG1 was also significantly increased in the FF + OVA group ($P < 0.05$) compared with the DR, DR + OVA*c*, DR + OVA*i*, and FF groups (Figure 1(b)). Importantly, OVA-specific IgG1 titers did not differ significantly between the DR groups regardless of oral OVA exposure. IgA was significantly greater in the DR, DR + OVA*c*, DR + OVA*i* groups ($P < 0.01$) and barely detectable in the FF and FF + OVA groups (Figure 1(c)). IgA levels did not differ between the DR groups.

3.3. TGF-β1 and Smad mRNA Expression in Spleen and Ileum. TGF-β1 mRNA expression in the spleen was significantly greater in the DR and DR + OVA*c* groups compared with the DR + OVA*i*, FF and FF+OVA groups ($P < 0.05$; Figure 2(a)). Splenic TGF-β1 mRNA expression did not differ significantly between the DR and DR + OVA*c* groups. TGF-β1 mRNA expression in the ileum was significantly greater in all DR groups regardless of OVA exposure compared with the FF and FF + OVA groups ($P < 0.05$; Figure 2(a)); however there were no significant differences in ileal TGF-β1 mRNA expression between the DR groups. Foxp3 mRNA expression in the spleen was significantly greater in the DR, DR + OVA*c*, and FF groups compared with the DR + OVA*i* and FF + OVA groups ($P < 0.05$; Figure 2(b)). Expression did not differ significantly between the DR, DR + OVA*c*, and FF groups. Although the mRNA expression of Foxp3 in the ileum did not differ between the DR groups, expression was significantly greater in DR groups compared with the FF and FF + OVA groups ($P < 0.05$; Figure 2(b)).

The Smad pathway was also investigated by analyzing the mRNA expression of Smad2, Smad4, and *i*Smad7 in the spleen and ileum. In the spleen, Smad2 mRNA expression was significantly greater in DR group compared with the DR + OVA*i* and FF + OVA groups ($P < 0.05$; Figure 3(a)). Expression of splenic Smad2 mRNA did not differ significantly between the DR, DR + OVA*c*, and FF groups. Smad 4 mRNA expression in the spleen was significantly greater in DR and FF groups compared with the DR + OVA*i* group ($P < 0.05$; Figure 3(b)). No significant difference in Smad4 mRNA expression was observed between the DR, DR+OVA*c*, FF, and FF + OVA groups. *i*Smad7 mRNA expression in the spleen was significantly greater in the DR and DR + OVA*c* groups compared with the DR + OVA*i* and FF + OVA groups ($P < 0.05$; Figure 3(c)). No significant difference in *i*Smad7 mRNA expression in the spleen was observed between DR, DR + OVA*c*, and FF rats. In the ileum, Smad2, Smad4 and *i*Smad7 mRNA expression was significantly greater in the DR groups regardless of OVA exposure compared with FF and FF + OVA groups ($P < 0.05$; Figures 3(d), 3(e), and 3(f)). There were no significant differences in Smad2, Smad4, and *i*Smad7 mRNA expression in the ileum between the DR, DR + OVA*c*, and DR + OVA*i* groups.

3.4. TGF-β1 and Smad4 Protein Expression in the Ileum. TGF-β1 staining was mainly localized to the enterocytes and occasional individual cells in the villus lamina propria of the ileum (Figures 4 (a), 4(b), 4(c), 4(d), and 4(e)). No TGF-β1 staining was evident at the base of the villus in the crypts or the surrounding lamina propria. Staining of Smad4 was localized throughout the enterocytes of the villi and in cells of the lamina propria in all rat groups (Figures 4(g), 4(h), 4(i), 4(j), and 4(k)). Smad4 was not detected in goblet cells or the longitudinal layer of smooth muscle. TGF-β1 and Smad4 staining was consistently more abundant in the DR groups regardless of OVA exposure when compared to staining in sections from FF or FF + OVA rats. No background staining was detected in negative controls (Figures 4(f) and 4(i)).

4. Discussion

We investigated the immune response profile after early oral OVA exposure in DR and FF rat pups. Early oral OVA exposure in rat pups, regardless of the dosage regime, in the presence of maternal milk maintained a similar immune response profile to that observed in DR unchallenged rats, with low levels of circulating OVA-specific IgE; and IgG1. In contrast to the low OVA IgG1 response seen in the rat pups fed formula alone (no OVA challenge), the IgE response to OVA was high (not significantly different from that seen in the OVA challenged formula fed rat pups). We have previously shown that formula feeding induces an overall increase in total serum IgE, this increased IgE response may contain cross-reactive antibodies to OVA [16]. Formula fed groups were only included as controls in this study, as we have previously shown sensitization after early oral antigen feeding in formula-fed pups [16]. The results seen for OVA-specific IgG1 are similar to our previous published data relating to feeding cow's milk allergen, β-lactoglobulin (BLG), where we showed that sensitization was prevented in maternal-milk-fed pups given oral BLG early in life. Importantly we showed that this regulated immune profile persisted into postweaning age [16, 23]. In contrast, immune

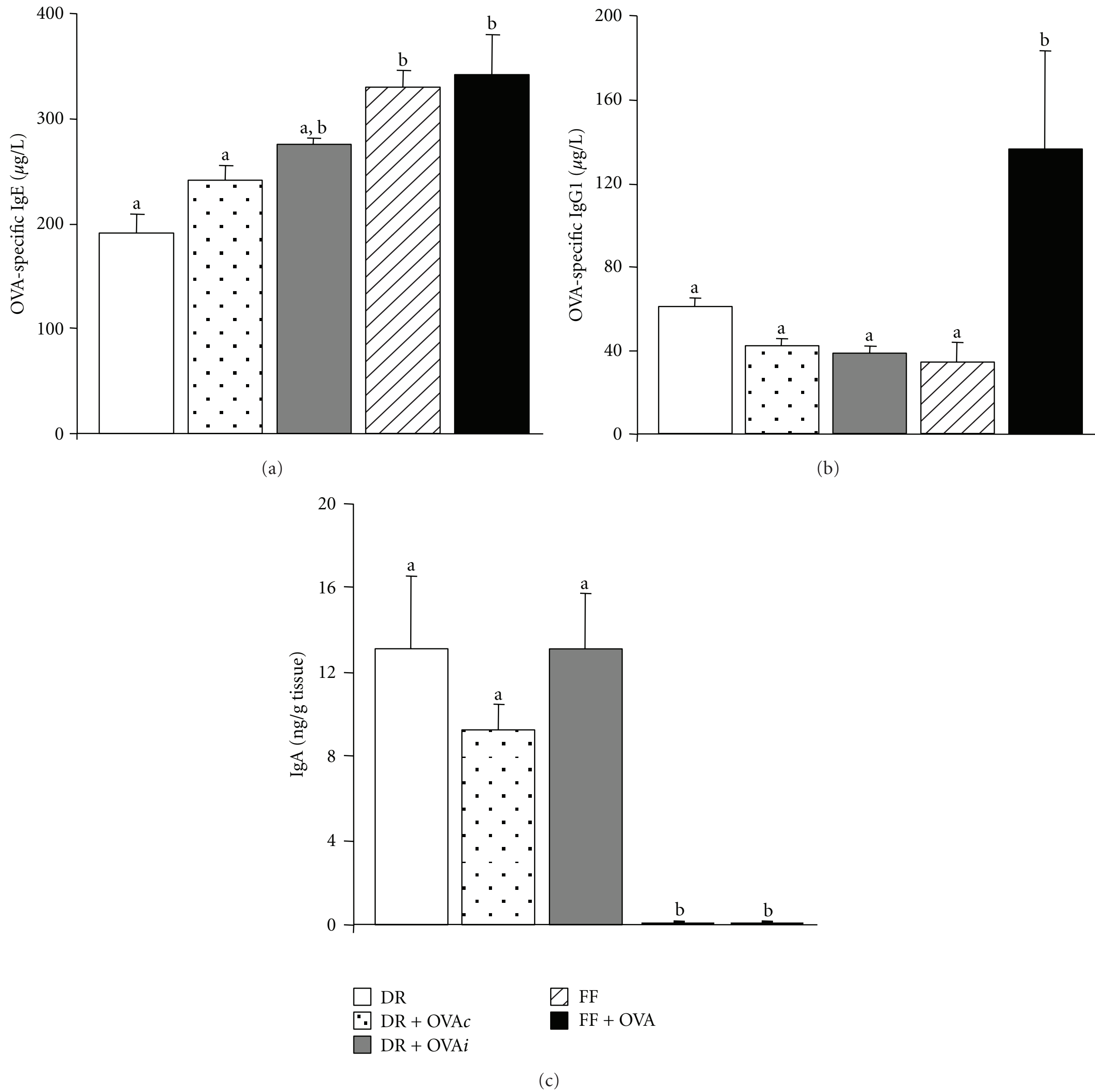

Figure 1: OVA-specific IgE, OVA-specific IgG1, and ileal IgA after oral OVA commenced at day 4 in DR or FF rat pups. Bars are mean + SEM, $n = 8$/group. Means without a common letter differ, $P < 0.05$.

activation and allergy development resulted when BLG was fed in the presence of formula [16].

TGF-βs are an important family of growth factors involved in maintaining homeostasis in the intestine, regulating inflammation and allergy development and promoting oral tolerance development in infants [31]. TGF-β is the predominant cytokine present in human and rodent milk [40, 42]. TGF-β predominately signals through the Smad protein family. Smad2 and Smad3 are phosphorylated after activation of TGF-β receptors, forming a complex with Smad4. Once translocated into the nucleus, this complex then binds to the Smad binding element in the promoter region of TGF-β target genes and regulates transcriptional responses in conjunction with DNA-binding partners [43, 44]. By also assessing Smad genes involved in the pathway we have been able to further elucidate the function of TGF-β1 in the development of immune responses in the gut when OVA was introduced. In the DR + OVA*c* group, TGF-β1 mRNA expression in both the spleen and the local gut environment did not differ significantly from that in unchallenged DR rats. However, DR rats receiving oral OVA intermittently displayed decreased TGF-β1 and Smad2, Smad4, and *i*Smad7 mRNA expression in the spleen. Collectively, we have shown that the DR + OVA*i*, FF, and FF + OVA groups exhibited the greatest systemic impairment of the TGF-β1/Smad pathway with lower mRNA expression of the TGF-β1/Smad genes. However, in the intestine, which is the first site of exposure to food antigens we observe a different

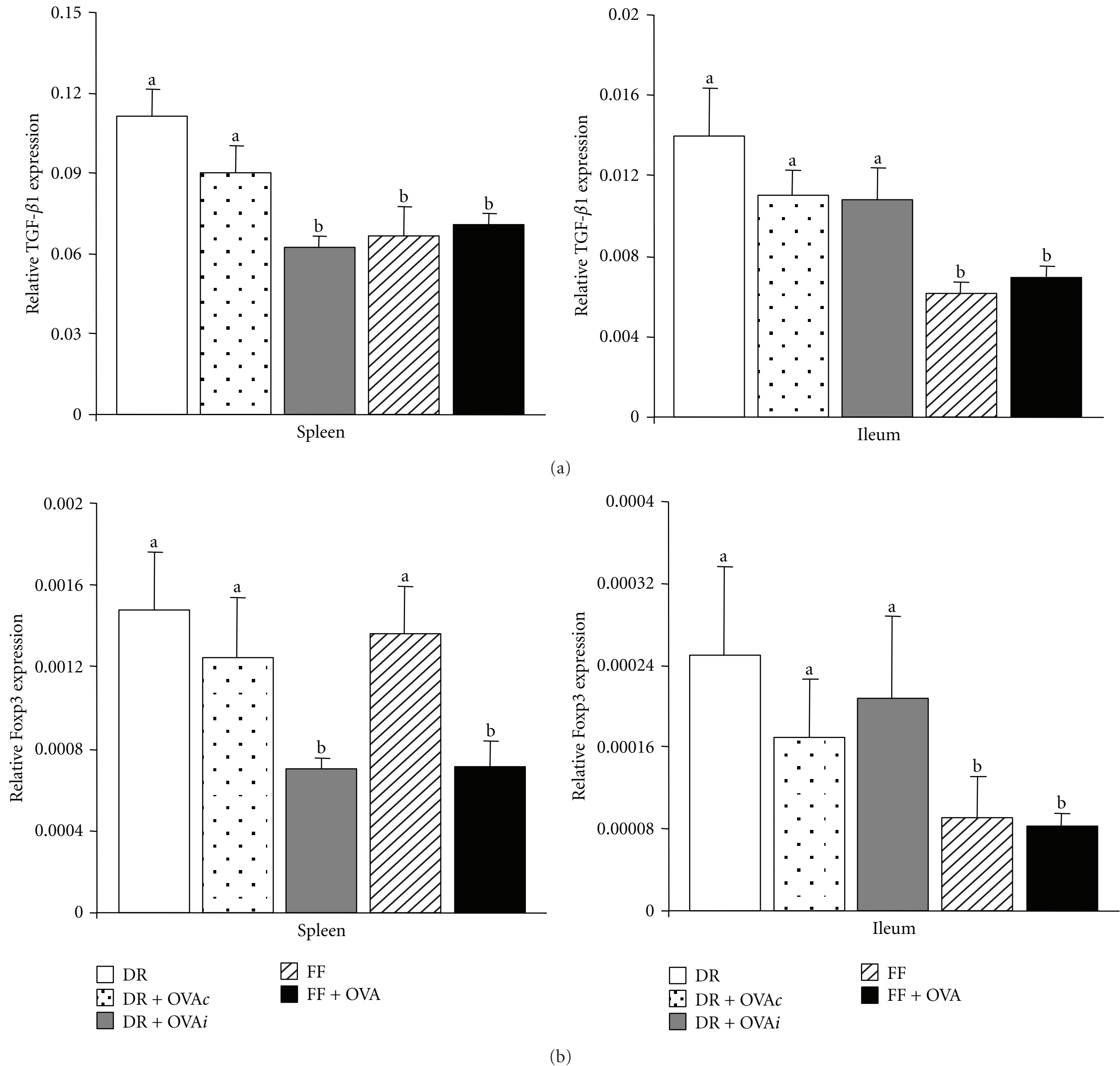

Figure 2: Splenic and ileal cytokine mRNA expression in DR and FF pups at day 14 with or without daily/intermittent treatment with OVA. TGF-β1 mRNA (a) and Foxp3 mRNA (b) as determined by real-time PCR. Bars are mean + SEM, $n = 7$-8. Means without a common letter differ, $P < 0.05$.

pattern of expression. DR rats exposed to OVA intermittently maintained a TGF-β1/Smad mRNA expression profile similar to the unchallenged DR group. One possible explanation is that external sources of TGF-β, provided by maternal milk, are sufficient to maintain the expression of these genes in the local gut environment. Our current data also shows that in the ileum of FF rats, with or without OVA exposure, lower levels of all Smad mRNA's were present when compared to the DR groups. High levels of TGF-β are present in rat milk during early lactation, with the highest levels detected just after birth. TGF-β levels then decrease toward weaning. In contrast in maternal-fed rat pups, the number of TGF-β1-producing cells and mRNA in the intestine is low after birth, but levels increase over the weaning period [40]. As TGF-β is essential for maintaining homeostasis in the intestine and promotion of T regulatory cells, our data suggests that the local gut environment in FF pups is impaired with regard to the potential for developing regulated immune responses to food antigens. We have shown in previous studies that when BN rat pups are fed a formula supplemented with physiological levels of TGF-β, markers associated with allergy development are reduced and the immune response profile to the cow's milk allergen, BLG, is not significantly different to that seen in unchallenged maternal-milk-fed pups. This regulated immune response profile extended out to postweaning ages, highlighting the importance of TGF-β in

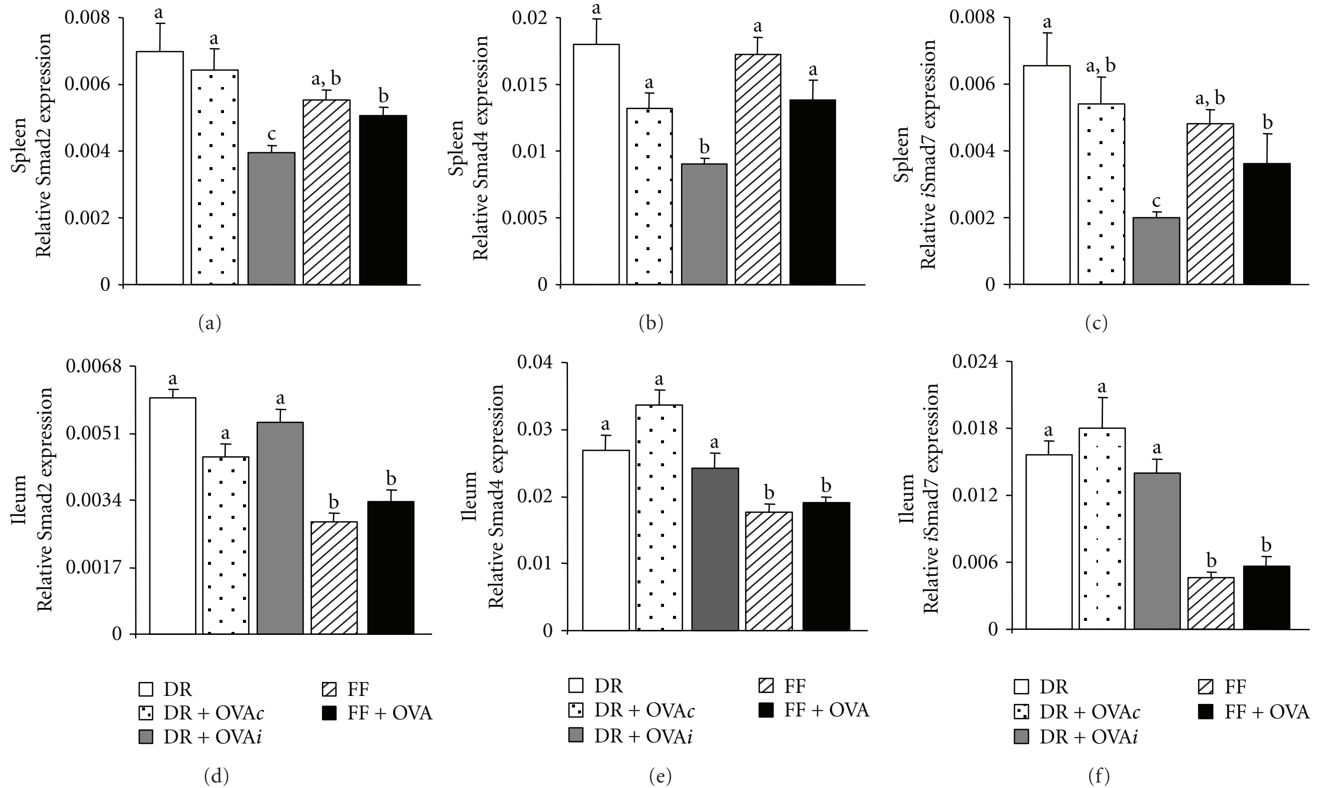

FIGURE 3: Splenic and ileal mRNA expression of Smad pathway genes in DR and FF pups at day 14 with or without daily/intermittent treatment with OVA. Smad2, 4, and 7 mRNA levels in spleen (a, b, and c) and Smad2, 4 and 7 mRNA levels in ileum (d, e, and f) as determined by real-time PCR. Bars are mean + SEM, $n = 7$-8. Means without a common letter differ, $P < 0.05$.

developing and preventing sensitization to food antigens [23].

TGF-β1 signals are controlled by inhibitory *i*Smads, predominantly *i*Smad7 [43, 44]. We have shown, particularly in the ileum, that TGF-β1 and *i*Smad7 mRNA levels maintain a homeostatic balance, possibly by forming a negative feedback loop. It has been documented that the transcription of *i*Smad7 can be turned on by TGF-β itself in the TGF-β/Smad signalling pathway [45]. This suggests that even in the presence of early oral antigen challenge the mucosal immune system can develop and maintain such regulatory mechanisms.

TGF-β signaling promotes T-cell tolerance and helps maintain normal homeostasis throughout the lifespan. TGF-β preferentially increases IgA antibody responses by directing isotype switching to IgA in Peyer's patches [46]. Ogawa et al. [47] showed in a study of newborn infants during their first month of life that an increase of serum IgA correlated with levels of both TGF-β1 and TGF-β2 in maternal colostrum [47]. Our data supports the role of TGF-β in regulating IgA levels during early food introduction in the presence of maternal milk. We have shown that early oral OVA exposure in the presence of maternal milk, as compared to formula, maintained IgA levels. In the FF groups, IgA levels were only slightly above the detection limit of the assay.

As well as being involved in epithelial growth, IgA production, DC maturation, and Treg cell differentiation, TGF-βs inhibit inflammation and regulate inflammatory responses in the intestine [17, 48–51]. In the adult intestine, TGF-β1 is the predominant isotype present in epithelial and lamina propria cells [52]. We assessed the localization pattern of both TGF-β1 and Smad4 in the intestine of DR and FF rats with or without antigen exposure. More abundant staining of TGF-β1 and Smad4 was observed in all the DR groups, regardless of antigen exposure compared with the FF groups. The histology supports our TGF-β1 and Smad4 mRNA expression data in the ileum and again highlights the potential for sensitization in FF rat pups. We have demonstrated in the local gut environment that formula feeding early in life results in an overall suppression of TGF-β1 and the signaling genes involved in its pathway, namely, Smad2, Smad4, and *i*Smad7.

TGF-β is also required for induction of Tregs, which play a critical role in maintaining immune homeostasis in the intestine. Foxp3$^+$ (CD4$^+$CD25$^+$Foxp3$^+$) regulatory cells are necessary for the development of oral tolerance [53–56]. Foxp3 mRNA expression was maintained in the ileum of DR rats receiving OVA daily with expression levels similar to that seen in the ileum of unchallenged DR rats. Ileal Foxp3 mRNA expression in the DR + OVA*i* group did not differ from the DR or DR + OVA*c* groups but a decrease in splenic Foxp3 mRNA was observed in the DR + OVA*i* group. A decrease in Foxp3 mRNA expression was also observed in both the ileum and spleen of FF rat pups receiving OVA. We have previously shown that this decrease

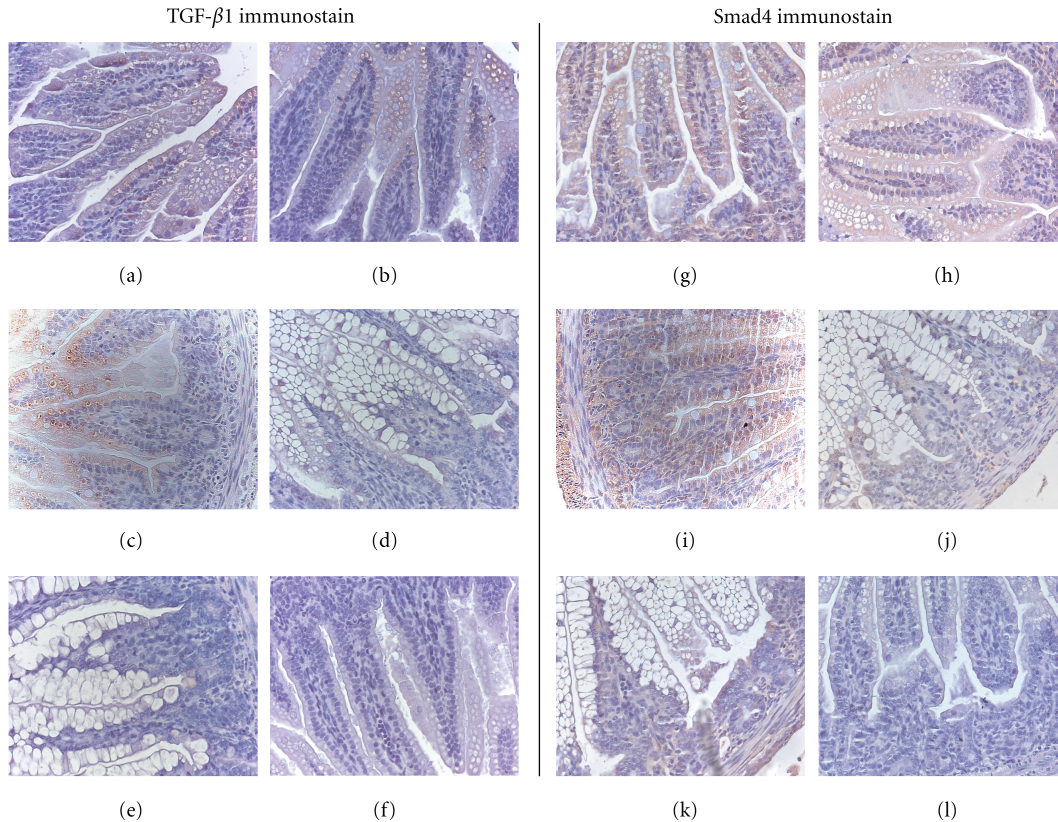

FIGURE 4: Mucosal immunolocalization of TGF-β1 and Smad4 in DR and FF pups at day 14 with or without daily/intermittent treatment with OVA. Representative images are shown for all groups. Positive staining is indicated as a brown color. TGF-β1 (a–f: DR (a), DR + OVA*c* (b), DR + OVA*i* (c), FF (d), FF + OVA (e), and negative control (f)) and Smad4 (G-L: DR (g), DR + OVA*c* (h), DR + OVA*i* (i), FF (j), FF + OVA (k), and negative control (l)).

in FoxP3 mRNA expression was also noted in the mesenteric lymph node of FF BN rat pups at day 14, and that the frequency of Foxp3$^+$ cells was greater in maternal-fed BN rat pups receiving a continuous dose of BLG [16]. The role of Foxp3$^+$/CD25$^+$/CD4$^+$ Tregs in development of food allergy is at present unclear. It has been shown that Foxp3$^+$cells are present in the intestine of food allergic children, but Foxp3 transcription levels are low [57]. In contrast, other studies report lower expression of Foxp3 and defects in transcription [55]. It has been shown that repeated small doses of antigens are necessary for the development of oral tolerance mediated by Treg cells [58]. Our results suggest that continuous as opposed to intermittent antigen exposure in the presence of maternal milk maybe required to promote Treg cells and Foxp3 expression in the periphery. A daily repeated exposure to OVA as compared to an intermittent (occasional) exposure may allow the immature immune system time to "practice" and therefore help to prime for development of a regulated immune response to prevent sensitization, potentially enhancing later tolerance development.

In infants with a predisposition toward allergy development, delaying the feeding of solids until after 6 months may program the developing immune response toward sensitization [15, 59]. Factors such as the duration of exclusive breastfeeding, timing of introduction, and the type of other foods (allergens) in the diet are also thought to influence the switch between tolerance and sensitization [15]. In support of this, in a previous time course study of early cow's milk allergen exposure (BLG was commenced at day 4 of life) we showed a reduction in the levels of markers associated with allergy development at day 10, 14, and 21 of life after BLG exposure in the presence of maternal milk [16].In our current study assessing an early weaning time point (day 14 of life) we show an upregulation of the levels of markers associated with immuno-regulatory mechanisms after early OVA exposure. Daily but not intermittent oral OVA exposure commenced on day 4 during maternal milk feeding created an immune environment with the potential to decrease sensitization to food antigens. Foxp3 mRNA expression, TGF-β1 mRNA and protein expression, and expression of the Smad genes involved in TGF-β signaling were maintained in both the microenvironment of the gut and the periphery. Early regular exposure to food antigens (OVA) in the presence of maternal milk in early life maintains immune regulatory mechanisms preventing allergic sensitization.

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Review Article

Shining a Light on Intestinal Traffic

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Inflammatory bowel disease (IBD), encompassing Crohn's disease and ulcerative colitis, is associated with enhanced leukocyte infiltration to the gut, which is directly linked to the clinical aspects of these disorders. Thus, leukocyte trafficking is a major target for IBD therapy. Past and emerging techniques to study leukocyte trafficking both *in vitro* and *in vivo* have expanded our knowledge of the leukocyte migration process and the role of inhibitors. Various strategies have been employed to target chemokine- and integrin-ligand interactions within the multistep adhesion cascade and the S1P/S1PR1 axis in leukocyte migration. Though there is an abundance of preclinical data demonstrating efficacy of leukocyte trafficking inhibitors, many have yet to be confirmed in clinical studies. Vigilance for toxicity and further research is required into this complex and emerging area of IBD therapy.

1. Introduction

Leukocyte migration is fundamental to immunologic mobilization in response to insult and injury. A coordinated series of molecular events underpins the trafficking of lymphocytes and granulocytes into stressed tissue with participation of adhesion molecules, chemokines, and their receptors.

The inflammatory bowel diseases (IBDs), Crohn's disease (CD) and ulcerative colitis (UC), are distinct syndromes, but both are associated with enhanced leukocyte trafficking to the inflamed gut. Thus, targeting the multistep leukocyte adhesion cascade has been employed as a therapeutic strategy. Here, we focus on the contribution of leukocyte trafficking to the pathogenesis of IBD. An overview of strategies employed to target leukocyte recruitment and of emerging models used to test these targets is presented.

2. IBD

Crohn's disease and ulcerative colitis are recurring, relapsing, and remitting disorders characterized by chronic inflammation of the intestinal mucosa. Though they share some common clinical symptoms such as diarrhea and abdominal pain, CD and UC possess very distinct features. Crohn's disease manifests as a transmural inflammation that can potentially develop anywhere along the gastrointestinal tract but primarily occurs in the terminal ileum and proximal colon. Ulcerations, granulomas, and bowel fistulas are characteristic histopathological features. In contrast, ulcerative colitis seldom ulcerates and is a relatively superficial inflammation of the mucosa that is diffuse, continuous, and restricted to the colon, usually extending proximally from the rectum. It is characterized by significant goblet cell depletion and crypt abscesses, but granuloma development is not a feature. In both CD and UC, leukocyte infiltration into the inflamed intestine is fundamental to disease development and perpetuation. The infiltrated effector cells resist apoptosis and persistently release harmful inflammatory cytokines causing tissue damage. In CD, the characteristic granulomas form upon dense accumulation of activated T cells and macrophages. Ulcerative colitis is characterized by excessive mucosal infiltration of T cells and neutrophils, the latter forming the characteristic crypt abscesses [1]. The pivotal role of T cells, neutrophils, and their proinflammatory cytokines in the pathogenesis of IBD has been reviewed elsewhere [2, 3].

Genetic and environmental risk factors have been implicated in IBD (for reviews see Xavier and Podolsky [1], Melgar and Shanahan [4], and Cho and Brant [5]). Despite extensive research, current therapeutic options in IBD remain limited, often varying in their maintenance, toxicity, and tolerability [6–8]. Novel therapeutic strategies for IBD are needed.

3. Leukocyte Trafficking

3.1. In Vitro and In Vivo Models of Leukocyte Trafficking

3.1.1. Traditional Methods. The concept of lymphocyte recirculation and homing was first demonstrated by Gowans and Knight in 1964, when they followed the migration of radiolabeled lymphocytes in rats using autoradiography and scintography [9]. They and others found that while naïve lymphocytes migrated to all secondary lymphoid tissues, activated lymphocytes preferentially migrated back to the tissue in which they had been exposed to the antigen. In order to devise rational therapeutic strategies that target leukocyte migration, it is important to elucidate leukocyte trafficking patterns *in vivo.* Techniques employed to study leukocyte trafficking frequently involve *ex vivo* labeling of donor cells and adoptive transfer into recipient animals. Subsequently, the distribution of the labeled cell population in recipient tissues is assessed using a variety of imaging methods, such as histological analysis of fixed tissues. This technique has provided us with valuable mechanistic knowledge, but it does not permit direct examination of dynamic processes at single-cell level or provide temporal or spatial information within the physiological environment of lymphoid tissues. Furthermore, labeling of cells *ex vivo* has been associated with variable labeling efficiency, alteration of cellular functions, and label elution postadoptive transfer. Radiolabeling techniques, such as white cell scintigraphy, have been used to successfully study neutrophil migration in clinical IBD studies [10]. However, such methods are hampered by radioactive decay, poor resolution, and cellular toxicity [11]. Myeloperoxidase (MPO) assays are commonly used to study neutrophils and quantitate neutrophil influx, but they do not distinguish between neutrophils and macrophages and can be problematic to carry out. Similarly, while *in vitro* chemotaxis assays are regularly employed to analyze the effects of potential inhibitors on cell migration, there is no guarantee that the cells will respond in the same way to the test compound *in vivo.* Multiphoton intravital microscopy has been widely used to image the dynamic movement of lymphocytes by tracking fluorescently labeled cells in exposed or explanted lymph nodes (LNs) of living animals. This technique has provided valuable insights into the dynamics of T- and B-cell homing to LNs [12, 13] and allows single-cell tracking, in conjunction with high-resolution images. Nonetheless, intravital microscopy is an invasive technique, and the surgery required may interfere with the flow of blood and lymph creating experimental artifacts.

3.1.2. Molecular Imaging Techniques. Molecular imaging is defined as "the visualization, characterization and measurement of biological processes at the molecular and cellular levels in humans and other living systems" [14]. Over the past decade, a number of such techniques have been adapted to small animal imaging, offering dynamic imaging methods to localize leukocytes *in vivo*. Positron emission tomography (PET) [15] and single-photon emission-computed tomography (SPECT) [16, 17] use scintillation cameras and other devices to detect radioactive emission from radiolabeled cells within the body. Though these nuclear imaging methods have excellent tissue penetration and cell quantitation capability, they too are subject to the drawbacks associated with radiotracers and exogenous labeling of cells. Magnetic resonance imaging (MRI), which relies on the nuclear resonance of protons in tissues upon scanning with radio frequency radiation, has shown great promise to track the recruitment of, for example, antigen-specific $CD8^+$ T cells to target tumors *in vivo* [18]. However, the time required for imaging using this technique makes it unsuitable for tracking fast-moving cells [19]. The substantial expense of MRI and its relatively poor sensitivity and quantitative capability have also hindered its use as a basic research tool. Optical fluorescence imaging has been frequently used to track T cells and has provided us with invaluable data on their migration patterns. The pitfalls with using fluorescent labels, such as CFSE and GFP, include signal loss due to label dilution upon cell division and limited sensitivity and specificity due to endogenous tissue autofluorescence and light scattering and absorption [20].

3.1.3. Bioluminescence Imaging. To illuminate cell trafficking *in vivo* and to test specific inhibitors of this migration, we used bioluminescence imaging (BLI) technology [21–23]. This form of optical imaging has several advantages over other techniques for tracking cell migration. It eliminates the necessity to prelabel cells, avoiding problems with exogenous cell labeling. It allows direct *in vivo* and *ex vivo* visualization, with no further processing of tissues required, and is, therefore, a less complicated and less labor intensive technique than most other *in vitro/ex vivo* methods. Unlike other molecular imaging methods, BLI combines high sensitivity with relatively low cost while providing quantitative, spatial, and temporal data. However, this technology has limitations. Since light transmission through animal tissues is wavelength dependent, loss of photon signal can occur with tissue depth and light sources closer to the surface of the animal can appear brighter. In addition, validation of potential therapeutics using BLI should be carried out in conjunction with *in vitro* mechanistic assays and *in vivo* efficacy studies. BLI has successfully monitored trafficking of bone marrow mononuclear cells in ischemic myocardium [24] and $CD4^+$ T cells in an experimental model of multiple sclerosis (MS) [25]. These and other studies have shown robust and reliable correlation between cell numbers and bioluminescence signals. We employed BLI to track both neutrophils and lymphocytes in murine models of experimental colitis and to test potential inhibitors of their migration Figure 2 [21, 22].

3.2. The Leukocyte Trafficking Cascade

3.2.1. Selectins, Integrins, and Their Ligands. Circulating leukocytes are subjected to extreme conditions with the flow of blood exerting a shearing stress on the cells dislodging any that touch the vascular wall. To leave the circulation and home to specific tissues, leukocytes must engage several adhesion pathways involving intimate interaction with endothelial cells [26]. Whether during physiological recirculation or inflammatory conditions, the mechanisms

involved in leukocyte trafficking are effectively the same. The leukocyte trafficking cascade is depicted in Figure 1. Leukocyte recruitment begins with tethering and rolling of the cells along the microvascular endothelium via three selectins: L-selectin, expressed by leukocytes, and E-selectin and P-selectin, expressed by inflamed endothelial cells on the blood vessel wall [27]. The ligand P-selectin glycoprotein ligand-1 (PSGL-1) binds all three selectins and plays an important role in leukocyte recruitment under inflammatory conditions [28]. L-selectin is constitutively present on T cells and interacts with its counter receptors, peripheral lymph node addressin (PNAd) and mucosal addressin cell adhesion molecule (MAdCAM)-1, acting as a mechanical anchor or tether to the high endothelial venules (HEVs). This allows the lymphocytes to roll along the vascular lining at a much slower pace than erythrocytes. Selectins engage rapidly and form strong bonds to secure contact. These bonds allow chemokines and their ligands to transmit activating signals for the next step in the migration cascade. Targeting selectins and their ligands as a treatment strategy for inflammatory disorders has been reviewed elsewhere [29].

Selectin bonds are unable to arrest cells at the vessel wall. Firm leukocyte adhesion is achieved through bonds formed downstream by the secondary adhesion molecules, integrins. Integrins are diversely expressed on different leukocyte subpopulations and are composed of noncovalently linked α and β chains. The α_4 integrins, $\alpha_4\beta_1$ and $\alpha_4\beta_7$, play a regulatory role in lymphocyte homing and recruitment to inflammatory tissues, particularly to the inflamed intestine. Two decades ago, it was revealed that memory T cells from the gut preferentially homed to the gut [30]. This phenomenon is linked to the expression of unique adhesion molecules within the mucosa [31, 32]. Both $\alpha_4\beta_1$ and $\alpha_4\beta_7$ are expressed by lymphocytes that reside in the gut and gut-associated lymphoid tissues (GALTs), and their respective ligands vascular cell adhesion molecule (VCAM)-1 and MAdCAM-1 are expressed within the HEV's in Peyer's patches (PPs) and the flat-welled venules of the lamina propria [33]. While the $\alpha_4\beta_7$-MAdCAM-1 interaction is restricted to leukocyte trafficking to the gut and GALT, the $\alpha_4\beta_1$-VCAM-1 pathway can also mediate leukocyte homing to the central nervous system, specifically to the inflamed brain [34]. Interestingly, mice deficient in the β_7 integrin gene are unable to form proper PPs and possess decreased numbers of lamina propria $CD4^+$ T cells and B cells [35]. The β_2 integrins are also prominent participants in leukocyte trafficking, mediating firm adhesion, particularly in the case of neutrophils [36]. The β_2 integrin lymphocyte function-associated antigen (LFA)-1 is predominantly expressed by lymphocytes and neutrophils and binds to its endothelial cell ligands intercellular adhesion molecule (ICAM)-1 and ICAM-2. In addition to leukocyte arrest, integrins can participate in leukocyte rolling. Under inflammatory conditions, lymphocytes can skip the selectin-mediated phase and bind directly to endothelial cells via $\alpha_4\beta_7$ [37].

Abundant evidence reveals that IBD is associated with enhanced leukocyte trafficking to the gut mucosa and altered expression of adhesion molecules [38]. Cytokines such as interleukin (IL)-1β, IL-6, and TNF-α, produced upon stimulation of innate immune cells at inflammatory sites [39], upregulate adhesion molecules and chemokines, enhance leukocyte recruitment, and amplify the inflammatory cascade. Expression of MAdCAM-1 is upregulated in animal models of colitis [40, 41] and in active IBD [42–44]. ICAM-1 and LFA-1 have also been implicated in a number of experimental animal models of IBD [45, 46]. The importance of adhesion molecules in IBD is evident from preclinical colitis studies, where their blockade ameliorated disease severity [47]. Table 1 summarizes preclinical and clinical data reported so far. Similar results have been reported in animal models of autoimmune disease including MS and rheumatoid arthritis [48].

3.2.2. Chemokines and Their Receptors. Chemokines mediate cell migration under normal physiological conditions and leukocyte recruitment to tissues during innate and adaptive immune responses. These small heparin-binding proteins come from a diverse family that is classified into four major subfamilies, CC, CXC, C, and CX_3C, based on structural and functional differences. The two most important subgroups in terms of leukocyte trafficking to inflamed tissues are the CC chemokines for dendritic cell (DC) and lymphocyte recruitment and the CXC chemokines for recruitment of neutrophils and monocytes. Chemokines exert their biological effects on target cells by binding to specific G-protein-coupled transmembrane receptors (GPCRs) on the cell surface and activating an intracellular signaling cascade. Consequently, an activating signal is sent to the integrin switching it into a high-affinity/high-avidity state so that the rolling leukocyte can arrest itself and firmly adhere to the HEVs, a step which is essential for leukocyte extravasation into the target tissue [74]. For example, binding of CCR7 on naïve T cells to its chemokine ligand CCL21 on HEVs in turn activates binding of $\alpha_4\beta_7$ and LFA-1 to their endothelial ligands MAdCAM-1 and ICAM-1, respectively [38]. In addition, the combined expression of chemokine receptors and adhesion molecules by naïve and memory T lymphocytes govern their selective homing patterns. For instance, while L-selectin and CCR7 regulate naïve T-cell migration to peripheral LNs, expression of $\alpha_4\beta_7$ in conjunction with CCR9 allows T-cell migration to the skin and gut. The encounter between CCR7 and its ligands CCL19 and CCL21 bridges the gap between innate and adaptive immune responses. Upregulation of CCR7 on antigen-laden DCs facilitates their migration into LNs for T-cell priming. In addition, enhanced expression and binding of CCR7 on naïve T cells to CCL19 on DCs and CCL21 on HEVs mediate transmigration from peripheral tissues into LNs. In contrast, downregulation of CCR7 allows activated T cells to exit the LN area and migrate to target tissues to carry out effector functions [75, 76]. Furthermore, elevated expression of CXCR5, the ligand of CXCL13, on certain $CD4^+$ T cells directs their migration to the follicle to provide B-cell help [77]. Upregulated mucosal expression of numerous chemokines and their counter receptors including CXCL8 (IL-8)/CXCR2, CXCL9,10,11/CXCR3, CCL25/CCR9, CCL19,21/CCR7, and CCL20/CCR6 is evident in active IBD and in models of colitis [78–80]. Thus, chemokines orchestrate the activation,

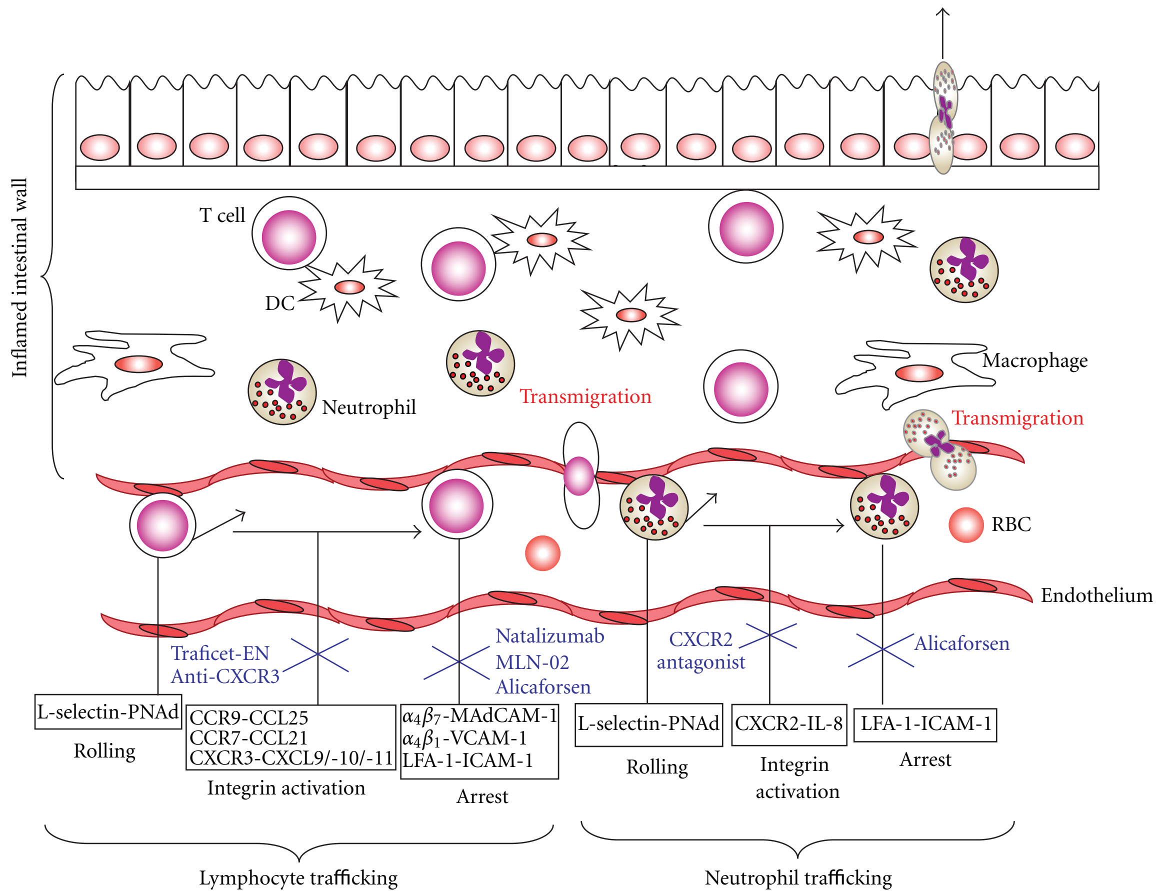

Figure 1: Schematic view of the leukocyte trafficking adhesion cascade in IBD. Leukocytes tether and roll along the vascular endothelium via selectin-mediated adhesion. They are then activated by chemokines into a high avidity, high affinity state so that integrin-mediated strong adhesion and arrest can take place. This prepares the leukocyte for transmigration through the blood vessel wall into the inflamed colon. Chemokine activation can be inhibited by various chemokine/chemokine receptor inhibitors such as the CCR9 small molecule antagonist Traficet-EN, a monoclonal antibody to CXCR3 or a CXCR2 antagonist (shown in blue). Additionally, antagonists of integrin firm adhesion include the anti- α_4 integrin monoclonal antibody Natalizumab, the selective $\alpha_4\beta_7$ small molecule antagonist MLN-02 and the antisense intercellular adhesion molecule-1 (ICAM-1) oligonucleotide Alicaforsen (shown in blue). KO, knock out; LFA-1, Lymphocyte function-associated antigen 1; MadCAM-1, mucosal addressin-cell adhesion molecule 1; PNAd, Peripheral lymph node addressin; RBC, red blood cell; V-CAM-1, vascular-cell adhesion molecule 1.

recruitment, and retention of leukocytes, and, the more insight we gain into their vital role, the more attractive they become as potential therapeutic targets.

3.2.3. The Role of DCs in Lymphocyte Homing to the Gut. The specific homing receptors expressed by activated T cells are determined by DCs. Several murine studies show that DCs from mesenteric lymph nodes (MLNs) or PPs imprint gut tropism on antigen-experienced T cells, by inducing expression of the gut homing receptors $\alpha_4\beta_7$ and CCR9 [81–83]. In the "steady state" mouse intestine, the ability to confer gut homing specificity is restricted to the CD103$^+$ intestinal DC subset [84–86]. The vitamin A metabolite retinoic acid (RA) plays a central role in the process of DC imprinting within lymphoid tissues. For instance, mice deficient in vitamin A have decreased numbers of T cells in the intestine [37, 87, 88]. However, the dependency of DC-T-cell imprinting on retinoic acid receptor (RAR) signaling is complex and not yet fully characterized [89]. Also, the key factors that induce DC imprinting activity have yet to be identified; for example, germ-free studies have indicated that gut bacteria are not required for intestinal DC imprinting of $\alpha_4\beta_7$ expression [90]. The specific role played by intestinal DC imprinting in IBD remains elusive, but presumably it influences the increased numbers of $\alpha_4\beta_7^+$ and CCR9$^+$ lymphocytes evident in the inflamed intestine [91]. Since the CD103$^+$ intestinal DC subset favors the generation of Foxp3$^+$ T-regulatory cells (Tregs) over Th17 cells, via a TGF-β- and RA-dependent mechanism [92], targeting leukocyte trafficking at the DC imprinting level may represent a potential therapeutic strategy for IBD. Notably, a diet low in vitamin A protects against colitis in mice, and this protection is associated with increased levels of Tregs in the gut mucosa. In this case, the reduced availability of RAR ligands affects lymphocyte homing to the gut by decreasing entry of $\alpha_4\beta_7$ and CCR9$^+$ T cells in favor of Tregs [93].

Table 1: Targeting leukocyte trafficking in inflammatory bowel disease.

Target	Drug type	Preclinical efficacy	Therapeutic	Clinical efficacy	
Adhesion molecules					
	Antisense MAdCAM-1 oligonucleotide	TNBS colitis [49, 50]	Natalizumab (humanized IgG4 mAb anti-α_4 integrin)	CD (Phase IV) [51, 52]	Prevented relapse, induced remission
	Anti-VCAM-1 mAb			UC [53]	Pilot study
α_4 Integrins/ligands	Anti-MAdCAM-1 mAb	DSS colitis [54, 55]	AJM300 (orally available anti-α_4 integrin mAB)	CD (Phase II) [56]	Reduced disease activity, good safety profile
	Small molecule α_4 integrin antagonist	DSS colitis [22]			
	Anti-β_7 and anti-MAdCAM-1 mAb	T-cell transfer colitis [57]	Vedolizumab/MLN-02 (humanized IgG4 mAb $\alpha_4\beta_7$ integrin)	CD [58], UC Phase II [59, 60]	Induced clinical response and remission, good safety profile
	Anti-MAdCAM-1 mAb	SAMP1/Yit mice [61]			
	Anti-$\alpha_4\beta_7$ mAb	Cotton top tamarin model [62]			
ICAM-1/LFA-1	Anti-ICAM-1 mAb Antisense ICAM-1 oligonucleotide	DSS colitis [63, 64]	Alicaforsen (ISIS2303) (antisense ICAM-1 oligonucleotide)	UC (Phase II) [65, 66]	Reduced disease activity, good safety profile
	Anti-ICAM-1 mAb	SAMP1/Yit mice [67]			
Chemokines					
CCR9/CCL25	Anti-CCR9/CCL25 mAb Traficet-EN (CCX282-B)	SAMP1/Yit mice [68] TNF (DeltaARE) mice [69]	Traficet-EN/CCX282-B (small molecule CCR9 antagonist)	CD (Phase III) [70]	Induced clinical remission, good safety profile
CXCR3/CXCL10	Anti-CXCL10 mAb	IL-10 KO [71, 72] DSS colitis [73]	MDX-1100 (humanized anti-CXCL10 mAb)	UC (Phase II)	NCT00295282 NCT00656890

CD: Crohn's disease; DSS: dextran sodium sulphate; ICAM-1: intercellular adhesion molecule 1; MadCAM: mucosal addressin-cell adhesion molecule 1; senescence accelerated mice (SAMP1/Yit); TNBS: trinitrobenzene sulfonic acid; UC: ulcerative colitis; V-CAM-1: vascular-cell adhesion molecule 1.

3.2.4. S1P and Control of Leukocyte Egress from Tissues. While chemokines control naïve T-cell migration to and within LNs, the natural bioactive lipid sphingosine-1-phosphate (S1P) regulates lymphocyte egress. S1P is formed upon the phosphorylation of sphingosine by sphingosine kinase and mediates a number of fundamental biological events including endothelial barrier enhancement, lymphocyte differentiation and immune cell trafficking [94]. Levels of S1P in the blood and lymph are constitutively high while they are low in tissues, increasing substantially upon inflammation. Although synthesized in most cells, tissue levels of S1P are tightly controlled due to intracellular degradation by S1P lyase or phosphorylation by S1P phosphatases. Five S1P receptors have been described so far, S1PR1–5, and are expressed in a cell-type specific manner within different tissues [95]. S1PR1–3 are ubiquitously expressed in mammals [96], while S1PR4 expression is restricted to lymphoid tissues [97] and lung, and S1PR5 to brain, skin, and natural killer (NK) cells [98]. The S1P/S1PR1 axis is essential for lymphocyte egress from the thymus and spleen into the blood and from the LNs into the lymph [99]. During T-cell priming, elevated expression of CD69 on activated lymphocytes promotes the temporary downregulation of S1PR1 expression on the cell membrane, disabling ligation to S1P and consequently trapping them within the LNs [100]. Concurrent binding of CCR7 to its ligand CCL21 mediates competitive retention signals. Following clonal expansion, CCR7 expression is lost and S1PR1 expression is upregulated once again, allowing the effector T cells to leave the lymphoid tissues, reenter the systemic circulation, and rapidly migrate to sites of inflammation [101]. The S1P/S1PR interaction also regulates the movement of DCs [102], neutrophils [103], and NK cells [104]. S1P signaling and functions in immunity have been reviewed elsewhere [105].

The discovery that the S1P/S1PR1 axis is essential for lymphocyte egress added a new potential target for blocking leukocyte migration in inflammatory diseases. Disruption of this S1P gradient has been reported in numerous inflammatory and/or autoimmune disorders including asthma [106] and rheumatoid arthritis [107]. The novel immunosuppressant FTY720 (Fingolimod) [108] is structurally similar to S1P and poses as an S1P analog. Like S1P, it is phosphorylated *in vivo* and binds with high affinity to 4 of the 5 S1P receptors S1PR1, S1PR3, S1PR4, and S1PR5 [109]. FTY720 interferes with S1P signaling and blocks the response of lymphocytes to egress signals from the lymphoid organs, sequestering them within the LNs and PPs. The result is a rapid and dramatic peripheral blood lymphopenia with depletion of

circulating T and B cells. In contrast, FTY720 increases the number of DCs in the blood and simultaneously reduces their numbers in secondary lymphoid organs. In addition, it can modulate DC cytokine signaling potentially affecting T-cell responses [110]. The mechanism of action of FTY720 is complex, and it is currently unclear whether it acts as an agonist or functional antagonist or both during regulation of lymphocyte recirculation *in vivo*. Since FTY720 inhibits cell migration to inflammatory sites, it has shown great potential as a treatment for inflammatory disorders [108]. In clinical studies, FTY720 successfully prevented kidney transplant rejection [111–114] and proved highly effective in treating MS. It was recently approved by the US Food and Drug Administration (FDA) as a first-line treatment for relapsing forms of MS. In terms of IBD, FTY720 ameliorated experimental colitis arising as a result of chemical induction [115–117], T-cell transfer [118], and IL-10 deficiency [119], suggesting it may be a potential candidate for IBD treatment. However, reports of side effects such as bradycardia and increased susceptibility to opportunistic infections [108] dictate that the use of FTY720 therapeutically should be approached with caution and that perhaps using more selective drugs to target the S1P receptor pathway may be a safer option and yield less side effects. Indeed, the specific agonist of S1PR1, SEW2871, has shown promise in preclinical kidney transplantation studies [120] and exhibited anti-inflammatory effects in mice administered TNF-α [121]. In addition, another selective S1PR1 agonist KRP-203 showed therapeutic potential in IL-10-deficient mice [122].

4. Preclinical and Clinical Evidence of Targeting Adhesion Molecules

Figure 1 and Table 1 summarize the data presented under Sections 4 and 5.

4.1. Natalizumab. Promising preclinical data [22, 49, 50, 54, 55, 57, 61, 62] (also see Table 1) led to the use of humanized α_4 integrin antibodies in clinical trials. The most well-known anti-α_4 drug is Natalizumab (Tysabri), a humanized pan-α_4 monoclonal antibody. Natalizumab blocks the ability of $\alpha_4\beta_1$ and $\alpha_4\beta_7$ to bind to their respective ligands on the endothelium, preventing lymphocyte transendothelial migration. This α_4 antagonist was approved by the FDA in 2004 and is highly effective in treating the symptoms of MS [123, 124] and in preventing relapse and increasing remission rates in sufferers with moderate to severe CD [51, 52]. Natalizumab therapy has been associated with cases of progressive multifocal leukoencephalopathy (PML) in a small number of patients, which is induced by the JC virus, an opportunistic infection of the brain [125]. Though rare, PML is a serious and often fatal disease. The approval and relative success of Natalizumab have heightened interest and greatly encouraged further research into targeting integrins and their counter adhesion molecule ligands as a novel treatment strategy for chronic inflammatory diseases, including IBD.

4.2. MLN-02. Vedolizumab (MLN-02) is a recombinant humanized IgG1 monoclonal antibody selective for the gut-specific integrin $\alpha_4\beta_7$. By binding to $\alpha_4\beta_7$, MLN-02 inhibits the adhesion and migration of leukocytes into the gastrointestinal tract, preventing intestinal inflammation. MLN-02 treatment ameliorated disease in the cotton top tamarin model of colitis [126] and safely and effectively induced clinical response and remission in two double-blinded placebo-controlled clinical trials of patients with active CD and UC [58–60]. The selectivity of MLN-02 makes it less likely to impair systemic immunity and more attractive as a therapeutic target for IBD.

4.3. Alicaforsen- (ISIS2302-) and LFA-1-Targeting Drugs. Alicaforsen, a human ICAM-1 antisense oligonucleotide, inhibits ICAM-1 production preventing T-cell adhesion, extravasation, and subsequent migration to inflamed areas [127]. Blocking ICAM-1 ameliorated colitis in a number of preclinical models [63, 64, 67]. In clinical studies, this approach has had variable success and in general has yielded disappointing results in the treatment of CD [128–130]. However, there have been promising results with an enema formulation of Alicaforsen in the treatment of UC [65] and refractory pouchitis [131]. Efalizumab, a humanized monoclonal IgG1 antibody treatment for plaque psoriasis, is FDA approved and also acts by blocking the LFA-1/ICAM-1 interaction. By doing this it inhibits T-cell migration to the inflamed dermal and epidermal tissues. However, similar to Natalizumab, serious adverse effects, such as the Epstein-Barr virus-associated B-cell lymphoma development, were reported following treatment [132].

4.4. Small Molecule Antagonists. The immunogenicity of antibody therapies has increased research into the use of nonpeptide small molecule antagonists to block leukocyte trafficking. Such therapeutics are less likely to elicit the undesirable and serious immunogenic responses associated with monoclonal antibody therapy, and, unlike antibodies, they can be taken orally and are less expensive to produce. We analyzed the leukocyte trafficking blockade effect of a small molecule α4 integrin antagonist in a preclinical model of IBD. We confirmed the therapeutic efficacy of the compound in dextran sodium sulphate- (DSS-) induced acute colitis and demonstrated its ability to inhibit leukocyte trafficking to the inflamed gastrointestinal tract *in vivo* using bioluminescence imaging, as shown in Figure 2 [22]. Previous studies using small molecule integrin antagonists in other models of inflammatory disease have also shown promising results [133, 134].

5. Preclinical and Clinical Evidence of Targeting Chemokines

5.1. CCR9. The chemokine CCR9 is exclusively expressed by gut homing leukocytes and interaction with its counter ligand CCL25 is essential for T-cell homing to the small intestine [135]. The CCR9/CCL25 interaction specifically contributes to the pathophysiology of small bowel CD [136]. Antibody blockade of this interaction reduced inflammation in early stages of chronic ileitis in senescence accelerated

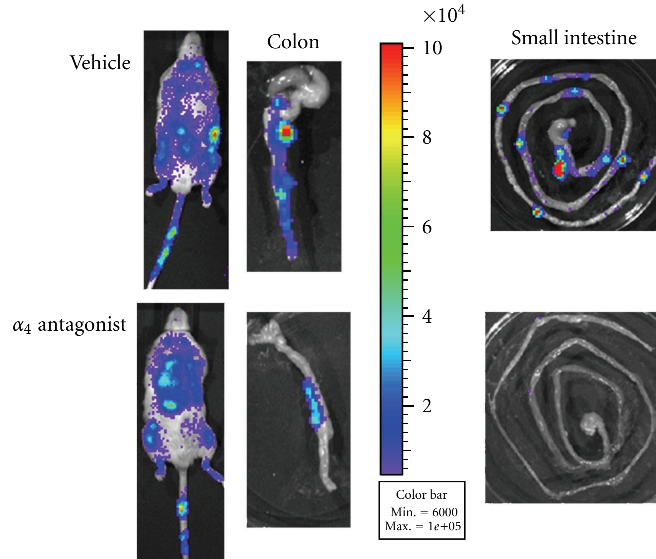

Figure 2: Inhibition of leukocyte migration by an α_4 integrin antagonist in experimental colitis. Leukocytes were isolated from mesenteric lymph nodes (MLNs) of β-actin luciferase mice and injected into recipient mice with dextran sodium sulphate- (DSS-) induced colitis. The recipient mice received vehicle or α_4 antagonist, 1 hour pre cell transfer. Whole body and organs *ex vivo* (colon and small intestine) were imaged using an IVIS 100 charge-coupled device imaging system 4 hours following transfer. The pseudocolored images represent light intensity, where red is the strongest, and violet is the weakest signal. Inhibition is detected in the colon and in Peyer's patches of the α_4 antagonist-treated mice.

(SAMP1/Yit) mice [68]. Additionally, pre- or post administration of a small molecule CCR9 antagonist (CCX282-B/Traficet-EN) reduced gut inflammation in TNFΔARE mice, an experimental model of CD [69]. Interestingly, Wermers et al. recently demonstrated that blockade of CCR9 exacerbated chronic ileitis in these mice, by inhibiting recruitment of Tregs to the small intestinal lamina propria and MLNs [137]. The exact role of CCR9/CCL25 in large intestinal inflammation remains unclear, and studies have yielded conflicting results. Preliminary clinical data using Traficet-EN demonstrated a beneficial therapeutic effect in both patients with ileal and colonic CD, by significantly reducing proinflammatory cytokine levels and disease scores and maintaining clinical remission [70]. This is surprising, since there is little or no expression of CCL25 in the colon [138, 139]. However, recent data showed that, although colonic levels of CCL25 are low in healthy mice, they are significantly upregulated upon DSS-induced colitis. In addition, CCR9 knock-out (KO) mice with acute DSS colitis exhibit enhanced severity of clinical symptoms and tissue injury and display delayed recovery. Exacerbation of disease was associated with an imbalance in DC subpopulations and increased macrophage infiltration into the colon [138]. These data suggest that use of CCR9 blockade therapy in, for example, strictly colonic UC, could have detrimental effects.

5.2. CXCR3. CXCR3 is expressed by monocytes, T cells, and NK cells and can mediate their recruitment to inflammatory sites by binding to its ligands CXCL9, CXCL10, and CXCL11. CXCR3 engagement with these chemokines mediates the rapid arrest of effector T cells *in vitro* [140] and selectively mobilizes high-CXCR3-expressing Th1 cells to sites of mucosal inflammation [141]. Expression of CXCR3 and its chemokine ligands is elevated in both preclinical and clinical models of IBD [142]. CXCL10 is considered the most crucial and potent chemokine in CXCR3-mediated chemotaxis, as it is highly upregulated and its expression robustly correlates with disease severity in inflammatory disorders such as IBD, MS, and arthritis [143–145]. Moreover, the ability of CXCL10 to preferentially attract Th1 cells emphasizes its contribution to these diseases [146]. CXCL10 antagonism prevented or ameliorated inflammation in numerous preclinical models of inflammatory disease [142]. More specifically, neutralization of CXCL10 using monoclonal antibody therapy proved effective in various experimental models of IBD [71–73]. In contrast, in a more recent study, though antibody blockade of CXCL10 reduced intestinal epithelial cell proliferation and $CXCR3^+$ cell migration *in vitro* and *in vivo*, it had no significant effect on disease in several preclinical models including IBD, arthritis, and MS [147]. The reasons for the discrepancies between these studies are unclear, but differing methods of antagonism and disease induction may play a part. For instance, Byrne et al. used T-cell ($CD4^+CD45RB^{Hi}$) transfer to induce colitis, while earlier studies employed the IL-10KO and DSS-induced colitis models. Future clinical trials are likely to resolve these uncertainties (NCT01294410).

5.3. CXCR2. Since neutrophil influx into the intestinal mucosa and resulting tissue damage is a major characteristic of active IBD, especially UC, neutrophil-specific chemokine receptors, and their ligands also represent potential therapeutics. Neutrophils exclusively use integrins of the β_2 family to arrest on the endothelium and antibodies against

these integrins reduced tissue damage in experimental colitis [148]. Engagement of the chemokines human IL-8/CXCL8 and the murine functional homologs CXCL1 and CXCL2 with their receptors CXCR1 and CXCR2 triggers numerous signal transduction cascades, which in turn activate neutrophil recruitment to target tissues [149]. Inflammatory mediators such as bacterial lipopolysaccharide (LPS), TNF-α, and IL-1 stimulate the production of IL-8. Upregulation of these chemokines in conjunction with polymorphonuclear cells (PMN) infiltration into the inflamed intestinal mucosa correlates well with the degree of active inflammation and tissue injury in human and experimental models of IBD [78, 150, 151]. CXCR2 is a well-established mediator of PMN recruitment in preclinical models of inflammatory disease [152–154]. A small molecule CXCR2 antagonist (SB225002) was effective in ameliorating trinitrobenzene sulfonic acid- (TNBS-) induced colitis in mice, as was an anti-CXCL1 antibody [155]. We used bioluminescence imaging of adoptively transferred luciferase-expressing neutrophils to study the kinetics of neutrophil migration in acute DSS-induced colitis. This enabled demonstration of preferential recruitment of the neutrophils to the inflamed colon and the blockade effect of an anti-CXCL1 antibody on the trafficking neutrophils [21].

5.4. The "Redundancy" Issue. The promise of drug inhibition of chemokines and their receptors in IBD has not yet been realized. The chemokine system is "redundant," and the same biological function can be carried out by several chemokines and their receptors *in vivo*. This questions their suitability as anti-inflammatory drug targets. However, it was recently pointed out that the lack of progress in chemokine strategies may not be due to "redundancy," but rather to the shortcomings in the approaches employed to target them [156]. Clinical efficacy of a small molecule CCR9 antagonist was demonstrated in patients with moderate to severe CD [157], proving that targeting one chemokine receptor can be therapeutically successful in the treatment of chronic inflammation.

6. Future Directions: MicroRNAs

Recent studies suggest that microRNAs (miRNAs) play a specific role in the posttranscriptional regulation of leukocyte trafficking. miRNAs are small (21−23 nucleotide) noncoding RNAs that control gene expression. By targeting complementary messenger RNAs (mRNAs) for degradation or translational repression, they suppress the expression of protein-coding genes. Blockade of miR-126 function using an antagomir, a single-stranded antisense-like molecule, suppressed the Th2 response and subsequently the development of disease in an experimental model of allergic asthma [158]. Interestingly, miR-126 was identified as one of a set of miRNAs expressed in endothelial cells [159, 160] and was shown to inhibit TNF-induced endothelial expression of VCAM-1, thus blocking leukocyte adhesion via its lymphocyte integrin ligand $\alpha_4\beta_1$ [161]. In terms of neutrophil trafficking, downregulation of E-selectin and ICAM-1 by miR-31 and miR-17-3, respectively, controlled neutrophil binding to endothelial cells [162]. Inhibition of these miRNAs using specific antagonists increased neutrophil adhesion to endothelial cells *in vitro*, while transfecting with mimics (agonists) of these miRNAs had the opposite effect. This study suggests that miRNAs negatively regulate inflammatory processes. In another study, miR-7 downregulated expression of CD98, a lymphocyte receptor that regulates integrin signaling [163]. CD98 levels were increased in the inflamed colons of patients with CD, while miR-7 levels were decreased. Taken together, the biological importance of miRNAs in the pathogenesis of IBD is becoming clearer, and targeting miRNAs in the context of leukocyte trafficking may be a safer approach for future therapeutic opportunities.

7. Concluding Remarks

Targeting leukocyte migration is a realistic strategy, and Natalizumab shows proof of principle. Compounds inhibiting a single chemokine, such as CCR9, have also shown promise, but awareness is necessary. Targeting molecules such as miRNAs, histone deacetylases [164], or the family of bromodomain proteins [165], which regulate gene expression programs that govern endothelial cell function in inflammatory settings, may represent a new generation of drugs for IBD.

Abbreviations

BLI: Bioluminescent imaging
CD: Crohn's disease
DC: Dendritic cell
DSS: Dextran sodium sulphate
GALT: Gut-associated lymphoid tissues
GPCRs: G-protein-coupled transmembrane receptors
HEV: High endothelial venules
ICAM-1: Intercellular adhesion molecule 1
IBD: Inflammatory bowel disease
IL: Interleukin
KO: Knock out
LFA-1: Lymphocyte-function-associated antigen 1
LN: Lymph node
LPS: Lipopolysaccharide
MadCAM: Mucosal addressin-cell adhesion molecule 1
MLNs: Mesenteric lymph nodes
MPO: Myeloperoxidase
MRI: Magnetic resonance imaging
mRNA: Messenger RNA
miRNAs: MicroRNAs
MS: Multiple sclerosis
NK: Natural killer
PET: Positron emission tomography
PMN: Polymorphonuclear cells
PNAd: Peripheral lymph node addressin
PPs: Peyer's patches
PSGL-1: P-selectin glycoprotein ligand 1
RA: Retinoic acid
RAR: Retinoic acid receptor

SPECT: Single photon emission computed tomography
S1P: Sphingosine-1-phosphate
TNF-α: Tumor necrosis factor-α
TNBS: Trinitrobenzene sulfonic acid
Tregs: T-regulatory cells
UC: Ulcerative colitis
V-CAM-1: Vascular-cell adhesion molecule 1.

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Research Article

Dietary Supplementation with Omega-3-PUFA-Rich Fish Oil Reduces Signs of Food Allergy in Ovalbumin-Sensitized Mice

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We investigated the effect of dietary supplementation with n-3 PUFA (fish oil source) in an experimental model of food allergy. Mice were sensitized (allergic group) or not (nonallergic group) with OVA and were fed with OVA diet to induce allergy signals. Mice were fed with regular diet in which 7% of lipid content was provided by soybean (5% of n-3 PUFA) or fish (25% of n-3 PUFA) oil. Allergic group mice had increased serum levels of antiovalbumin IgE and IgG1 and changes in small intestine, characterized by an increased edema, number of rolling leukocytes in microcirculation, eosinophil infiltration, mucus production, and Paneth cell degranulation, in comparison to non-allergic group. All these inflammatory parameters were reduced in mice fed high-n-3-PUFA diet. Our data together suggest that diet supplementation with n-3 PUFA from fish oil may consist of a valid adjuvant in food allergy treatment.

1. Introduction

The normal immune response to dietary proteins is associated with the induction of oral tolerance, which involves a modification of the antigen in the lumen by gastrointestinal enzymes, the posterior contact with specific antigen-presenting cells with distinct activation requirements, and activation of regulatory T cells. It is well accepted that a breakdown in oral tolerance mechanism or a failure of induction of oral tolerance results in food allergy [1, 2].

Food allergies are disorders that affect about 20–30% of the human population in developing countries, making them some of the most common chronic diseases [3]. It is generally accepted that 6–8% of all children below 3 years of age present food allergy reactions [4, 5], specially IgE-mediated hypersensitivities [6]. Milk, eggs, peanuts, chestnuts, and shrimp are commonly related to food allergy episodes [4, 7]. In these atopic patients, continuous involuntary exposure to a food allergen may induce a mild and persistent allergic condition involving skin, gastrointestinal, and respiratory tracts disorders or trigger a multiple-organ system reaction with cardiovascular collapse [8]. Therefore, there is considerable interest in identifying interventions that are able to prevent or modify this pathological condition.

The main treatment strategy for most food allergies is based on allergen avoidance, which may present potential adverse nutritional deficiencies related to inadequate growth, neurological development, and cardiovascular

health [9, 10]. Therapeutic strategies under study include oral immunotherapy [11], vaccines [12], Chinese herbal medicines [13], and dietary supplementation strategies with antioxidants [14]. Another available therapeutic option is the use of essential fatty acids for the prevention and treatment of symptoms of allergies, once the increased prevalence of allergies has been associated with modern dietary style (increased consumption of n-6 polyunsaturated fatty acids (n-6 PUFA) and decreased n-3 polyunsaturated fatty acids intake (n-3 PUFA)) [15, 16]. However, there is no clear evidence regarding modulation of immunological profile with use of n-3 PUFA during allergy.

Based on this, in the present study we evaluated the effect of chronic intake of n-3 PUFA in a murine model of food allergy. In order to simulate this persistent food allergy situation, we used an experimental model of food allergy in which ovalbumin- (OVA-) sensitized BALB/c mice are given the antigen orally [17]. This model mimics several pathological changes that occur in patients with food allergy including increased anti-OVA IgE and IgG1 production, intestinal edema, and eosinophil infiltration in the small intestine [18].

2. Materials and Methods

2.1. Animals. Female BALB/c mice at four weeks of age were obtained from our animal facility (ICB/UFMG). All mice have received water and food *ad libitum*. The procedures were in accordance with the Ethical Principles in Animal Experimentation, adopted by the Ethics Committee in Animal Experimentation of our institution (CETEA/UFMG).

2.2. Diet. Two different diets were prepared in accordance with AIN-93 components [19], varying in fatty acid composition. Mice were fed with regular diet in which 7% of lipid content was provided by soybean (Control group: 5% of n-3 PUFA) or fish oil (n-3 PUFA group: 25% of n-3 PUFA). The diet consumption was started just after weaning in 4-week-old mice (Figure 1). The fatty acid composition of the diet is showed in Table 2. Fresh diet was given every 2 days to avoid lipid oxidation. All diets contained 14% of protein (casein before antigen challenge and ovalbumin for the antigen challenge; Tables 1 and 2). The fatty acids profile of fish oil used in this study was examined by the Chemistry Department of the Exact Sciences Institute (ICEx/UFMG) using gas chromatography. The fatty acids profile of the soybean oil used (Lisa) was obtained by Sanibal and Mancini Filho, 2004 [20].

2.3. Mice Sensitization and Oral Challenge. After 21 days (week 3 of experiment) of diet consumption (without OVA), allergic group (OVA^+) received 0.2 mL saline (0.9%) with adjuvant (1 mg $Al(OH)_3$) and 10 μg OVA (five times crystallized hen's egg albumin; Sigma, St. Louis, Mo, USA). The nonallergic group (OVA^-) received only saline and adjuvant. After 14 days (week 5), an immunological booster was given with 10 μg OVA to allergic group. At the same time, nonallergic mice received saline. All injections were performed subcutaneously. After 7 days of booster (week 6), the two different diets remained with the same composition regarding lipids levels; however, for all groups casein was replaced by ovalbumin (lyophilized egg white-Salto's, Belo Horizonte, MG, Brazil) to induce allergic manifestations in sensitized mice during 7 days (Figure 1).

Table 1: Mouse chow ingredients (based on AIN-93G diet).

Ingredient	g/kg diet
Protein source	
Casein (day 0–21)	200.000
or	
Ovalbumin (day 21—end)	200.000
Lipid source	
Soybean oil	70.000
or	
Fish oil	70.000
Cornstarch	529.500
Sucrose	100.000
Fiber (cellulose)	50.000
Mineral mix (AIN-93G-MX)	35.000
Vitamin mix (AIN-93-VX)	10.000
L-cystine	3.000
Choline bitartrate (41.1% choline)	2.500
Tert-butylhydroquinone	0.014

Table 2: Fatty acid composition of mouse diets.

Fatty acid	Fish oil (%)	Soy oil (Lisa) (%)
Sum of SFA	30.8	15.24
Sum of MUFA	29	22.69
C18 : 2 n-6c (LA)	1.3	55.83
C18 : 3n6 (GLA)	0.2	
C20 : 4 n-6c (AA)	0.0	
Sum of n-6PUFA	1.5	55.83
C18 : 3n3 (ALA)	0.6	4.79
C20 : 3n3	1.3	
C20 : 5 n-3 (EPA)	15.9	0.0
C22 : 6 n-3 (DHA)	7.9	0.0
Sum of n-3 PUFA	25.7	4.79
SD	13	0.07
Total	100	100

SFA: saturated acid; MUFA: monounsaturated acid; LA: linoleic acid; GLA: γ-linolenic acid; AA: arachidonic acid; ALA: α-linolenic acid; DHA: docosahexaenoic acid; EPA: eicosapentaenoic acid; SD.

2.4. Serum Antibody Evaluation. After 7 days of continuum challenge with OVA diet (week 7), all mice were anesthetized with an i.p. injection of a mixture of 10 mg/kg xylazine and 200 mg/kg ketamine hydrochloride. Serum was collected for anti-OVA IgG1 and IgE analyses. ELISA for IgG1 was carried out using plates coated with OVA and 100 μL of 1 : 8000 diluted mouse sera, with goat anti-mouse IgG1 (Southern

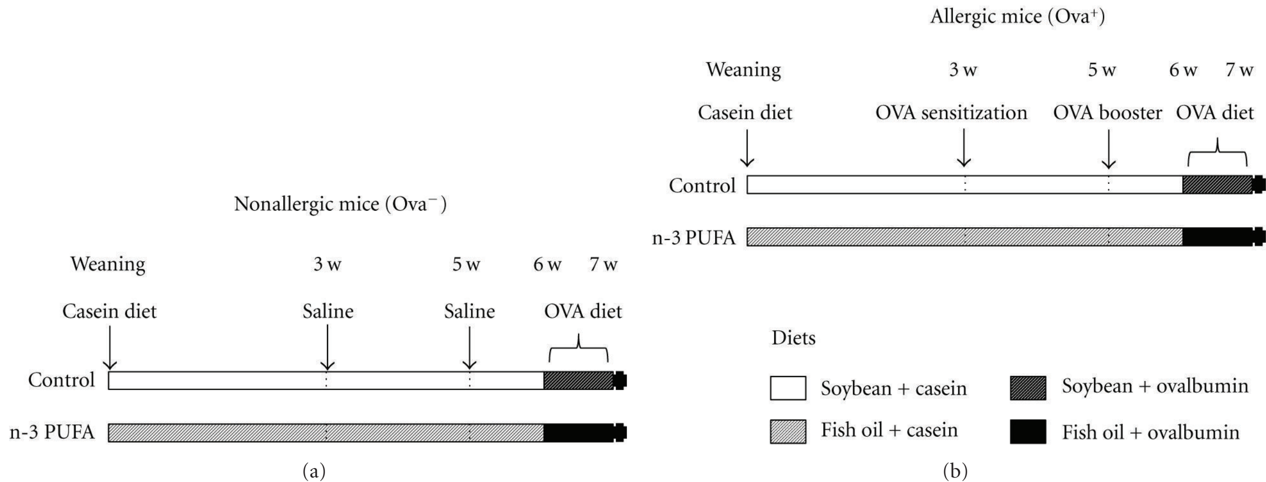

Figure 1: Experimental protocol. BALB/c mice were separated in 4 groups: control/nonallergic, control/allergic, n-3 PUFA/nonallergic, and n-3 PUFA/allergic. According to the diet, mice received 5% n-3 PUFA (control group) or 25% n-3 PUFA (n-3 PUFA group) as source of lipids in their diet since the beginning of the experiment (after weaning) until the end (7th week). According to the immunological procedures, mice were sensitized and received a booster (allergic group) or not (nonallergic group) with ovalbumin. Seven days after the booster, all mice received OVA diet (the diet remained with the same profile of lipids but the source of the protein was changed from casein to ovalbumin). After 7 days, the mice were sacrificed and the serum and tissues collected for analyses.

Biotechnology Associates, Birmingham, Ala, USA) and rabbit anti-goat labeled with peroxidase (Southern Biotechnology Associates, Birmingham, Ala, USA). The plates were developed with o-phenylenediamine and H_2O_2 and were read at 492 nm on an automated ELISA reader (EL800, Bio-Tek Instruments, Inc., Winooski, Vt, USA). Anti-OVA IgE was measured by capture-ELISA using plates coated with rat anti-mouse IgE, 50 μL of serum, and biotinylated OVA, as previously described [21]. The results for both antibodies are reported in arbitrary units (1000 A.U) according to the standard curve obtained with serial dilutions of pooled serum from OVA-hyperimmunized BALB/c mice.

2.5. Histological Analysis. After 14 days of oral challenge, the mice were sacrificed by cervical dislocation. The proximal jejunum was taken for histological analysis. It was fixed in 10% formalin in PBS, embedded in paraffin and cut into 5 μm thick sections. The sections were stained with periodic acid Schiff (PAS) for mucus analysis or with hematoxylin-eosin to evaluate eosinophil infiltration. Ten fields from hematoxylin-eosin-stained sections were randomly chosen at 40x (53.333 μm^2/field) in order to count the number of eosinophils, and the data are reported as number of eosinophils/field. For mucus analysis, three sections of the jejunum stained with periodic acid Schiff were submitted to morphometric analysis using an image analysis program running on an IBM computer. Images were obtained at 40x (53.333 μm^2/field) with a JVC TK-1270/RGB microcamera and analyzed with the KS300 software built in a Kontron Eletronick/Carl Zeiss image analyzer. For the determination of goblet cell volume, all pixels with green hues were selected for the creation of a binary image and subsequent calculation of the total area, and data were reported as a percentage of mucus area/total area.

2.6. Intravital Microscopy of Intestine Microcirculation. To study leukocyte recruitment *in vivo*, animals were anesthetized and the abdomen was opened via a midline incision. The mice were maintained in constant temperature (34°C). A segment of small intestine was chosen and placed onto a stage, and the microcirculation was imaged using intravital microscopy by fluorescence microscopy (OLYMPUS BX41). Rhodamine 6G was used for visualization of rolling and adhered cells in intestinal microcirculation. Rolling was measured by counting the number of cells that passed for a given point during 3 minutes (cells/min) and cells that remained stopped for 30 seconds in the same point were counted as an adherent cell.

2.7. Statistical Analysis. The results were expressed as the mean ± SEM, as indicated in the figure legends. Significance was determined by the ANOVA-Tukey and Student t-tests, with $P < 0.05$ defining significance over the control group.

3. Results

3.1. Evaluation of Serum Anti-OVA IgG1 and IgE Antibodies. The experimental allergy protocol induced a significant increase in serum levels of specific anti-OVA IgE and IgG1. Interestingly, n-3-PUFA-supplemented mice had significant lower levels of specific anti-OVA IgE compared to control group (Figure 2).

3.2. Intestinal Histology Analyses. The ingestion of OVA diet induced submucosal edema and increased degranulation of Paneth cells and inflammatory cell infiltration in mucosa. There was 10-fold increase in the number of eosinophils in control allergic group compared to nonallergic mice. Interestingly, edema and eosinophil infiltration were significantly

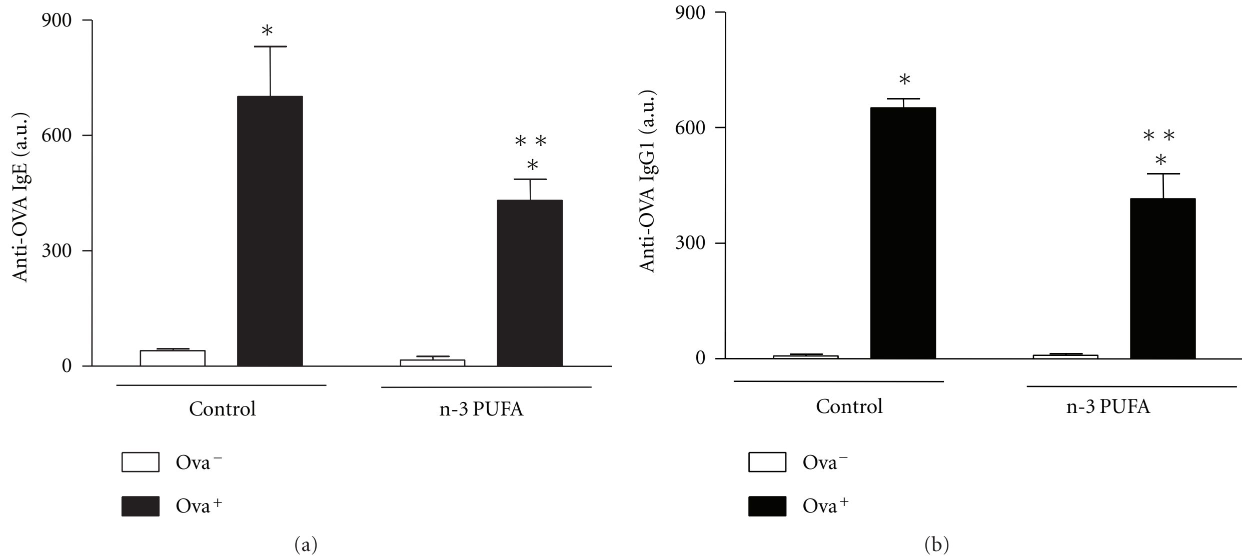

Figure 2: Dietary supplementation with n-3 PUFA decreases serum concentrations of anti-OVA IgE and IgG1 in BALB/c-sensitized mice. BALB/c mice received 5% n-3 PUFA (control group) or 25% n-3 PUFA (N-3 PUFA group) as source of lipids in their diet 21 days before the sensitization. BALB/c mice were sensitized (allergic, OVA^+) or not (nonallergic, OVA^-) with OVA. Seven days after the booster, all mice received OVA diet. After 7 days, the mice were sacrificed and the serum was collected for measurement of anti-OVA IgE and IgG1 by ELISA. Data are reported as means ± SEM for 5 animals/group. $^*P < 0.05$ compared to nonallergic group (OVA^-) with the same diet, and $^{**}P < 0.05$ compared to allergic control group (ANOVA-Tukey).

reduced in mice fed increased n-3 PUFA diet. Also, Paneth cells from n-3 PUFA-supplemented mice displayed a regular profile of degranulation, similar to controls (Figure 3).

3.3. Evaluation of Intestinal Mucus by Goblet Cells. Continuous exposure to the antigen induced a significant increase in mucus production by goblet cells in the small intestine of sensitized wild-type BALB/c mice when compared to nonsensitized animals. On the other hand, antigen ingestion induced no increase in mucus secretion in the small intestine of mice fed with n-3 PUFA diet (Figure 4).

3.4. Intravital Microscopy of Intestine. Leukocyte recruitment is a hallmark feature of the inflammatory response, and it involves a sequential series of molecular interactions between the leukocyte and endothelial cells [22]. Once we have detected a reduced eosinophil infiltration in n3-PUFA group, we decided to investigate, by using intravital microscopy, in which step of leukocyte recruitment this diet was interfering. Allergic group had an increase in rolling and adhered leukocyte number in intestinal microvasculature after OVA diet. However, mice fed n-3 PUFA diet displayed a reduction in the total number of rolling leukocytes (Figure 5(a)) with no differences in the number of adherent cells (Figure 5(b)).

4. Discussion

Immunoglobulin-E-dependent food allergy typically affects the gastrointestinal tract with different degrees of eosinophilic inflammation and edema [8]. Food allergy treatment is mostly based on pharmacological approach (mainly antihistaminic and corticoids) and food antigen avoidance, being the last one the only efficacious alternative in several refractory patients. In this sense, supplements with ability to decrease or avoid allergic reactions against food contents may be promising. In the food allergy model used in this study, when ovalbumin-sensitized mice were given OVA (antigen) in the diet, several signs of food allergy were observed, including increased serum antiovalbumin IgG1 and IgE and marked histological findings of intestinal inflammation (mucus hypersecretion, eosinophil infiltration, Paneth cell degranulation, and edema) [17]. In the present work, we have shown that OVA allergic mice had a less severe allergic response when the polyunsaturated fatty acid omega 3 was increased in the diet. We provided evidence that reduced OVA-specific IgE production by n-3-PUFA-supplemented diet led to reduced eosinophil infiltration into gut mucosa, with mild intestinal inflammatory response in mice. These data together suggest that food supplementation with n-3 PUFA may consist of a promising venue to treat food-associated allergic disorders despite food avoidance.

The known mechanism involved in IgE-dependent food allergy is attributed to the generation of Th2 cells that produce IL-4 with further generation of IgE and IgG1 antibodies [8]. These immunoglobulins bound in mast cells via its high affinity receptor (FcεRI), leading to release of a large number of proinflammatory mediators and proteases into adjacent tissues [23]. Also, these activated mast cells produce Th2-type cytokines, including IL3, IL-5, and IL-13, leading to the accumulation of eosinophils which promote the expansion of Th2 cells in inflamed tissues and release of proinflammatory mediators with upregulation of adhesion systems, modulation of cellular trafficking, activation and

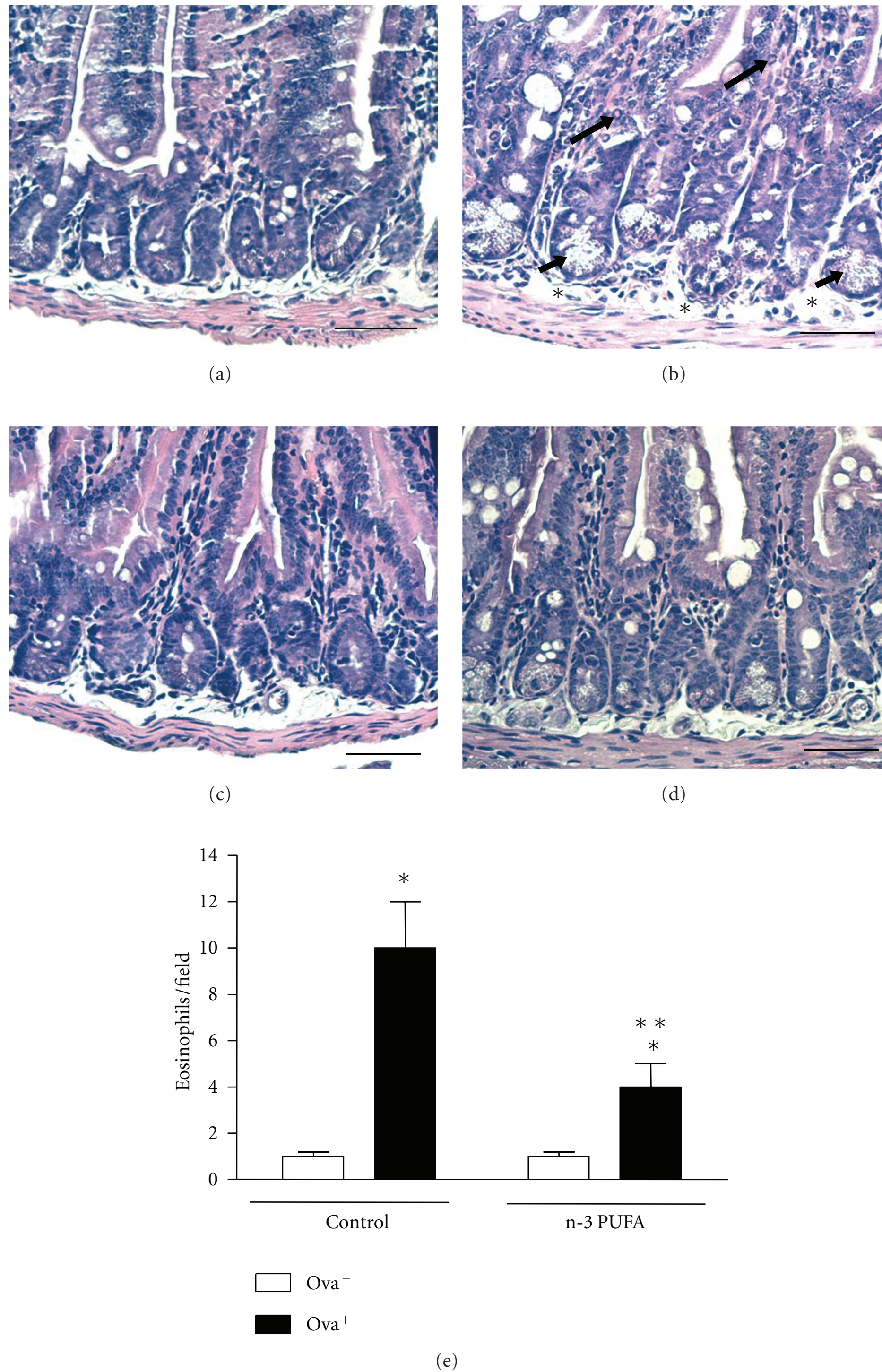

FIGURE 3: Dietary supplementation with n-3 PUFA decreases histological inflammatory parameters in jejunum of BALB/c-sensitized mice. BALB/c mice received 5% n-3 PUFA (control group) or 25% n-3 PUFA (N-3 PUFA group) as source of lipids in their diet 21 days before the sensitization. BALB/c mice were sensitized (allergic, OVA^+) or not (nonallergic, OVA^-) with OVA. Seven days after the booster, all mice received OVA diet. After 7 days, the mice were sacrificed and the intestine was taken for histology analyses. (a) Nonallergic control; (b) allergic control; (c) nonallergic n-3 PUFA; (d) allergic n-3 PUFA. Bar = 50 μm. In (b), long arrows show eosinophils, short arrows show Paneth cells, and asterisks show submucosal edema. In (e), data are reported as means ± SEM of number of eosinophils for 5 animals in each group. $^*P < 0.05$ compared to nonallergic group (OVA^-) with the same diet, and $^{**}P < 0.05$ compared to allergic control group (ANOVA-Tukey).

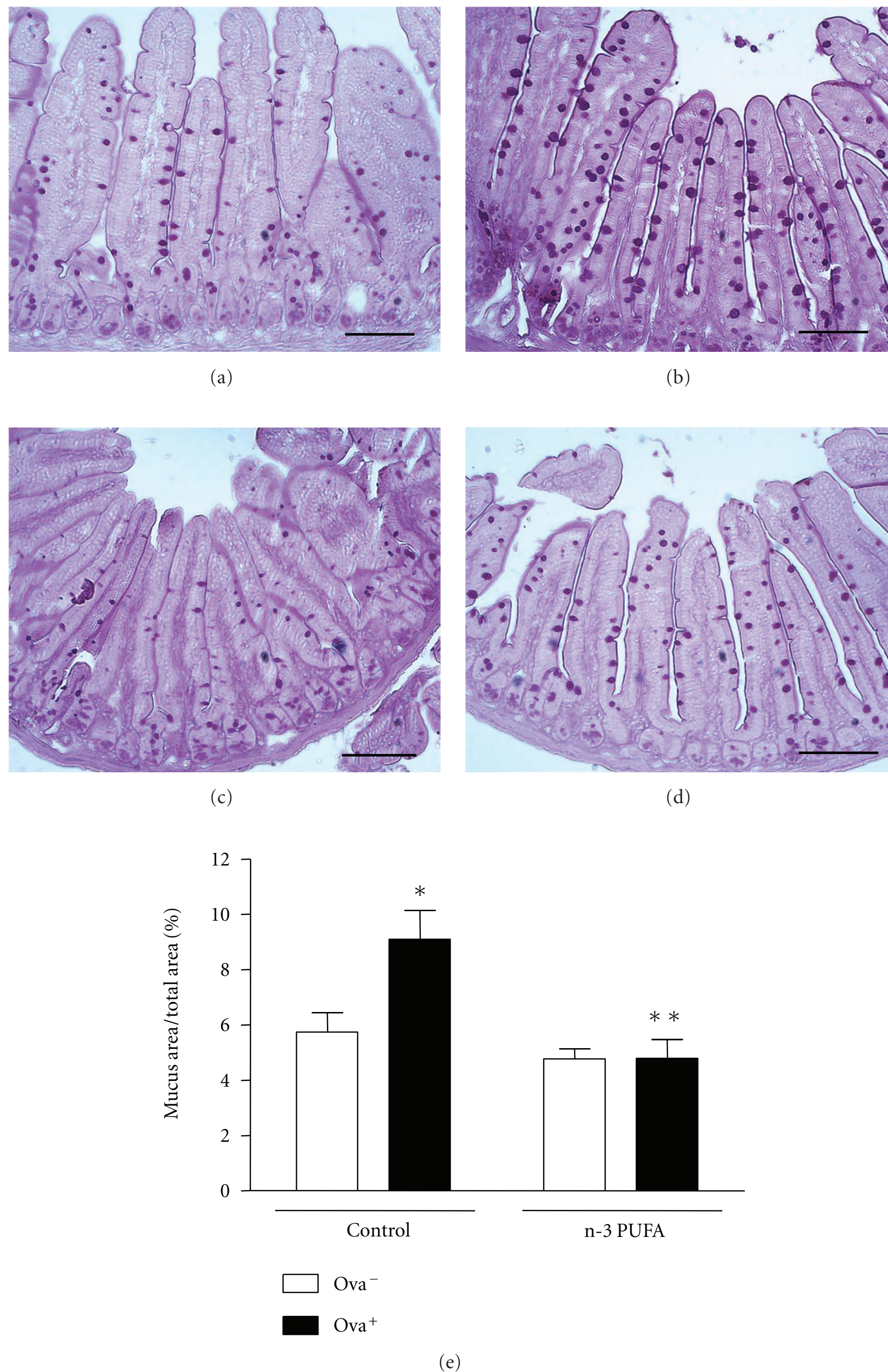

FIGURE 4: Dietary supplementation with n-3 PUFA decreases mucus production in small intestine of BALB/c-sensitized mice. BALB/c mice received 5% n-3 PUFA (control group) or 25% n-3 PUFA (N-3 PUFA group) as source of lipids in their diet 21 days before the sensitization. BALB/c mice were sensitized (allergic, OVA^+) or not (nonallergic, OVA^-) with OVA. Seven days after the booster, all mice received OVA diet. After 7 days, the mice were sacrificed and the intestine was taken for histology analyses. (a) Nonallergic control; (b) allergic control; (c) nonallergic n-3 PUFA; (d) allergic n-3 PUFA. Bar = 50 μm. In (e), data are reported as means ± SEM of mucus production for 5 mice in each group. $^*P < 0.05$ compared to nonallergic group (OVA^-) with the same diet, and $^{**}P < 0.05$ compared to allergic control group (ANOVA-Tukey).

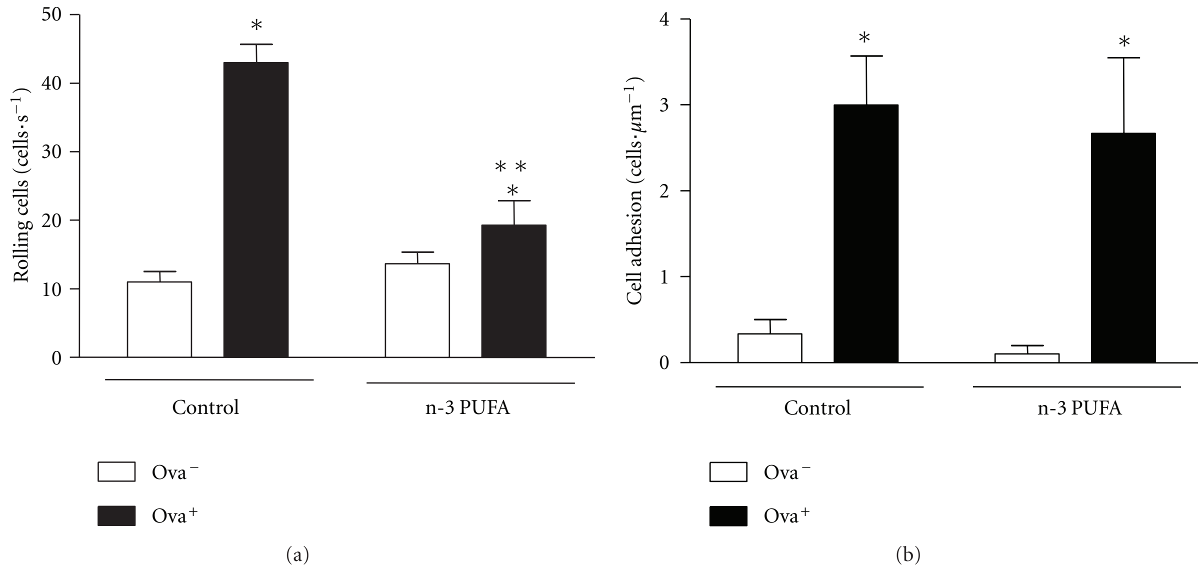

Figure 5: Dietary supplementation with n-3 PUFA decreases leukocyte rolling in small intestine of BALB/c-sensitized mice. BALB/c mice received 5% n-3 PUFA (control group) or 25% n-3 PUFA (n-3 PUFA group) as source of lipids in their diet 21 days before the sensitization. BALB/c mice were sensitized (allergic, OVA^+) or not (nonallergic, OVA^-) with OVA. Seven days after the booster, all mice received OVA diet. After seven days of ingestion of the ovalbumin- (OVA-) containing diet, the leukocyte rolling (a) and adhesion (b) to the microvasculature of intestine were assayed by intravital microscopy. Data are reported as means ± SEM for 3 mice in each group. $^*P < 0.05$ compared to control group (ANOVA-Tukey).

regulation of vascular permeability and mucus secretion, and tissue damage [24]. The mucosal immune system accounts for a number of mechanisms to avoid an uncontrolled immune response against food antigens, including the presence of regulatory T cells in the lymphoid tissue of the gut [25] and mucus (a physical barrier against antigens) [26]. Consistent with this, a reactive increase of mucus production is expected during food allergy model, which is reduced in the absence of IL-4 and IgE [27]. Additionally, gut mucosa cells play a key role in the digestive tract homeostasis. Paneth cells are secretory cells in the epithelium of the small intestine, which reside in small clusters at the base of crypts of Lieberkühn, and they are the main source or antimicrobial peptides in gut [28]. These peptides are described to act protecting gut mucosa against enteric bacterial pathogens, participating as a key homeostatic role in establishing and maintaining the intestinal microbiota [29]. In our study, histological evaluation showed that during food allergy a degranulation profile is observed in Paneth cells. Although there is no known direct correlation between Paneth cells and food allergy, we suggest that these cells may be involved in allergy-induced gut mucosa inflammation.

The polyunsaturated acids (including n-3 and n-6 PUFA) are precursors of several eicosanoids, such as prostaglandins (PG), leukotrienes, thromboxanes, and hydroxyeicosatetraenoic acids. Consumption of n-6 PUFA leads to the formation of specific eicosanoids with a proinflammatory profile, which has an essential role in allergic inflammation. Thereby, linoleic acid (LA; C18 : 2 n-6), one of the major dietary n-6 PUFA, is converted to arachidonic acid (AA; C20 : 4 n-6) and is incorporated into membrane phospholipids [30]. During metabolism, AA is released by phospholipases and metabolized to PGE2, driving a Th2 subset response. In this sense, imbalanced dietary intake of n-6 PUFA may increase the predisposition to atopic disorders with IL-4 and consequent IgE production. In sharp contrast, an increased n-3 PUFA ingestion will lead to a metabolic competition with n-6 PUFA metabolism, culminating in decreased synthesis of PGE2, decreased IL-4 and IgE, as seen in our model [27]. Additionally, n-3 PUFA supplementation in allergic subsets may be beneficial, since a less inflammatory environment may be achieved during fatty acid metabolism [31].

In fact, n-3 PUFA supplementation leads to a less severe inflammation in gut mucosa from allergic mice and decreased production of IgE. This statement may be strengthened by three major findings. First, lower levels of IgE were observed in supplemented mice. Mast cells, which are extremely activated by IgE, can release several mediators upon IgE activation, IL-5 and eotaxin being potent chemoattractants to eosinophils. This may explain the marked reduction in eosinophils observed in n-3-PUFA-supplemented mice. In fact, a previous report has shown that both DHA and EPA are able to decrease the chemotactic and chemokinetic responses of eosinophils in a dose-dependent fashion [31]. Second, we observed less mucus production and Paneth cells degranulation in n-3-PUFA-supplemented mice, which is a clear histological indication of a mild gut inflammatory response [27]. And finally, intravital microscopy of intestinal microvasculature revealed that food enriched with n-3 PUFA by fish oil led to a reduction in the total number of rolling leukocytes, as

an indication of reduced endothelial-leukocyte interaction. Several reports have shown that consumption of n-3 PUFA in fish oil may reduce the inflammatory response in several chronic inflammatory diseases characterized by leukocyte accumulation such as atherosclerosis, asthma, systemic lupus erythematosus, inflammatory bowel disease, and rheumatoid arthritis [32–35]. In a previous report it was shown that oxidized EPA is a potent inhibitor of leukocyte interaction with the endothelium. The proposed mechanism responsible for this effect seems to be the activation of nuclear receptor peroxisome proliferator-activated receptor α (PPARα) and subsequent downregulation of leukocyte adhesion receptor expression [36].

Our results are consistent with previous works such as that performed by Watanabe et al. which showed that the IgE antibody response against egg albumin was significantly lower in the mice fed with safflower seed oil [37]. Also, Yamashiro et al. have shown that the mucosal damage induced by intestinal hypersensitivity reactions to ovalbumin is regulated by omega-3-fatty-acid enriched diet [38]. On the other hand, depending on the model or on the feeding design, the results can be controversial. For example, Johansson et al. have shown that, during the airway hypersensitivity (Th2), mice fed with fish oil produced high levels of OVA-specific IgE and had slightly high eosinophil infiltration into the lungs. Contrastingly, chronic n-3 PUFA consumption (as shown in our study) or lipid-based allergy prevention performed since in the uterus, via maternal diet [39, 40], provide evidence of benefic immunological modulation and less inflammatory tissue damage. Further basic investigation may provide guidelines for new trials, since meta-analysis studies have not confirmed the beneficial role of n-3 or n-6 PUFA supplementation as a strategy for the primary prevention of food allergy [41].

In conclusion, we have shown that diet supplementation with n-3 PUFA from fish oil led to a reduction of gut inflammatory response against food antigen, which suggests that n-3 PUFA may modulate the allergic immune response.

Acknowledgments

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Research Article

Regulatory T Cells Accumulate in the Lung Allergic Inflammation and Efficiently Suppress T-Cell Proliferation but Not Th2 Cytokine Production

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$Foxp3^+CD25^+CD4^+$ regulatory T cells are vital for peripheral tolerance and control of tissue inflammation. In this study, we characterized the phenotype and monitored the migration and activity of regulatory T cells present in the airways of allergic or tolerant mice after allergen challenge. To induce lung allergic inflammation, mice were sensitized twice with ovalbumin/aluminum hydroxide gel and challenged twice with intranasal ovalbumin. Tolerance was induced by oral administration of ovalbumin for 5 consecutive days prior to OVA sensitization and challenge. We detected regulatory T cells ($Foxp3^+CD25^+CD4^+$ T cells) in the airways of allergic and tolerant mice; however, the number of regulatory T cells was more than 40-fold higher in allergic mice than in tolerant mice. Lung regulatory T cells expressed an effector/memory phenotype ($CCR4^{high}CD62L^{low}CD44^{high}CD54^{high}CD69^+$) that distinguished them from naive regulatory T cells ($CCR4^{int}CD62L^{high}CD44^{int}CD54^{int}CD69^-$). These regulatory T cells efficiently suppressed pulmonary T-cell proliferation but not Th2 cytokine production.

1. Introduction

Regulatory T (Treg) cells have been implicated in the mechanisms that govern peripheral dominant tolerance. From autoimmunity, transplantation, and cancer to mucosal tolerance, the presence of functional Treg cells, either thymus-derived naturally occurring or peripherally-induced adaptive Treg cells have been associated with the control of inflammation [1].

Allergic asthma is a chronic inflammatory disease characterized by airway eosinophilia, airway hyperreactivity (AHR), mucous hypersecretion, and high titers of IgE [2]. In asthmatic patients, $CD4^+$ T lymphocytes upon allergen challenge secrete type-2 cytokines such as IL-4, IL-5, IL-9, and IL-13 that in turn mediate the Th2-associated inflammatory network and IgE production [3]. It has been suggested that insufficient immune regulation by Treg cells might lead to aberrant Th2 response [4–7]. Conversely, mucosal exposure to nonpathogenic antigens results in a state of hyporesponsiveness, known as mucosal tolerance that efficiently inhibit pulmonary and systemic Th2-mediated response [8–12].

Different subtypes of regulatory T cells or suppressive cytokines have increasingly been defined as important in mediating T-cell unresponsiveness by mucosal tolerance [9, 13–15]. For instance, TGF-β-producing Th3 cells and

IL-10-producing Tr1 cells were proposed to mediate oral and nasal tolerances, respectively [9, 16, 17]. Other Treg cells involved in mucosal tolerance have been characterized as $CD4^+CD25^+CD45RB^{low}$ T cells that also express glucocorticoid-induced TNF receptor (GITR), CTLA-4, and Foxp3 [13, 14, 18–23].

The involvement of Treg cells in the control of allergic responses was clearly established in double T/B transgenic mice [7], a mice that harbor monoclonal $CD4^+$ T-cell population specific to OVA and monoclonal B cells specific to hemagglutinin A (HA). These animals when devoid of natural Treg cells develop hyper-IgE response upon OVA-HA sensitization and challenge [7]. Previously, we have shown that oral tolerance induced by OVA feeding prevented the development of hyper-IgE production and asthma-like responses in these animals [24]. We found that oral OVA exposure induced the development of adaptive OVA-specific Treg cells that displayed suppressive activity *in vivo* and *in vitro* in a TGFβ-dependent manner [24] indicating that Tregs are quite efficient in preventing priming of naive T cells.

Natural or adaptive Treg cells can be further characterized as naive or effector Treg cells by the expression of chemokine receptors and adhesion molecules responsible for their preferential localization in lymph nodes or in inflamed tissues [25]. The suppressive effect of Treg cells in lymph nodes is well documented, whereas their role at sites of allergen challenge is still elusive. It has been reported that the resolution of allergic airway disease induced by long-term allergen challenge (inhalational tolerance) is associated with local accumulation of Treg cells [26]. Previous studies that employed oral or nasal tolerance to suppress OVA-induced allergic lung disease did not investigate the migration of Treg cells to the lung [23, 24].

In the present work, using the murine OVA model of asthma-like responses, we investigated whether Treg cells migrate to the site of allergen challenge in allergic mice or in mice made tolerant by OVA feeding before sensitization (oral tolerance). Because we found that $Foxp3^+$ Treg cells as well as Th2 inflammatory cells and high levels of suppressive cytokines accumulated in the airways of allergic but not in tolerant mice, we further characterized the phenotype of these Treg cells. Upon allergen challenge, Treg cells accumulated into airways of allergic mice and showed upregulation of the chemokine receptor CCR4 and substantially downregulation L-selectin. These two surface markers could, at least, distinguish Treg cells present in the airways ($CCR4^{high}CD62L^{low}$) from those present in the draining lymph nodes ($CCR4^{int}CD62L^{high}$). In addition, airway Treg cells also upregulated molecules associated with effector/memory T cells such as CD54, CD44, and others [27, 28]. Interestingly, the increased frequency of $Foxp3^+$ Treg cells in the allergic lung expressed CD69, whereas the majority of lung Treg cells from tolerant mice were $Foxp3^+CD69$-negative. Finally, airway $CD4^+CD25^+$ Treg-like cells from allergic mice exhibited strong and efficient antiproliferative activity on lung $CD4^+CD25^-$ T cells but were unable to suppress type 2 cytokine production. Indeed, experiments with highly purified green fluorescent Foxp3 Treg cells confirmed the inability of these cells to suppress cytokine production by Th2 cells.

2. Materials and Methods

2.1. Mice. Female BALB/c and C57BL/6 mice at 8–12-week old, housed under specific pathogen-free conditions at the Department of Immunology, Biomedical Science Institute, University of São Paulo, Brazil, were used throughout the experiments. Foxp3-green fluorescence protein knockin (*Foxp3gfp*.KI) mice were already described elsewhere [29]; these animals were kindly provided by Howard L. Weiner (Center for Neurologic Diseases, Brigham and Women's Hospital, Harvard Medical School) and were bred at the Department of Microbiology, Immunology and Parasitology of Federal University of São Paulo. Mice were treated according to Animal Welfare guidelines of the Biomedical Science Institute (ICB-USP).

2.2. OVA Sensitization and Airway Challenge. Mice were sensitized and boosted by subcutaneous route with 4 μg chicken OVA/1.6 mg of aluminum hydroxide gel in 0.2 mL of sterile PBS at days 0 and 7. For the induction of airway inflammation, mice receive two intranasal (i.n.) challenges with 10 μg OVA in 40 μL of sterile PBS at days 14 and 21. Experiments were performed 24 h after the last i.n. OVA challenge (day 22).

2.3. Oral Tolerance Induction. Oral tolerance to OVA was induced by spontaneous intake of 1% OVA (grade V, Sigma-Aldrich, St. Louis, Mo USA) solution dissolved in sterile drinking water for 5 consecutive days before sensitization as previously described [24].

2.4. Bronchoalveolar Lavage (BAL). Mice were deeply anesthetized, trachea was cannulated, and lungs were rinsed with 1.0 mL of cold PBS. Total and differential cell counts of BAL fluid were determined by hemocytometer and cytospin preparation stained with Instant-Prov (Newprov, Brazil).

2.5. Determination of Respiratory Pattern. Respiratory pattern was determined before and after increasing doses of inhaled methacholine (3, 6, 12, and 25 mg/mL) in conscious unrestrained mice using whole-body plethysmograph (Buxco Electronics Inc. Wilmington, NC, USA) as previously described [12, 30]. The enhanced pause (Penh), a dimensionless value that takes into account box pressure recorded during inspiration and expiration and the timing comparison of early and late expiration was used to define the respiratory pattern.

2.6. Flow Cytometry Analysis. Single cell suspensions were preincubated with FcBlock for 10 min at room temperature (BD PharMingen, San Diego, Calif, USA). Cells were then incubated in staining buffer (PBS containing 2% fetal calf serum and 0.1% NaN_3) for 30 min at 4°C with the antibody cocktails. Samples were analyzed in FACSCalibur or FACSCanto II instruments (Becton Dickinson, San Diego,

Calif, USA). Anti-mouse CD4-FITC, CD4-PerCP, CD4-Pacific Blue, CD25-PerCP-Cy5.5, CD25-FITC, CD62L-PE, CD69-FITC, CTLA-4-PE, GITR-PE, IgG2a$^{\kappa}$-PE, IgG2a$^{\kappa}$-FITC, IL-10-PE, IL-5-PE, and streptavidin-PE-Cy5 were purchased from BD Pharmingen (San Diego, Calif, USA). Anti-mouse Foxp3-APC and Foxp3-FITC antibodies were purchased from e-Biosciences (San Diego, Calif, USA). Affinity-purified biotinylated goat anti-TGF-β-bound precursor cytokine latency-associated peptide (LAP) polyclonal antibodies were purchased from R&D Systems (Minneapolis, Minn, USA). The remaining antibodies CCR4-APC, CD44-PE, CD54-PE, and CCR7-PerCP-Cy5.5 were purchased from BioLegend (San Diego, Calif, USA).

2.7. Intracellular Staining for Foxp3, CTLA-4, and Cytokines. After stimulation with 2 μg/mL anti-CD3 for 8 h in the presence of Monensin (Sigma-Aldrich) at 37°C, cells were first surface stained and then permeabilized for 30 min with Cytofix/Cytoperm kit (BD Pharmingen). After washing, cells were stained with anti-IL-10 and IL-5 antibodies for 45 min at 4°C. For Foxp3 and CTLA-4 intracellular staining, an additional permeabilization was performed using a Foxp3 Staining Buffer Set (eBiosience) for 30 min at 4°C. Samples were analyzed in a FACSCalibur or FACSCanto II instruments (Becton Dickinson, San Diego, Calif, USA).

2.8. Lung Digestion and Cell Sorting. After bronchoalveolar lavage, pieces of lung tissue were digested with collagenase (2 mg/mL) and DNase (1 mg/mL) (Sigma-Aldrich) at 37°C for 30 min. Lung CD4$^+$CD25$^-$ and CD4$^+$CD25$^+$ cells were isolated using magnetic cell sorting (Miltenyi Biotec). First, CD4$^+$ cells were negatively isolated using MicroBeads to MHCII, CD8a, and B220 (Miltenyi Biotec). Negative cells were then magnetically labeled to CD25 and isolated CD4$^+$CD25$^-$ (>95%) and CD4$^+$CD25$^+$ (>90%) cells assessed by flow cytometry. In selected experiments, lung cells from allergic *Foxp3gfp*.KI mice were staining for CD4-Pacific Blue and sorted into CD4$^+$Foxp3-GFP$^-$ and CD4$^+$Foxp3-GFP$^+$ using a FACSAria cell sorter (Becton Dickinson).

2.9. In Vitro Suppression Assay. The suppression assay was performed with CD4$^+$CD25$^+$ cells purified by magnetic sorting or with highly purified FACS-sorted CD4$^+$ Foxp3-GFP$^+$ obtained from *Foxp3gfp*.KI mice. For this, CD4$^+$CD25$^-$ and CD4$^+$CD25$^+$ cells were purified using magnetic sorting. Proliferation assays were set up in 96-well round-bottom plates and contained, per well, 2×10^4 responder cells (CD4$^+$CD25$^-$ cells from sensitized and challenged BALB/c mice), 4×10^4 APCs (Mitomycin C-treated spleen cells from TCR$\alpha\beta$-deficient BALB/c mice or from *nude* mice), and anti-CD3 (145-2C11) antibody at a 1 μg/mL. Cells were cocultured at CD25$^-$/CD25$^+$ ratios of 1 : 1, 1 : 0.3, and 1 : 0.1. Proliferation was determined by adding ^{3}H-thymidine on the third day of culture and determining incorporation 6 h later. Suppression assay with CD4$^+$ Foxp3-GFP$^+$ was performed with lung CD4$^+$ Foxp3-GFP$^+$ or Foxp3-GFP$^-$ T cells that were FACS-sorted from allergic *Foxp3gfp*.KI mice. Responder cells (CD4$^+$Foxp3-GFP$^-$) were labeled with 5 μM of *Cell Proliferation Dye eFluor-670* (eBiosciences, San Diego, Calif, USA) according to the manufacturer's recommendations. Dye labeled CD4$^+$Foxp3-GFP$^-$ T cells (2×10^5) were than cultured without or with CD4$^+$Foxp3-GFP$^+$ Treg cells at ratios of 1 : 1, 1 : 0.3, and 1 : 0.1 in the presence of 4×10^5 APCs (spleen cells from RAG$^{-/-}$ mice) and anti-CD3 (1 μg/mL) for 72 h. The proliferation was determined by reduction of the fluorescence intensity of Dye eFluor-670 using a flow cytometry instruments. For analysis of IL-4 and IL-5 production, responder cells (2×10^4 CD4$^+$Foxp3-GFP$^-$) were cocultured without or with CD4$^+$Foxp3-GFP$^+$ Treg cells in the presence of 35 Gy-irradiated lung MHCII$^+$ MACS-purified cells (4×10^4) from *Foxp3gfp*.KI mice and anti-CD3 (1 μg/mL). Cytokine concentrations were quantified by sandwich kit ELISA according to the manufacturer's recommendations as previously described [8].

2.10. Determination of OVA-Specific IgE and IgG1 Antibodies. OVA-specific antibodies were assayed by sandwich ELISA as previously described [8]. For OVA-specific IgE determinations, plates were coated overnight at 4°C with 2 μg/mL of goat anti-mouse IgE antibody (Southern Biotechnology). Serum samples were added followed by addition of biotin-labeled OVA. Bound OVA-biotin was revealed by Streptavidin Peroxidase conjugate (Sigma) as previously described [8]. Hyperimmune serum from OVA/Alum-immunized BALB/c mice was used as IgE standard and arbitrarily assigned as 10.000 U/mL. For OVA-specific IgG1 antibodies, serum samples were plated on 96 wells previously coated with OVA (2 μg/well). The bound antibodies were revealed with goat anti-mouse IgG1 followed by peroxidase-labelled rabbit anti-goat antibodies (all from Southern Biotechnology). The concentration of OVA-specific antibody was estimated by comparison with IgG1 standards run in parallel as previously described [8].

2.11. Cytokine Determinations. The levels of IL-4, IL-5, IL-10, IL-13, and TGF-β in the BAL fluid or supernatants from lung cells culture were assessed by a sandwich kit ELISA according to the manufacturer's recommendations as previously described [8]. Values are expressed as pg/mL deduced from standards run in parallel with recombinant cytokines. Purified and biotinylated antibodies to IL-4, IL-5, and IL-10 kits were from BD OptEIA, San Diego, Calif, USA. IL-13 kit was from R&D Systems and TGF-β1 from Promega, Madison, Wis, USA.

2.12. Lung Histology. Lungs were perfused via the right ventricle with 10 mL of cold PBS, removed, and immersed in 10% phosphate-buffered formalin for 24 h and then in 70% ethanol until embedding in paraffin. Tissues were sliced and 5 μm sections were stained with hematoxylin/periodic acid-Schiff (PAS) for analysis of cellular inflammation and mucus production.

2.13. Statistical Analysis. ANOVA was used to determine the levels of difference between all groups. Comparisons of all

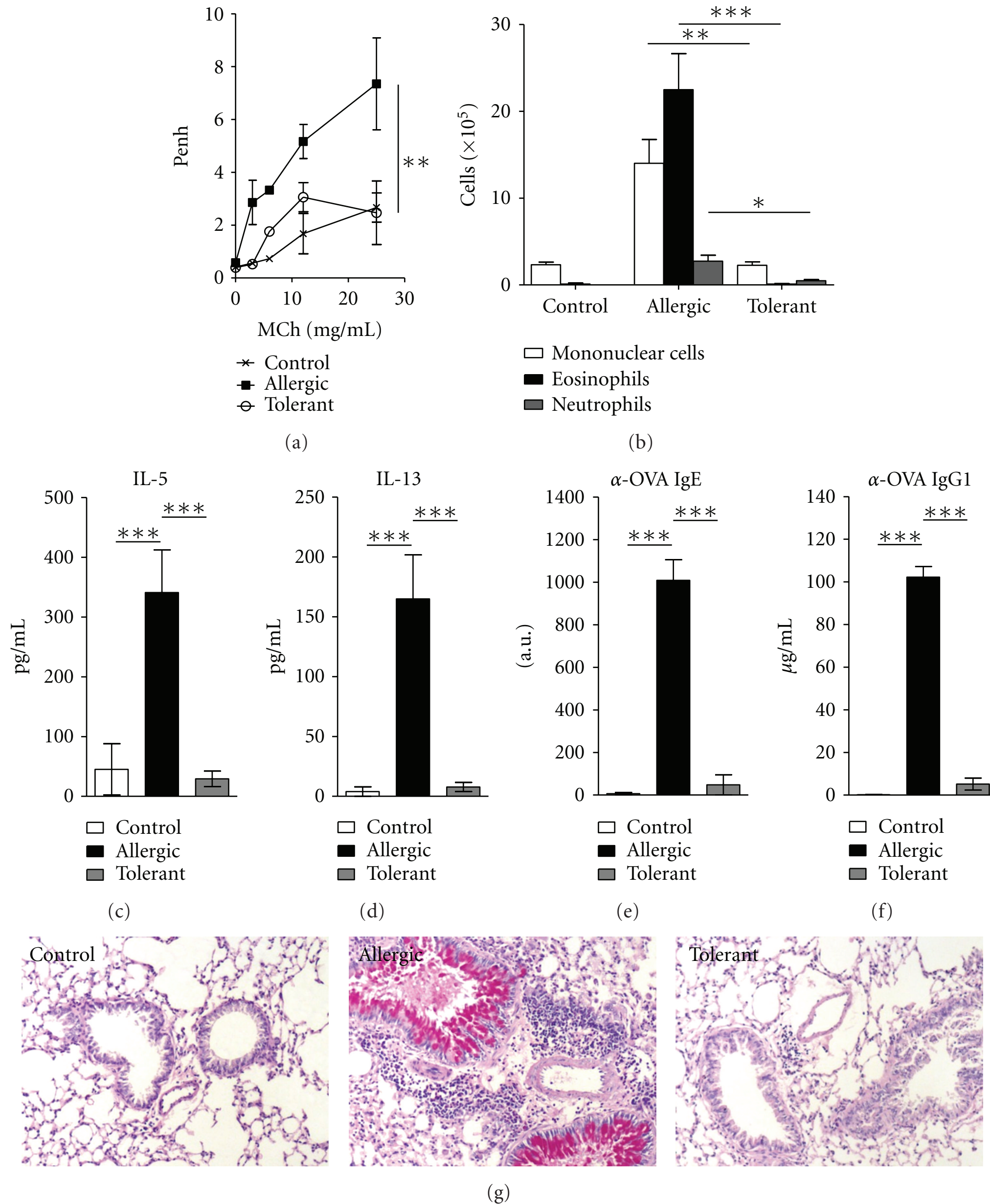

Figure 1: Oral tolerance prevents airway allergic disease. (a) Respiratory pattern to increasing dose of methacholine (MCh) in control, allergic, or tolerant BALB/c mice 24 h after the last OVA challenge. (b) BAL differential cell counts. Quantification by ELISA of (c) IL-5, (d) IL-13 in the BAL fluid, and (e) anti-OVA IgE, (f) IgG1 in the serum. (g) Histology of lung sections at 100x. Lung parenchyma inflammation and mucus production by goblet cells are shown in representative lung sections stained with hematoxylin/PAS. Values represent the means ± SEM for groups of five mice and are representative of more than three experiments. Significant differences $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ are shown.

pairs were performed by Tukey-Kramer honestly significant difference test. Values for all measurements are expressed as mean ± SEMs, and the P values for significance were set to 0.05.

3. Results

3.1. Oral Tolerance Prevents the Development of Asthma-Like Responses. OVA-sensitized and -challenged mice (*Allergic*) developed an enhanced ventilation as revealed by Penh values to increasing doses of methacholine (MCh) compared to untreated mice (*Control*). Conversely, prior oral administration of OVA (*Tolerant*) prevented the increase in ventilation (Figure 1(a)). Differential cell counts showed an increased number of mononuclear cells, neutrophils, and mainly eosinophils in allergic mice compared to control mice. In tolerant mice, the influx of inflammatory cells was almost completely absent (Figure 1(b)). The levels of type-2 cytokines IL-5 and IL-13 in the BAL (Figures 1(c) and 1(d)) and the serum levels of OVA-specific IgE and IgG1 antibodies

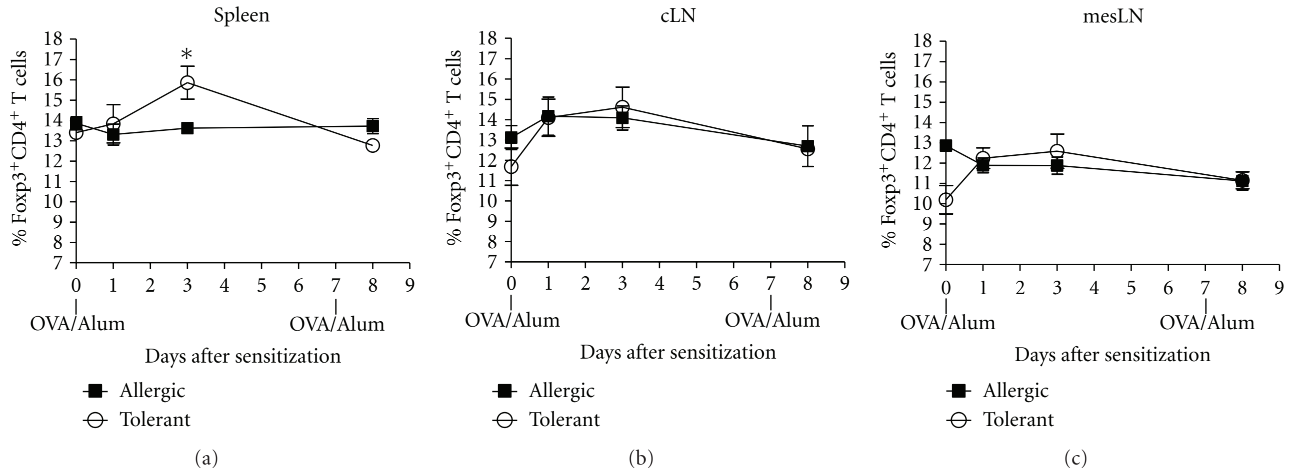

Figure 2: Regulatory T cells in lymphoid organs. Frequency of Foxp3$^+$CD4$^+$ T cells in (a) spleen, (b) cervical lymph nodes (cLN), and (c) mesenteric lymph nodes (mesLN) of C57BL/6 fed or not with OVA before and after OVA/Alum sensitization. Cells recovered from the different lymphoid organs were stained for CD4 and Foxp3 and gated in CD4-positive cells. Values are representative of two independent experiments with pooled cells from three animals per group.

(Figures 1(e) and 1(f)) were also significantly increased in allergic mice than those orally OVA exposed. Furthermore, lung histology of allergic mice showed intense peribronchial and perivascular inflammatory infiltrates and mucus hypersecretion, determined by PAS staining (Figure 1(g)). In contrast, tolerant mice exhibited lung histology similar to control group (Figure 1(g)). These data show and confirm [12] that OVA-feeding before sensitization efficiently suppresses airway allergic responses and systemic IgE antibody production.

3.2. OVA-Feeding Increase Regulatory T Cells in Spleen after Antigen Sensitization. We and others have previously shown that adaptive CD4$^+$CD25$^+$ (Foxp3$^+$) regulatory T (Treg) cells increase in peripheral lymphoid organs after oral OVA administration in mice with monoclonal OVA-T-cell receptor repertoire [13, 14, 18–23]. Here we were interested in determining whether oral OVA in mice with polyclonal T-cell repertoire could also increase the frequency of Treg cells. For this we monitored the frequency of CD4$^+$Foxp3$^+$ Treg cells detected in spleen, mesenteric lymph nodes (mesLN), and cervical-draining lymph nodes (cLN) before and after OVA sensitization in mice that received previously OVA or not in the drinking water. We found that the frequency of CD4$^+$Foxp3$^+$ Treg cells increased at day 3 (d.3) after s.c. OVA sensitization in the spleen of tolerant but not allergic mice and decreased thereafter (Figure 2(a)). No differences were observed between tolerant and allergic mice when the percentages of CD4$^+$Foxp3$^+$ Treg cells were quantified in cLN and mesLN (Figures 2(b) and 2(c), resp.). These results show that oral OVA administration leads to an increased frequency of spleen Treg cells even in mice with polyclonal T-cell repertoire.

3.3. Regulatory T Cells Accumulate in the Airways of Allergic but Not in Tolerant Mice. To monitor the appearance of Treg cells in the airways the number of CD4$^+$CD25$^+$Foxp3$^+$ cells present in the BAL and in the lung tissue were determined from days 14 to 22 (before and after OVA challenges) in mice that received or not OVA in the drinking water. Interestingly, we found an increased number of Foxp3$^+$ Treg cells in the BAL of allergic but not tolerant mice. An apparent increase of these cells was found at day 17, that is, 48 h after the first OVA challenge and a significant increase was detected after the second OVA challenge (d.22) (Figure 3(a)). As expected, the number of effector (CD4$^+$CD25$^+$Foxp3$^-$) T (Teff) cells in allergic mice also increased after the first (d.17) and second OVA challenge (Figure 3(b)). Similar results were found in the lungs of allergic group where the frequency and number of both Treg and Teff cells increased after first and second OVA challenge (Figures 3(c), 3(d), and 3(e)). In allergic group at day 22, the number and frequency of Teff cells in the BAL and lung tissue were more than 4-fold higher than Treg cells (Figures 3(a), 3(b), and 3(c)). These results clearly document that Treg cells are recruited at sites of allergen challenge only in mice experiencing allergic inflammation.

3.4. Lung Infiltrating Regulatory T Cells Expresses an Effector/Memory Phenotype. Because Treg cells were recruited to the airways of allergic mice, we reasoned that these cells might have acquired a migratory phenotype similar to Th2 cells that infiltrate lung tissue [31, 32]. Therefore, we analyzed several T-cell surface molecules associated with T-cell migration and/or activation. As shown in Figure 4(a) by mean fluorescence intensity (MFI) into each FACS-histogram, the BAL CD4$^+$Foxp3$^+$ Treg cells from allergic mice upregulated the chemokine receptor CCR4 but not CCR7, downregulated L-selectin (CD62L) and upregulated ICAM-1 (CD54) when compared with CD4$^+$Foxp3$^+$ Treg cells from lung draining lymph nodes (dLN) (Figure 4(a) upper histograms). To further characterize the phenotype of these Treg cells, we determined the expression of activation

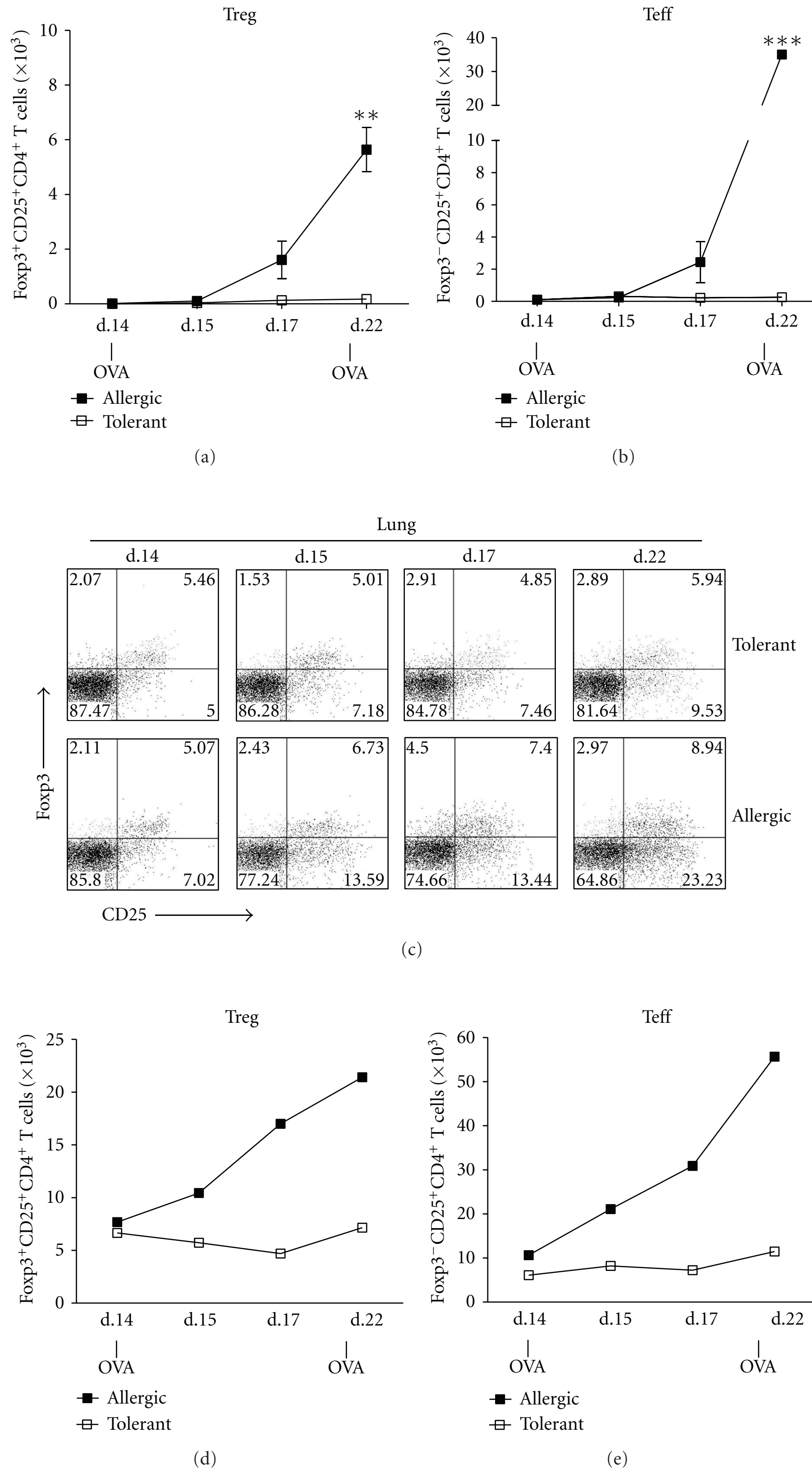

FIGURE 3: Regulatory T cells accumulate in the airways of allergic but not tolerant mice. Time course of (a) $CD4^{+}CD25^{+}Foxp3^{+}$ (Treg) and (b) $CD4^{+}CD25^{+}Foxp3^{-}$ (Teff) cells number in the BAL of allergic and tolerant mice. (c) Frequency and (d) number of $CD4^{+}CD25^{+}$ lung cells expressing or not Foxp3. Pooled cells from three mice recovered from BAL and lung were stained for CD4, CD25, and Foxp3 and gated in CD4-positive cells. Values in (a) and (b) represent the means ± SEM for groups of three mice and are representative of two experiments. The data in (c) show a representative experiment of two. Significant differences $^{**}P < 0.01$, $^{***}P < 0.001$ are related to tolerant group.

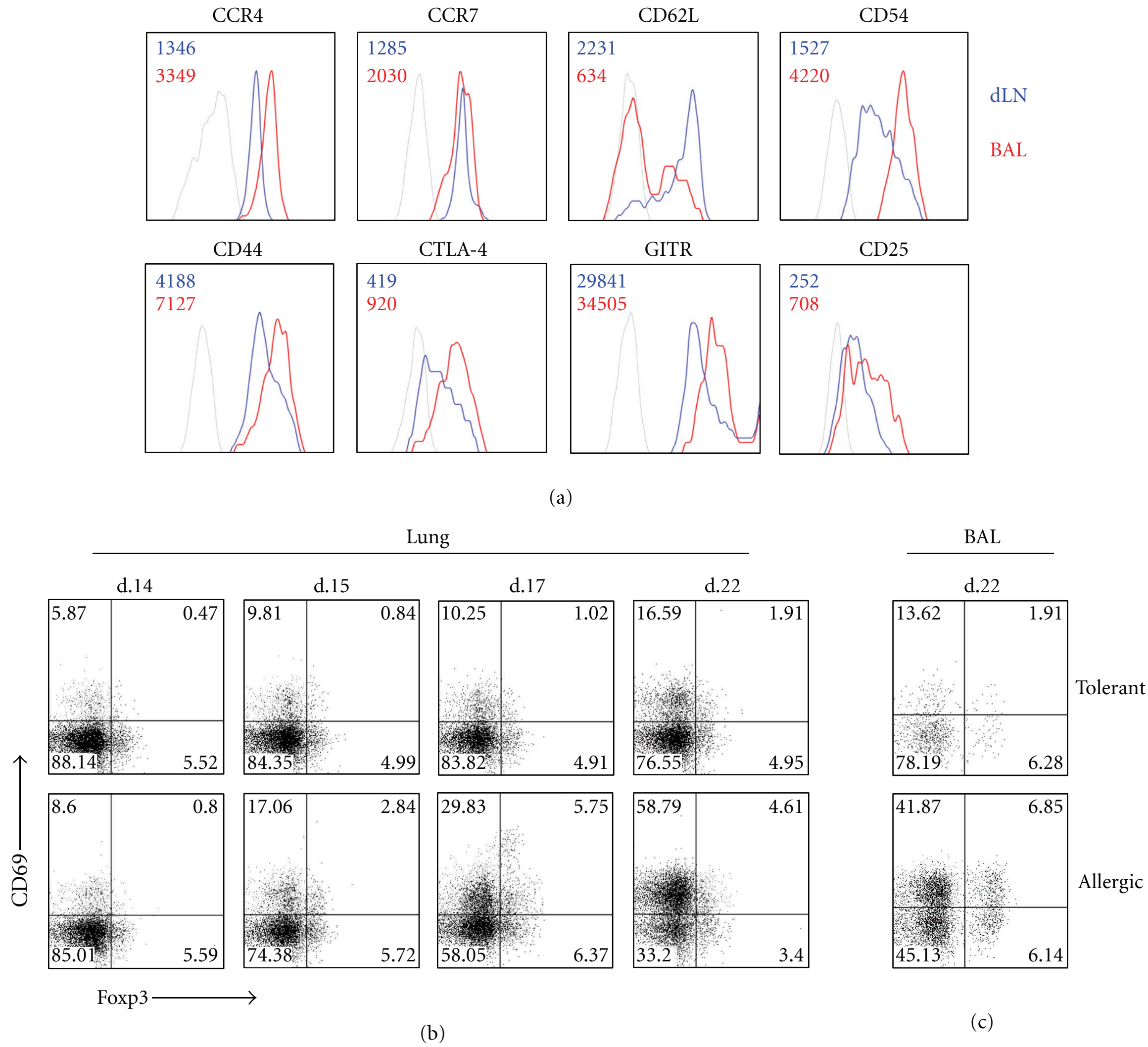

Figure 4: Airway regulatory T cells from allergic mice express a memory/effector phenotype. (a) FACS-Histograms of CD4$^+$Foxp3$^+$ cells from allergic mice expressing CCR4, CCR7, CD62L, CD54, CD44, CTLA-4, GITR, CD25 in BAL (red line), or mediastinal draining lymph nodes (dLN) (blue line). The numbers into each histogram represent the mean fluorescence intensity (MFI). Kinetic of lung CD4$^+$CD69$^+$ cells frequency expressing or not Foxp3. (c) Percentage of BAL CD4$^+$CD69$^+$ cells expressing or not Foxp3. Pooled cells from four mice recovered from lung or BAL were stained for CD4, CD69, and Foxp3 and gated in CD4-positive cells. The results are representative of two experiments with four mice per group.

markers. We found that BAL CD4$^+$Foxp3$^+$ Treg cells from allergic mice also upregulated CD44, CTLA-4, GITR, and CD25 (Figure 4(a) lower histograms). Moreover, in lung tissue the frequency of CD4$^+$Foxp3$^+$ Treg cells expressing CD69 molecule increased substantially after OVA challenge in allergic mice compared to tolerant mice (Figure 4(b)). As expected, the frequency of Foxp3-negative CD69$^+$ T helper (Teff) cells was drastically enhanced in allergic but not in tolerant group after OVA challenges (Figure 4(b)). Similar results were obtained with T cells present in BAL at day 22 (Figure 4(c)). Notably, the frequency of CD69$^+$ Treg cells in the lung and BAL of allergic mice was higher than CD69$^-$ Treg cells, whereas in tolerant mice we found an inverse relation (Figure 4(c)). Taken together, our findings clearly indicate that infiltrating Foxp3$^+$ Treg cells from allergic mice acquire an effector/memory phenotype distinguishing them from Treg cells present in lung-draining lymph nodes and from those present in the airways of tolerant mice.

3.5. Regulatory T Cells Recruited to the Airways of Allergic Mice Are Not the Principal Producers of Suppressive Cytokines. Interleukin-10 (IL-10) and transforming growth factor-β (TGF-β) have been implicated in suppression of inflammation by Treg cells [33–36]. Therefore, we investigated whether airway infiltrating Treg cells from allergic mice produce these cytokines. We first determined the levels of IL-10 and TGF-β in BAL fluid. We found that high levels of IL-10, total, and bioactive TGF-β were significantly increased in the BAL of allergic mice compared to control or tolerant groups (Figures 5(a), 5(b), and 5(c)). To ascertain whether Treg

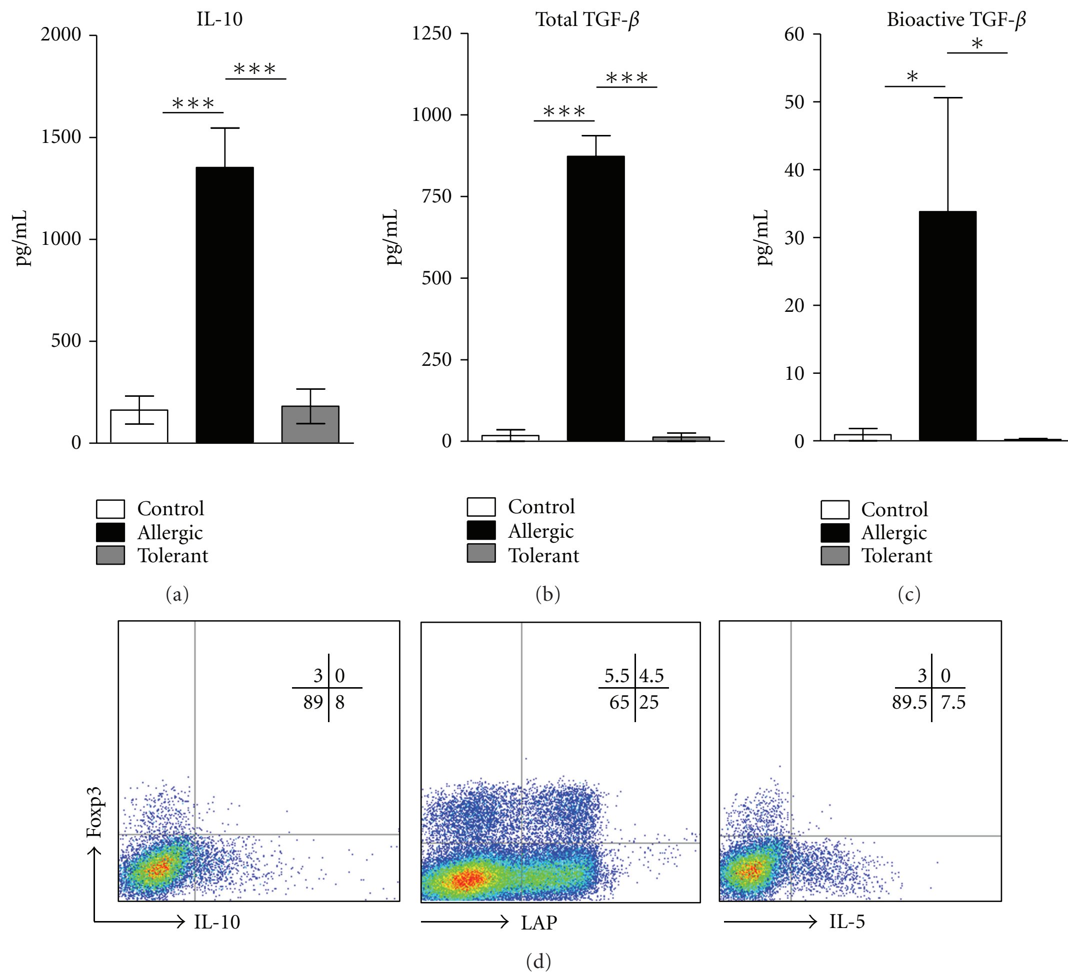

Figure 5: Regulatory T cells are not the major producer of suppressive cytokines in the BAL. Quantification by ELISA of BAL (a) IL-10, (b) total, and (c) bioactive TGF-β of BALB/c control, allergic and tolerant mice upon 24 h of the last OVA challenge. (d) IL-10, LAP, and IL-5 staining of BAL CD4$^+$Foxp3$^+$ cells from allergic mice. Pooled cells recovered from BAL of five allergic mice were stained for CD4, Foxp3, IL-10, LAP, and IL-5 and gated in CD4-positive cells. Values represent the means ± SEM for groups of five mice and are representative of two experiments. Significant differences $^*P < 0.05$, $^{***}P < 0.05$ are shown.

cells of allergic mice produce these suppressive cytokines, we stained CD4$^+$Foxp3$^+$ T cells for intracellular IL-10 or for latent-associated peptide (LAP) to indirectly detect TGF-β producing cells. TGF-β complexes with latency-associated peptide (LAP), and LAP expression correlates with TGF-β production in many cell types [37–39]. We found that only CD4$^+$Foxp3$^-$ cells stained positively for IL-10. The expression of LAP was found in both Foxp3$^-$ and Foxp3$^+$ cells, however, the majority (25%) of CD4$^+$ cells in the BAL expressing LAP were Foxp3$^-$ (Figure 5(d)). As expected, Foxp3$^+$ T cells did not produce IL-5 (Figure 5(d)). These results indicate that high levels of suppressive cytokines at site of allergen challenge are associated with lung allergic inflammation and that CD4$^+$Foxp3$^+$ Treg cells in the airways of allergic mice do not produce IL-10 and are not the major population of TGF-β producing cells.

3.6. Lung Treg Cells of Allergic Mice Exhibit Strong Antiproliferative Activity but Are Unable to Suppress Type-2 Cytokine Production. Finally, to address the role of Treg cells present in the lung of allergic mice, we performed a standard *in vitro* suppression assay [40, 41], as previously described [24]. First, we tested the proliferative activity of CD4$^+$CD25$^-$ (memory/effector T cells) cells from lung upon anti-CD3 stimulation in the presence or absence of CD4$^+$CD25$^+$ cells. As shown in Figure 6(a), lung CD4$^+$CD25$^-$ cells exhibited high proliferative response upon anti-CD3 antibody stimulation whereas lung CD4$^+$CD25$^+$ cells did not proliferate. Coculture of CD4$^+$CD25$^+$ with CD4$^+$CD25$^-$ cells showed that CD25$^+$ cells almost completely suppressed CD25$^-$ cell proliferation at ratio 1 : 1, partially at 0.3 : 1 but not at 0.1 : 1 (Figure 6(a)). Next, we evaluated the production of Th2-cytokine by lung CD4$^+$CD25$^-$ cells in the presence or absence of CD4$^+$CD25$^+$ cells. Albeit CD4$^+$CD25$^+$ cells efficiently suppressed T-cell proliferation, they were unable to inhibit IL-4 and IL-5 production upon anti-CD3 stimulation (Figures 6(b) and 6(c)). Similar data were found when these cells were stimulated specifically with OVA (data not shown). The lack of inhibition of IL-4 and IL-5 secretion by CD4$^+$CD25$^+$ cells might be due to

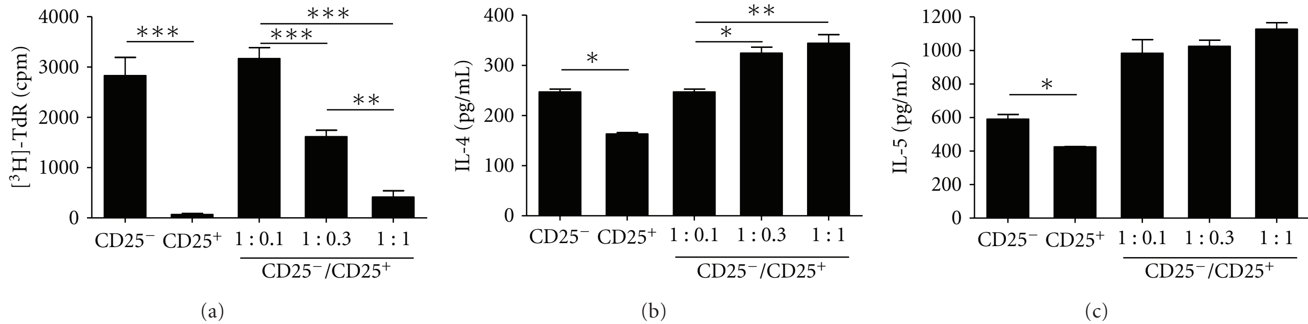

Figure 6: Lung CD4$^+$CD25$^+$ T cells from allergic mice suppress T-cell proliferation but not Th2 cytokine production. (a) Proliferation of MACS-purified lung CD4$^+$CD25$^-$ or CD4$^+$CD25$^+$ cells from BALB/c allergic mice alone or cocultured at different CD25$^-$/CD25$^+$ ratios determined by ^{3}H-thymidine (^{3}H-TdR) incorporation after anti-CD3 stimulation. ELISA assays for (b) IL-4 and (c) IL-5 in the culture supernatants showed in (a). Values represent the means ± SEM of triplicate wells. The results are representative of two experiments. Significant differences $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ are shown.

the fact that this cell population also contains effector T cells. Indeed, CD4$^+$CD25$^+$ cells produced significant amounts of type-2 cytokines (Figures 6(b) and 6(c)). In order to circumvent this problem and address more directly whether Treg cells affect type-2 cytokine production, we performed experiments in *Foxp3gfp*.KI mice that harbor fluorescent Treg cells [29]. Therefore, we induced airway allergic disease in *Foxp3gfp*.KI mice and sorted CD4$^+$ T cells expressing Foxp3-GFP$^+$ Treg cells and CD4$^+$GFP$^-$ T cells (Foxp3$^-$) present in the lungs. We found that only Foxp3$^-$ T cells produced significant amounts of type 2 cytokines upon anti-CD3 stimulation (Figures 7(a) and 7(b)). Notably, a highly purified (>98%) lung population of Foxp3-GFP$^+$ Treg cells could not suppress efficiently Th2 cytokine production by CD4$^+$Foxp3-GFP$^-$ T cells upon anti-CD3 stimulation (Figures 7(a) and 7(b)). Finally, through using purified lung Foxp3-GFP$^+$ Treg cells, we confirmed the suppression assay obtained with CD4$^+$CD25$^+$ T-cell by showing that they efficiently suppressed T effector (CD4$^+$GFP$^-$) cells proliferation at ratio 1 : 1 and 0.3 : 1 but not at 0.1 : 1 as evidenced by Dye eFluor-670 staining (Figure 7(c)). We conclude that lung Treg cells with regulatory phenotype present in the airways of allergic mice exhibit a strong antiproliferative activity but are unable to efficiently suppress type-2 cytokine production.

4. Discussion

A critical issue in immune regulation is where Treg cells exert their suppressive function. Their presence on lymphoid tissue appears to be required for efficient suppression of naive T-cell activation. Conversely, some data indicate that Treg cells are recruited to effector site in order to suppress the action of inflammatory T cells [25, 42, 43]. Previous reports showed a relationship between suppression of asthma-like responses by mucosal tolerance and the emergence of Treg cells in lymphoid organs [17, 21, 24]. We have previously shown in T/B receptors transgenic mice (T-Bmc) devoid of natural regulatory T cells that soon after mucosal antigen exposure, Foxp3-expressing Treg cells are generated in dLN and in spleen [24]. This early induction of Treg cells by prior oral antigen exposure appears to inhibit the development of polarized Th2 inflammatory cells in a TGF-β-dependent manner [24]. Indeed, using the T-Bmc model, we found that Treg cells are able to suppress early T-cell activation, 48 h after immunization with the cognate antigen [24]. However, after establishment of tolerance they became dispensable for its maintenance *in situ*. In the present study, we used a well-established model of mucosal tolerance to allergic lung inflammation [8, 12, 24, 44] to monitor the appearance of Treg cells in the airways after OVA challenge in mice with polyclonal repertoire. We found that only in OVA-fed mice, the frequency of spleen Treg cells increased at day 3 after OVA sensitization, a result resembles the T-Bmc model. However, here we were particularly interested in determining whether Treg cells migrate to airways of allergic or tolerant mice after administration of OVA. We found that allergic but not tolerant mice showed a striking increase in the number of Treg cells in the BAL compared to tolerant mice. Also, high levels of IL-10 and TGF-β were detected in the airways of allergic mice. Notably, we found that among CD4$^+$ T cells recruited to allergic inflammation only Foxp3-negative, but not Foxp3-positive T cells stained positively for IL-10. Moreover, the majority of LAP$^+$ cells were Foxp3-negative T cells. Our results are line with data obtained with T-cell infiltrates in *Shcistosoma mansoni* egg-induced Th2-mediated inflammation [45]. In concert with our findings, migration of Treg cells was also reported in a model of parasite egg antigens-induced inflammation [46], or other pathological conditions, such as arthritis, type 1 diabetes, sarcoidosis and transplants [25, 43, 47–52]. Therefore, it is plausible that the allergic inflammatory milieu triggers the migration of Treg cells into the airways. Accordingly, it has been shown that recruitment of Foxp3-expressing Treg cells to the site of allergic inflammation is dependent on chemokine receptors such as CCR4 [52] and CCR8 [53], where their ligands CCL17, CCL22, and CCL1 are high expressed during allergic lung inflammation [54, 55]. Our data demonstrated that the majority of Foxp3-expressing Treg cells present in the airways upregulated CCR4, CD44

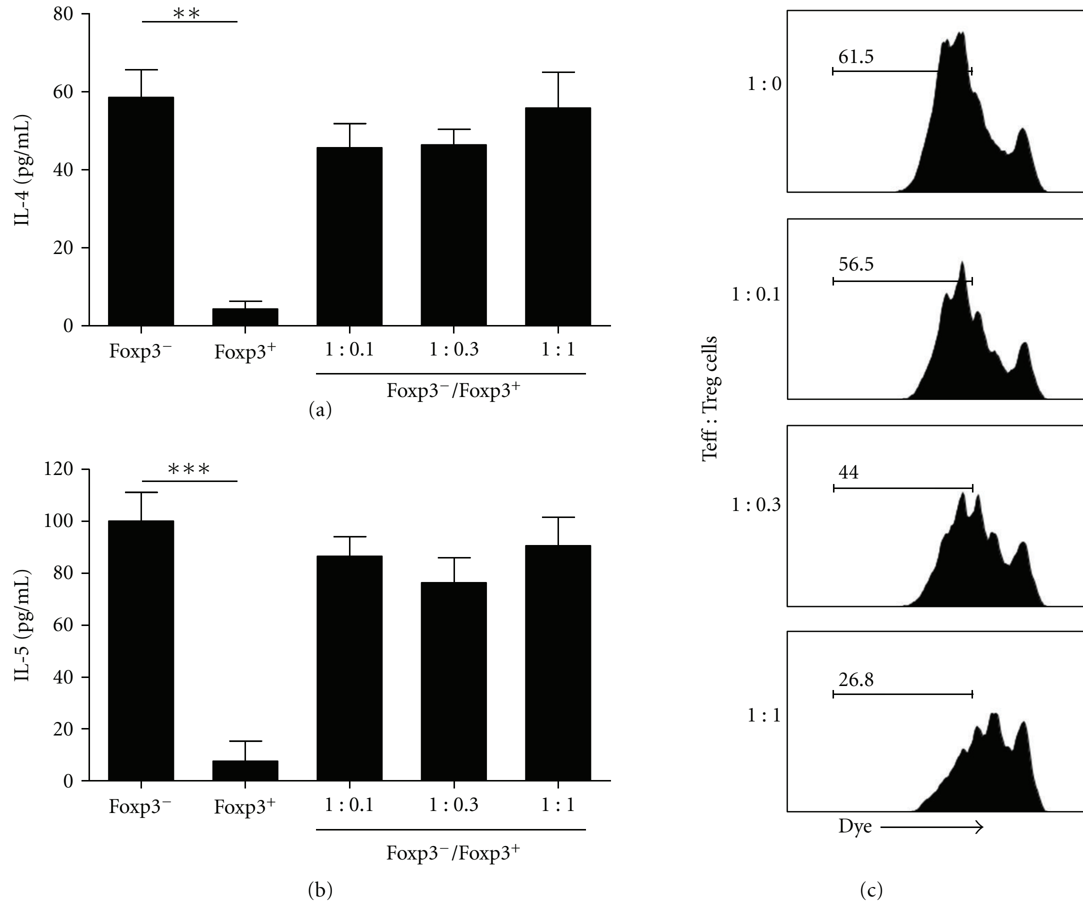

Figure 7: Lung Foxp3$^+$ Treg cells from allergic mice suppress T-cell proliferation but not type 2 cytokine production. (a) IL-4 and (b) IL-5 levels in the supernatants of FACS-sorted lung CD4$^+$Foxp3-GFP$^+$ (Foxp3$^+$) or CD4$^+$Foxp3-GFP$^-$ (Foxp3$^-$) cells from *Foxp3gfp*.KI allergic mice alone or cocultured at different Foxp3$^-$/Foxp3$^+$ ratios. (c) Proliferation of Foxp3-GFP$^-$ cells cocultured at different Foxp3$^-$/Foxp3$^+$ ratios was determined by flow cytometry. Sorted lung CD4$^+$Foxp3-GFP$^-$ were labeled with Dye eFluor-670 and the proliferation was determined by reduction of the fluorescence intensity of Dye. Representative FACS-histograms, which indicate the frequency of T-cell proliferation, are shown. Values represent the means ± SEM of triplicate wells. The results are representative of two experiments. Significant differences $^{**}P < 0.01$, $^{***}P < 0.001$ are shown.

and CD54 and drastically downregulated CD62L, a phenotype that resembles effector/memory T cells. Noteworthy, this phenotype could distinguish Treg cells present in the airways from those present in the lung-draining lymph nodes (dLN). In addition, we showed that Treg cells that accumulated in the airways of allergic mice also acquired activated phenotype, as revealed by increased expression of CTLA-4, GITR, and CD25 contrasting with Treg cells present in the dLN. Moreover, CD69, a marker of cell activation, was highly expressed in Treg cells present in the lung and BAL of allergic mice but not in tolerant group. These data suggest a functionally important activation step that accompanies Treg cell migration. The loss of CD62L and the increased of CD54 expression by Treg cells could also contribute to their migration to the lung [32]. A picture that emerges from our findings is that Treg cells get activated and are recruited to sites of allergic inflammation probably because at these sites CCR4-specific ligands are expressed at high levels [28, 56].

It was recently reported that the loss of CCR4 severely inhibited the accumulation of CD4$^+$CD25$^+$ T cells in the lung and skin [57]. CCR4 knockout mice also fail to develop allograft tolerance after administration of anti-CD154 with donor spleen cells, which is associated with a decreased of Foxp3$^+$ T cells in the graft [43]. Previous data indicated a division of labor between naive and activated Treg cells [58]. For instance, naive-like Treg cells use the chemokine receptor CCR7 for recirculation through lymph nodes where they control the priming phase of an immune response whereas CCR7 is dispensable in effector/memory-like Treg cells for their accumulation in inflamed sites and in fact CCR7-deficiency enhance Treg cells-mediated suppression of inflammation [58]. In our model, the role of CCR7 could not be established because activated lung Treg cells expressed similar levels of CCR7 when compared to naive dLN Treg cells [25]. Using an islet allograft model it was demonstrated that Treg cells first migrate from blood to the allograft where they become activated, and then they migrate to the dLN in a CCR7 fashion. This movement was essential for optimal suppression allograft rejection [25]. A similar situation was found by Graca et al. that found regulatory

T cells in skin allografts suggesting that T-cell suppression of graft rejection is an active process that involves the presence of regulatory T cells at the site of the tolerated transplant [59]. This scenario does not appear to operate in our model because we did not find Treg cells in dLN with an activated phenotype.

We first studied the suppressive activity of airway $CD4^+CD25^+$ T cells, putative Treg cells, in order to determine their role in lung inflammation. We clearly showed that $CD4^+CD25^+$ T cells containing activated $Foxp3^+$ Treg cells efficiently suppressed the proliferation of lung $CD4^+CD25^-$ memory/effector T cells. Strikingly, these $CD4^+CD25^+$ T cells did not suppress the secretion of IL-4 and IL-5 by anti-CD3 or OVA-activated $CD4^+CD25^-$ T cells. Because $CD4^+CD25^+$ T cells contain also effector Foxp3-negative T cells, it is likely that these cells were the source of the type 2 cytokines detected in the cultures. To circumvent this we purified lung fluorescent Foxp3 Treg cells from allergic *Foxp3gfp*.KI mice and tested their suppressive activity on type 2 cytokine production by Foxp3-negative $CD4^+$ T cells. In this situation, Foxp3-positive Treg cells did not secrete type 2 cytokines and did not suppress significantly type 2 cytokine production by Foxp3-negative $CD4^+$ T cells but did suppress $CD4^+$ T-cell proliferation. These results could explain why, despite the large infiltration of Treg cells, allergic mice still show Th2-associated pathological responses. Our results are in line with previous finding showing that in allergic patients, $CD4^+CD25^+$ T cells did not suppress the release of Th2 cytokines [6]. The inefficiency of Treg cell in suppressing inflammatory cytokines in established pathological conditions was also reported in sarcoid granulomas, in which Treg cells suppressed T-cell proliferation but were unable to inhibit TNF-α secretion [51]. Notably, in a model of autoimmune encephalomyelitis, Treg cells also expand after exposure to myelin antigens and infiltrate the central nervous system, but these infiltrating Treg cells were unable to suppress proliferation and inflammatory cytokine production of effector T cells from target tissue [60]. Based on our results and previous reports, it appears that the inflammatory milieu impairs Treg-cell functions.

In summary, we showed that oral tolerance was not associated with an increased number of Treg cells or suppressive cytokines in the airways. Conversely, allergic inflammation triggers the infiltration of Treg cells into the airways that efficiently suppress T-cell proliferation but not Th2 cytokine production. Our findings suggest that allergic inflammation renders the suppressive activity of Treg cells less stringent that, in turn, allows the manifestations allergic reactions mediated by type 2 cytokines.

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Author Contribution

Lucas Faustino and Daniel Mucida contributed equally to this work.

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Review Article

Induction of Immunological Tolerance by Oral Anti-CD3

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In recent years, our knowledge about immunoregulation and autoimmunity has significantly advanced, but nontoxic and more effective treatments for different inflammatory and autoimmune diseases are still lacking. Oral tolerance is of unique immunologic importance because it is a continuous natural immunologic event driven by exogenous antigen and is an attractive approach for treatment of these conditions. Parenteral administration of anti-CD3 monoclonal antibody is an approved therapy for transplantation in humans and is effective in autoimmune diabetes. Orally administered anti-CD3 monoclonal antibody is biologically active in the gut and suppresses experimental models of autoimmune diseases. Orally delivered antibody does not have side effects including cytokine release syndromes, thus oral anti-CD3 antibody is clinically applicable for chronic therapy. Here we review findings that identify a novel and powerful immunologic approach that is widely applicable for the treatment of human autoimmune conditions.

1. Introduction

Understanding how the immune system balances between tolerance and protective immunity is still a key challenge in immunology. Although several approaches have been used to treat autoimmune diseases, they usually involve nonspecific immunosuppression, which frequently comes along with several undesirable side effects. Thus, one of the major goals for the immunotherapy of these pathologies is the induction of regulatory T cells that mediate immunologic tolerance. In this scenario, the gut environment is particularly important, as tolerance induction is the default immune pathway at this site in physiological conditions [1]. The immunological tolerance to antigens that gain access to the body via the oral route has been termed "oral tolerance" [2] and it has been classically defined as the specific suppression of cellular and/or humoral immune responses to an antigen that was first administered by the oral route [1, 3, 4].

Studies of oral tolerance have classically involved the administration of oral antigen followed by challenge with the homologous antigen (usually in an adjuvant) to demonstrate antigen-specific tolerance. In this context, an experimental system that has been frequently used for the study of T-cell function in oral tolerance is the use of TCR transgenic (Tg) mice in which all T cells have a common TCR. Using TCR Tg mice, we administered the cognate antigens myelin basic protein (MBP) and ovalbumin (OVA) and investigated how oral administration of an antigen-affected specific T-cell subsets. In these studies, we demonstrated the dose-dependent induction of Tregs in MBP TCR Tg mice [5] and deletion following high-dose oral administration of OVA in OVA TCR Tg mice [6].

During the course of our experiments, we found that feeding OVA to OVA TCR Tg mice induced CD4+CD25+ Treg cells [7, 8]. Other investigators also showed that oral antigen induced CD4+CD25+ Tregs [9]. The CD4+ cells from OVA TCR Tg fed animals had greater suppressive properties *in vitro* than natural Tregs, mediated suppression in part by both TGF-β and IL-10, and presented increased expression of CTLA-4, a molecule known to be involved in Treg activity [6, 10]. Although, these findings demonstrated that oral antigen could induce/expand Tregs, administration of OVA to OVA TCR Tg mice is dependent on TCR Tg mice and not translatable to humans. We thus asked whether it was possible to trigger the TCR in the gut CD4+ T cells of wild-type mice and induce Tregs without using cognate antigen. This possibility will be discussed in more detail below.

2. Immune Tolerance and Anti-CD3 Antibody Treatment

Different mechanisms have been implicated in the induction and maintenance of immune tolerance including deletion, anergy, and active cellular regulation [11]. One approach that has been successfully used for the induction/restoration of immune tolerance is the administration of CD3-specific antibody. Different groups have demonstrated that parenteral administration of anti-CD3 is effective not only in animal models of autoimmunity, including autoimmune diabetes [12–14] and experimental autoimmune encephalomyelitis (EAE) [15, 16] but also in human trials of autoimmune diabetes [17–19] and psoriatic arthritis [20]. Furthermore, intravenous administration of anti-CD3 is an approved therapy for acute transplant rejection in humans although side effects related to cytokines release limit its chronic use [21, 22]. Humanized antibodies have been designed to reduce these side effects [23, 24] but the successful translation might require new therapies that would be more physiologic and less toxic and mucosal tolerance can be exploited in this direction.

2.1. Oral Anti-CD3. It is known that anti-CD3 binds to the ε chain of the TCR complex and when given intravenously deletes T cells and, as mentioned above, has been shown to be an effective treatment for type 1 diabetes in the non-obese diabetic (NOD) mouse [25]. We hypothesized that oral administration of anti-CD3 monoclonal antibody would replace the use of a cognate antigen to trigger the TCR and would thus induce Tregs when given orally. Monoclonal antibodies have not been given orally on the assumption that they would be degraded in the gut and thus would not be biologically active. Nonetheless, it is known that orally administered cytokines [26] and peptides [27, 28] are biologically active, demonstrating that orally administered proteins are not completely degraded in the gut.

Thus, to test this hypothesis, we administered hamster anti-mouse CD3 (2C11 clone) to SJL mice and immunized with PLP/CFA to induce EAE. We found that oral anti-CD3 suppressed both clinical and pathologic features of EAE both in the PLP and MOG EAE model [29]. There was a dose effect observed, with EAE suppression by oral anti-CD3 seen at lower (5 μg), but not higher doses (50 μg, 500 μg). These findings were consistent with the classic paradigm of oral tolerance in which induction of Tregs is seen at lower but not higher doses [1]. Furthermore, it demonstrates that induction of Tregs by oral anti-CD3 is not simply related to administering large amounts of antibody to overcome degradation of the antibody in the gut. Indeed, biologically active anti-CD3 could be isolated from intestinal eluates of animals that were given anti-CD3 orally [30] and we could also visualize anti-CD3 being taken up by gut epithelial cells and binding to gut DCs in intestinal loop experiments [1]. The extent to which these DCs that take up anti-CD3 play a role in the mechanism of action of oral anti-CD3 has still to be better studied. Of note, the Fc portion of anti-CD3 was not required, as anti-CD3 Fab′2 fragment is active orally and induces Tregs.

2.2. Differences between Intravenous and Oral Anti-CD3 Antibody. It is known that intravenous anti-CD3 enters the blood stream, modulates CD3 from the cell surface, and leads to the depletion of CD3+ T cells. Oral anti-CD3, on the other hand, does not enter the blood stream or modulate CD3 from the cell surface but acts locally in the gut to induce Th3 type CD4+CD25−LAP+ Tregs in the MLNs. As oral anti-CD3 does not enter the bloodstream, there is no cytokine release syndrome, one of the main problems with the intravenous administration of anti-CD3. In the EAE model, intravenous anti-CD3 is effective when given after disease manifests but not when given prior to disease induction. Oral anti-CD3, on the other hand, ameliorates EAE both when given prior to EAE induction and at the height of disease [29]. The explanation for this difference is related to the fact that intravenous anti-CD3 acts primarily by lysing disease effector cells (present only after disease induction), whereas oral anti-CD3 acts by inducing Tregs. Intravenous anti-CD3 has been reported to induce Tregs that act in a TGF-β-dependent fashion, but only after lysis of T cells [12]. Therefore, immunoregulation by oral anti-CD3 involves different mechanisms other than intravenous anti-CD3 and has the advantage of being safer as it is not associated with systemic side effects [31].

We found that oral anti-CD3 ameliorates disease in other autoimmune and inflammatory diseases including streptozocin-induced [30] and NOD autoimmune diabetes (unpublished data), type 2 diabetes in the Ob/Ob mouse [32], lupus prone SNF1mice [33], and collagen-induced arthritis [34]. A different group of investigators reported that oral anti-CD3 suppresses atherosclerosis in ApoE$^{-/-}$ mice [35]. In all these models, disease amelioration was related to the induction of TGF-β-dependent Tregs that express LAP on their surface. Indeed, *in vivo* treatment of animals with anti-LAP antibody [36], abrogated tolerance induction by oral anti-CD3 in the EAE model (da Cunha et al. [1], unpublished). We also found that nasal anti-CD3 ameliorates lupus but does so by inducing an IL-10-dependent CD4+CD25−LAP+ Treg as opposed to the TGF-β-dependent LAP+ Treg induced by oral anti-CD3 [37]. This is consistent with the observation that GALT DCs induce TGF-β-dependent Tregs versus IL-10-dependent Tregs induced in the bronchial associated lymphoid tissue [38]. Furthermore, these results demonstrate that mucosally administered anti-CD3 appears to act in a fashion analogous to mucosally administered cognate antigen [38].

The effects of oral anti-CD3 raise the question whether it is more advantageous to induce antigen-specific versus antigen nonspecific Tregs for the treatment of autoimmune and inflammatory diseases. It is assumed that the induction of antigen-specific Tregs is preferable, as one would have specific immune modulation with less potential side effects. Furthermore, because of the phenomenon of bystander suppression, which we first described in association with oral tolerance [39], cytokines such as TGF-β released from antigen-specific Tregs at the target organ would suppress reactivity to other autoantigens that developed in the course of epitope spreading. Of note, there may be target organ specificity even when antigen nonspecific Tregs are induced

with oral anti-CD3 as we observed increased numbers of Th3 type LAP+ Tregs in the pancreatic lymph nodes of autoimmune diabetic mice [30], and this has been suggested for atherosclerosis models [29]. Furthermore, in conditions such as type 2 diabetes, lupus, and atherosclerosis, there are not well-defined target antigens and in these conditions induction of antigen nonspecific Tregs by anti-CD3 may be preferable. Studies are in progress to test the combination of mucosal anti-CD3 given with antigen. As discussed below, oral anti-CD3 is currently being tested in humans.

3. Oral Anti-CD3 in Humans

Oral anti-CD3 has been tested in a phase 1 study in healthy human volunteers (3 per group) who were orally administered 0.2, 1.0, or 5.0 mg of mouse anti-human OKT3 mAb daily for 5 days [31]. Immunologic effects were observed in the peripheral blood and consisted of transient proliferation, suppression of Th1/Th17 responses, increased expression of Treg markers, increased TGF-β/IL-10, and decreased IL-23/IL-6 expression in dendritic cells. No side effects were observed. There were no human anti-mouse antibody responses, changes in CD3 cells in the blood, or modulation of CD3 from the surface of T cells. The optimal dose was found to be 1 mg.

Oral OKT3 mAb has also been recently tested in a single-blind randomized placebo-controlled phase 2a study in patients with nonalcoholic steatohepatitis (NASH) and altered glucose metabolism that included subjects with type-2 diabetes [40]. The study was performed at the Hadassah-Hebrew University Medical Center in Jerusalem, Israel. OKT3 or placebo was orally administered (9 per group) at doses of 0.2, 1.0, and 5.0 mg. In the NASH study 36 subjects were treated once daily for 30 days with final follow-up 60 days after the first dose.

Oral OKT3 was safe with no adverse effects or systemic toxicity as measured by blood hematology, chemistry, immunological safety markers, and physical signs. There were no changes in blood levels of CD3, CD4, or CD8-positive cells. Oral OKT3 induced regulatory T cells, which generally persisted to day 60 and trends in cytokine production consistent with effects observed in the phase 1 clinical study and in animal models. Positive trends in clinical parameters, some of which were statistically significant were also observed including a reduction in liver enzymes and reduced blood levels of glucose and insulin. Several of the positive efficacy trends persisted to day 60 following cessation of treatment at day 30. Some subjects had increased levels of serum antibodies directed against OKT3, which did not affect the positive trial results observed. These results suggest that oral anti-CD3 mAb may have clinical benefit for subjects with NASH or type-2 diabetes. Confirmatory studies are now needed, including studies with humanized antibodies. These results will provide the basis for investigating oral/nasal anti-CD3 in other autoimmune and inflammatory conditions in humans.

Recently, an important development that will facilitate studies related to the use of humanized anti-CD3 antibodies was the generation of transgenic mice expressing the ε chain of the human CD3 complex on the NOD background (NOD-huCD3ε). These mice develop spontaneous diabetes to the same extent as the wild-type NOD mice and when treated with a humanized anti-CD3 antibody, they exhibited a complete and durable disease remission [41]. This model will be very useful for studies evaluating anti-human CD3 antibodies, including its administration by oral route, and this will open new perspectives and clarify their potential clinical utility.

4. Summary

Mucosal tolerance is an attractive approach for the treatment of autoimmune inflammatory diseases as it is a clinically applicable physiologic manner to suppress inflammation through the induction of regulatory T cells. Its lack of toxicity and ease of administration are also important features desired for an effective immunotherapy. Several studies have demonstrated that oral (or nasal) administration of anti-CD3 monoclonal antibodies can be used to induce immune tolerance and is effective in the treatment of animal models of autoimmune diseases. These studies have also demonstrated that mucosal administration of anti-CD3 antibodies does not lead to detectable side effects including cytokine release syndrome or antiglobulin response and thus could be used as a continuous therapy for the treatment of these conditions. In summary, results presented here indicate that oral administration of anti-CD3 antibodies represents a new avenue that can be investigated for the treatment of autoimmune diseases.

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Review Article

Induction of Tolerance via the Sublingual Route: Mechanisms and Applications

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The clinical efficacy of sublingual immunotherapy (SLIT) with natural allergen extracts has been established in IgE-dependent respiratory allergies to grass or tree pollens, as well as house dust mites. Sublingual vaccines have an excellent safety record, documented with approximately 2 billion doses administered, as of today, in humans. The oral immune system comprises various antigen-presenting cells, including Langerhans cells, as well as myeloid and plasmacytoid dendritic cells (DCs) with a distinct localisation in the mucosa, along the *lamina propria* and in subepithelial tissues, respectively. In the absence of danger signals, all these DC subsets are tolerogenic in that they support the differentiation of Th1- and IL10-producing regulatory $CD4^+$ T cells. Oral tissues contain limited numbers of mast cells and eosinophils, mostly located in submucosal areas, thereby explaining the good safety profile of SLIT. Resident oral Th1, Th2, and Th17 $CD4^+$ T cells are located along the *lamina propria*, likely representing a defence mechanism against infectious pathogens. Second-generation sublingual vaccines are being developed, based upon recombinant allergens expressed in a native conformation, possibly formulated with Th1/T reg adjuvants and/or mucoadhesive particulate vector systems specifically designed to target oral dendritic cells.

1. Introduction

Following the pioneer studies by Noon and Freeman [1, 2] conducted a century ago, allergen-specific immunotherapy is presently the only curative treatment for type I allergies. Specifically, subcutaneous immunotherapy (SCIT) was shown to provide clinical benefit for patients with IgE-dependent allergies to either grass, weed, and tree pollens, dust mites (e.g., *Dermatophagoides pteronyssinus, Dermatophagoides farinae*), cat and dog epithelia, or moulds [3, 4]. Also, SCIT has become a reference treatment for venom allergies [3]. Although SCIT has been occasionally performed with soluble allergens, in most circumstances, subcutaneous vaccines include adjuvants such as aluminum hydroxide or calcium phosphate. Since SCIT requires multiple injections and can be associated with severe side effects, including anaphylactic shocks, safer and noninvasive mucosal routes of administration have been explored as an alternative [5, 6].

Most particularly, sublingual immunotherapy (SLIT) was investigated in allergic patients almost twenty years ago and is now established as a valid noninvasive alternative to subcutaneous immunization to treat type I respiratory allergies [5–8]. Although the sublingual route is the only mucosal route commonly used in humans for tolerance induction in allergic patients, other exploratory routes are being tested, including the oral, intranasal, epicutaneous and intralymphatic routes [9–20] (Table 1). This review focuses on the clinical indications, mechanisms of action, and future developments pertaining to sublingual allergy vaccines.

2. Sublingual Allergy Vaccines as a New Therapeutic Class

Sublingual immunotherapy (SLIT) represents a form of therapeutic vaccination aiming to a long-term allergen-specific immunomodulation in patients with allergic rhinoconjuctivitis, with or without moderate asthma [6]. It is performed by reiterated administration (over months or even years) of an allergen extract in the form of drops, fast dissolving

Table 1: Compared characteristics of sublingual versus other administration routes for allergy vaccines.

Routes	Current clinical indications	Comments	Ref
Sublingual (SLIT)	(i) Established as a safe and efficacious treatment for IgE-dependent respiratory allergies (rhinoconjunctivitis with or without moderate asthma) (ii) For adults and 5–15 year old children	(i) No adjuvants (ii) Dose 50 to 100 fold the one used for SCIT (iii) Treatments available as drops, fast-dissolving tablets, lyocs) (iv) Two sublingual grass pollen tablets (Grazax, Oralair) have been registered in Europe as pharmaceutical specialties) (v) Excellent safety record (mostly moderate local reactions). Systemic reactions are extremely rare) (vi) Efficacy documented by large scale double blind placebo controlled Phase III trials (evidence-based medicine)	[5–8]
Subcutaneous	(i) Same as SLIT (ii) Venom allergies (iii) Latex allergies	(i) Adjuvants (aluminum salts or calcium phosphate) are being used (ii) For effective immunotherapy, a 5 to 25 μg maintenance dose of allergen is necessary (iii) Efficacy documented by historical practice (reference route since 1911) (iv) Potential safety issues (besides acceptable local reactions, risk of infrequent but life threatening anaphylactic shocks).	[3, 4]
Exploratory routes (oral, nasal, epicutaneous, intralymphatic)	(i) None as of today (ii) Numerous clinical studies are being conducted in patients with respiratory allergies (mites, pollens) or food allergies (milk, egg, peanut)	(i) Encouraging results in small cohorts of patients (ii) Safety and efficacy remain to be confirmed in large scale clinical studies. (iii) Expected positive outcomes of those new routes include new applications for immunotherapy (e.g., food allergy for the oral or epicutaneous routes) or tolerance induction with a limited number of administrations (e.g., intralymphatic route)	[9–20]

tablets, or lyocs [6, 21]. Patients are asked to maintain the allergen(s) under the tongue for 1-2 mn to allow contact with the oral mucosa. The allergens are subsequently swallowed, as per the “sublingual-swallow” procedure. In all circumstances, high doses (usually 50- to 100-fold the ones used for SCIT) of the allergen(s) are being used in the absence of any adjuvant [6, 21]. Currently, sublingual vaccines are based on aqueous extracts prepared from natural allergenic materials such as animal hair, pollens, or lab cultures of house dust mites [22]. Less frequently, these allergens are treated with glutaraldehyde to form polymers precluding IgE reactivity, but the clinical efficacy of such “allergoïds” remains to be documented [3, 4].

Multiple studies further compiled in metaanalyses have demonstrated the efficacy of sublingual drops in adult and pediatric patients with allergic rhinoconjunctivitis to either pollens (from common grasses, ragweed, parietaria, birch, olive tree, cupressus), house dust mites, or cat dander [23–27]. More specifically, SLIT significantly reduces both rhinoconjunctivitis symptoms as well as the need for symptomatic medication. These studies, as well as the experience cumulated by allergists during their daily practice (which together account for around 2 billion doses of sublingual vaccines administered to human beings), have unambiguously documented the excellent safety profile of sublingual vaccines [6, 23–27]. Specifically, treatment-related adverse events include frequent (i.e., in more that 60% of patients) but moderate local reactions in the form of throat irritation, ear pruritus, or tongue oedema [6]. Those adverse events are mostly observed when SLIT is initiated, and are usually self resolving without any specific further treatment. In contrast to SCIT, systemic reactions, most particularly in the form of anaphylaxis, are extremely rare, and are thought to be linked to the nonrespect of commonly accepted medical guidelines (i.e., administration of overdoses or inappropriate allergen mixes, treatment of patients with uncontrolled asthma) [4, 6].

Recently, two sublingual grass pollen tablets have been developed and shown in multiple double-blind placebo-controlled Phase III clinical studies conducted in both adults and children to be highly efficacious, with an overall 30–40% improvement in rhinoconjonctivitis symptom scores when compared with placebo [28, 29]. Another large-scale study has also documented the clinical efficacy of SLIT in perennial allergy (i.e., to house dust mites) using a tablet containing extracts from the two common *D. pteronyssinus* and *D. farinae* mite species [30]. Other trials have demonstrated a long-term efficacy of sublingual immunotherapy, for example, following a three-year administration of grass pollen tablets [31, 32]. In addition, those allergic patients remained protected for at least two years after stopping the treatment, thus documenting a “disease-modifying” effect of SLIT. Based on those results, such sublingual tablets have been registered as pharmaceutical specialties (Grazax, Oralair) in Europe. The recommendation to use sublingual

immunotherapy has been endorsed by the World Health Organisation (WHO) in several position papers on allergen immunotherapy as well as by the allergic rhinitis and its Impact on asthma (ARIA) workshop group [3, 4, 6].

3. Specific Properties of the Oral Immune System

The sublingual route has been initially used for small synthetic drugs (e.g., nitroglycerine, opioid analgesics) for which a fast plasmatic release was needed [21]. In contrast to such small molecules, proteins do not cross the mucosa to reach the bloodstream, but are rather captured by professional antigen-presenting cells (APCs) within 15 to 30 minutes, which will subsequently migrate to draining cervical submaxillary lymph nodes within 12 to 24 hours (Figure 1) [21, 33]. This makes the sublingual route very interesting for clinical tolerance induction over other mucosal routes, including the oral route, in that the antigen is being captured and processed by APCs prior to significant proteolytic degradation, thus preserving the integrity of T and B cell epitope repertoires.

A detailed mapping of the oral immune system, most particularly of antigen-presenting cells (APCs), has been completed in mice [33, 34]. Specifically, three subsets of oral dendritic cells (DCs) exhibiting a distinct tissue distribution have been identified including (i) Langerhans cells (LCs) located in the mucosa itself, (ii) a predominant subpopulation of myeloid DCs (MDCs) located along the *lamina propia,* and (iii) plasmacytoid DCs (pDCs) found in submucosal tissues (Figure 1) [33]. In humans, LCs have similarly been described in the mucosa itself, whereas myeloid and plasmacytoid DCs are less abundant [35–38]. Noteworthy, all these DC subsets are thought to be tolerogenic, in that they produce both IL-10 and IL-12 cytokines and thus, drive the differentiation of naïve $CD4^+$ T cells towards a Th1/T Reg phenotype (Figure 1). Among those APCs, Langerhans cells and a subset of macrophage-like CD11b+CD11c− APCs are thought to be critical in capturing the antigen/allergen [33, 35, 36, 39].

Only few proinflammatory cells (i.e., mast cells (MCs) or eosinophils (Eos)) are found in oral tissues, and these cells are mostly located in muscular tissues (Figure 1) [34, 38]. In this context, most allergens are likely captured by tolerogenic dendritic cells in the upper layers of oral tissues prior to reaching proinflammatory mast cells, thus explaining the excellent safety profile of the sublingual route, with virtually no risk of severe systemic reactions when compared with the subcutaneous route [6, 21, 35]. Lastly, resident $CD4^+$ T lymphocytes are abundant in oral tissues, located in the vicinity of myeloid APCs along the *lamina propria*. These cells comprise both suppressive as well as effector T cells, including Th1, Th2, and Th17 lymphocytes, likely involved in defence against infectious pathogens. This explains why the sublingual route is also currently considered to elicit effector immune responses against pathogenic viruses [40]. Nonetheless, in the absence of any danger signal, the default response to an antigen administered via the sublingual route is tolerance induction following the induction of Th1/T reg $CD4^+$ T cells [21, 35].

4. Immune Changes Associated with Sublingual Immunotherapy

Allergen-specific immunotherapy is known to reduce both immediate- and late-phase allergen-induced symptoms, via both humoral and cellular mechanisms [41–43]. Immune mechanisms leading to clinical tolerance, described in more details below, are thought to be associated with both subcutaneous and sublingual immunotherapy. Most particularly, changes in the polarization of allergen-specific $CD4^+$ T-cell responses are considered to be central, in that variations in the patterns of cytokines produced significantly impact antibody responses as well as recruitment and activation of proinflammatory cells in target mucosae [21, 41–43].

4.1. Antibody Responses. After an initial rise, allergen-specific sublingual immunotherapy induces a prolonged decrease in seric IgE levels and prevents the seasonal increase in IgEs associated with exposure to environmental allergens [21, 41–43]. For example, pollen immunotherapy results in the blunting of the seasonal upregulation of specific IgEs, while eliciting allergen-specific IgG responses—mostly IgG1 and IgG4. Such IgG antibodies may act as “blocking” antibodies by competing with IgEs for allergen binding, thereby inhibiting IgE-mediated allergen presentation to T cells [44]. In addition, they can engage low-affinity Fc receptors for IgGs (CD32) known to down regulate mast cell and B cell activation [45]. A specific property of SLIT, when compared to SCIT, is further to elicit allergen-specific IgAs, both in serum and mucosal secretions [43, 46, 47].

4.2. Proinflammatory Cells. A reduced recruitment and activation of inflammatory cells in target mucosae has been observed following allergen-specific immunotherapy [48–50]. Specifically, successful SLIT has been associated with a decrease in the recruitment of mast cells (both tryptase/chymase+ or tryptase+ only), basophils, and eosinophils in the skin, nose, eye, and bronchial mucosae [21, 43].

4.3. T-Cell Responses. Sublingual immunotherapy shifts allergic-specific $CD4^+$ T-cells responses from Th2 to Th1, with the stimulation of IFNγ-producing T lymphocytes [21, 42, 43]. In addition, SLIT also induces regulatory T (T Reg) cells, thought to play a central role in inhibiting effector mechanisms associated with allergic inflammation [51, 52]. T Reg cells induced during immunotherapy are type 1 (Tr1) cells producing high levels of IL-10 and/or transforming growth factor-β (TGF-β), known to decrease IgE production and to enhance IgG4 and IgA production, respectively [42, 43]. In addition, both IL-10 and TGF-β lower the release of proinflammatory mediators and inhibit the production of Th2 cytokines [42].

5. Future Directions

5.1. Development of Sublingual Recombinant Allergy Vaccines. There is, as of today, no recombinant allergy vaccine commercially available. With the advent of molecular biology and the use of recombinant DNA technology, the possibility

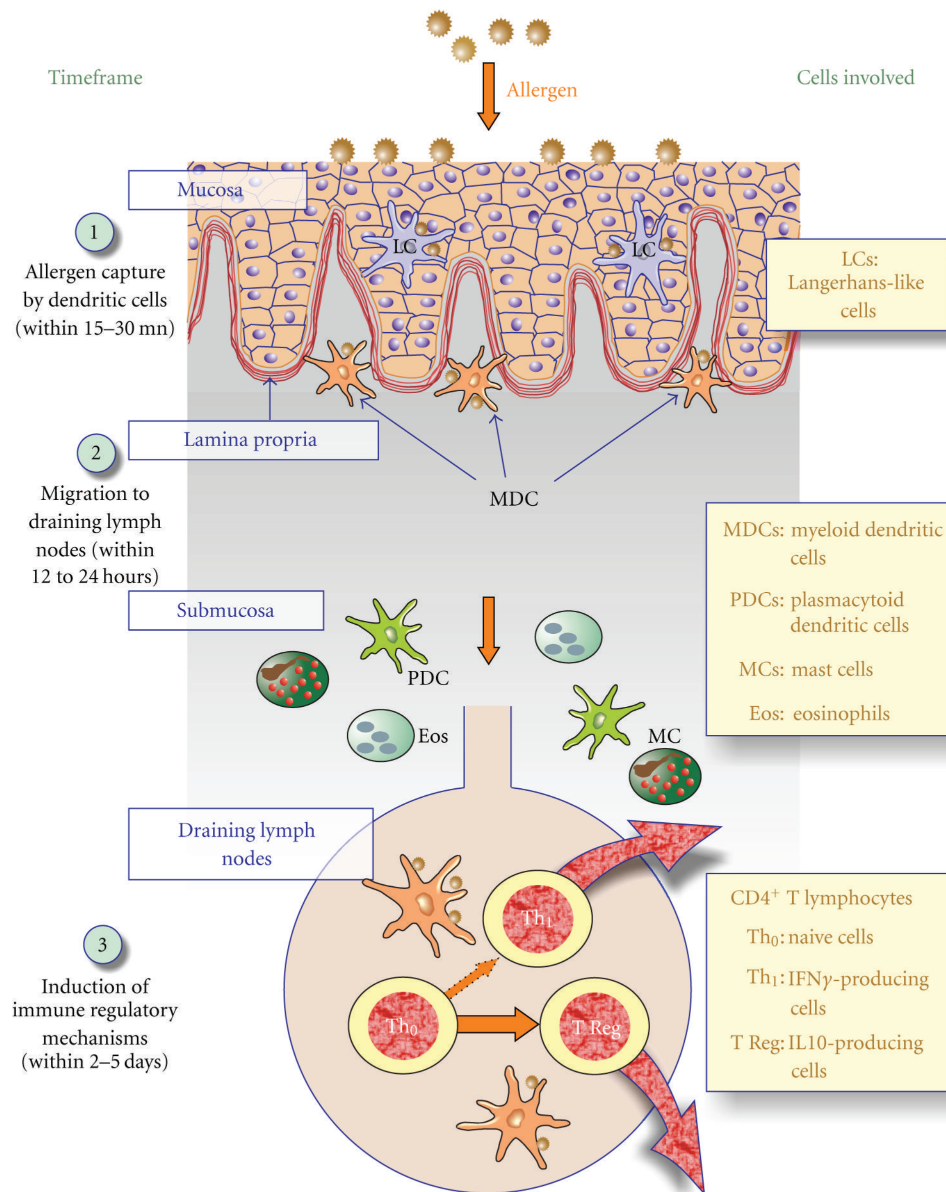

Figure 1: Fate of the allergen following sublingual administration. Following sublingual immunization, substantial amounts of the allergen bind to epithelial cells within minutes, then cross the mucosa between 15 and 30 minutes. The allergen is subsequently captured by dendritic cells (likely by Langerhans cells (LCs) within the mucosa itself and myeloid dendritic cells (MDCs) along the lamina propria) and processed as small peptides presented in association with MHC class I and class II molecules at the cell surface. Those DCs loaded with allergen-derived peptides reach cervical lymph nodes within 12 to 24 hours, where they interact with naive $CD4^+$ T cells to support the differenciation of Th1 and T Reg cells within two to five days. These $CD4^+$ T cells subsequently migrate into the blood and back to mucosal tissues, resulting in allergen tolerance with downregulation of preexisting Th2 responses.

of developing highly purified allergens for sublingual immunotherapy is raising considerable interest [22, 53]. Over the last decade, genes encoding the most important allergens have been cloned (cf., updated list at http://www.allergen.org/) and for a number of them, expressed as recombinant proteins. Such recombinant allergens offer the advantage over natural extracts of being better characterized and easier to produce in a consistent manner [22, 53].

Vaccines based on recombinant allergens are particularly suitable when the number of target allergens is limited, which is the case for birch (*Betula verrucosa*) pollen allergy [54]. Over 95% of patients allergic to birch pollen display IgE reactivity to the Bet v 1 allergen and up to 60% of these patients are sensitized to Bet v 1 only. A recombinant form of Bet v 1 (isoform a) has been produced in *Escherichia coli* and shown to be folded similarly to the native protein, with a compact and stable structure and a well-preserved anti-

genicity [54]. This rBet v 1 protein has been initially tested in a Phase I/II clinical trial via the subcutaneous route (using 15 ug/dose in association with Alum as an adjuvant). Under those conditions, the rBetv 1 allergen alone was as efficient as the total birch pollen extract in alleviating patients' symptoms during the pollen season [55]. Based on those results, rBet v 1 has been administered without any adjuvant via the sublingual route in a Phase II study and shown to decrease significantly rhinoconjunctivitis symptoms as well as the use of symptomatic medications, in comparison to placebo [56].

One pending question regarding the use of recombinant allergens for immunotherapy is whether IgE binding epitopes should be preserved in the molecule [53]. Hypoallergenic forms of recombinant allergens or peptide fragments have been produced which do not induce degranulation of IgE+ mast cells or basophils, while maintaining their capacity to elicit IgG and $CD4^+$ T responses [53]. However, while hypoallergens could in theory represent safer vaccines, there is, as of today, no evidence of their clinical efficacy. With respect to the sublingual route, oral DCs have been shown to express Fc receptors for IgEs, which thus can be used to better address the allergen onto APCs [37]. Interestingly, in the Phase II study described above, rBet v 1 administered sublingually was well tolerated at doses up to 50 ugs, besides the expected local reactions commonly associated with SLIT [56]. For those reasons combined, our working hypothesis is that recombinant allergens to be used sublingually should rather be produced in a wild-type (i.e., native) conformation in order to mimick the natural allergen [21, 22].

5.2. Adjuvants and Vector Systems for Sublingual Vaccines. Novel adjuvants and vector systems could be considered to further improve the efficacy of sublingual allergy vaccines [57]. Those immunopotentiators could as well allow to reduce the dose of allergens or simplify immunization schemes. Potential mucosal adjuvants which have been successfully tested in murine SLIT models to modulate T cell polarization include ligands for Toll-like receptors (TLRs) 2 (e.g., Pam3Cysk4) and 4 (i.e., synthetic lipid A analogs) [57–59]. Such TLR ligands enhancing tolerance induction via the sublingual route share in common a capacity to elicit mixed Th1/T reg $CD4^+$ T cell responses. In addition, dexamethasone + (1, 25) dihydroxyvitamin D3 as well as selected strains of probiotics (i.e., lactobacilli, bifidobacteria) represents potential T reg adjuvants, since they are powerful inducers of IL-10 production by immune cells. As such, these compounds enhance SLIT efficacy in murine models of OVA-induced asthma [58–61]. In humans, the only adjuvant which has been tested via the sublingual route is monophosphoryl lipid A (MPL), a TLR4 ligand-inducing Th1 responses. Coadministration of MPL enhanced IgG responses to the allergen when using high doses of adjuvant [62]. The clinical relevance of this enhancement of specific antibody responses remains to be established.

Mucosal vectors could also enhance SLIT efficacy, for example, by protecting the allergen(s) from degradation by local proteases or by targeting the allergen to antigen-presenting cells [57]. As an example, the genetically detoxified adenylate cyclase (CyaA) from *Bordetella pertussis* conjugated to OVA was shown to enhance tolerance induction via the sublingual route in OVA-induced asthmatic mice, as a consequence of a superior targeting of oral CD11b+ tolerogenic myeloid DCs [39]. In addition, positively charged polymers have been used to generate mucoadhesive particulate vectors which can enhance allergen interaction with negatively charged epithelial cells and as a consequence, contact duration with the mucosa. Formulations of allergens within a particle increase allergen uptake by antigen-presenting cells with a phagocytic activity [57]. For example, both nanoparticles made from polymerized maltodextrin [47] or chitosan-based microparticles [63] were found to enhance *in vitro* and *in vivo* allergen capture by tolerogenic oral DCs, thus resulting in a stronger tolerance induction via the sublingual route in murine asthma models. To date, no vector system has been evaluated sublingually in humans.

5.3. New Clinical Indications. Both subcutaneous and sublingual immunotherapies of patients with rhinoconjunctivitis appear to prevent subsequent sensitization and evolution towards asthma [24, 25, 27, 64]. In addition, several studies suggest a benefit of SLIT in controlling asthma associated with house dust mites [6, 65, 66]. Additional clinical trials in adult and pediatric patient populations are needed to further document a benefit of SLIT in this indication. Recently, SLIT has also been tested successfully in several new clinical indications, including allergies to latex and food (e.g., peach, kiwi, hazelnut) [67–69]. These studies conducted on small cohorts of patients provided encouraging results, both in terms of safety and clinical efficacy (e.g., increase in amounts of food allergens tolerated by the patients). Similarly, SLIT has been recently shown to decrease atopic dermatitis symptoms linked with mite exposure, in patients with mild-to-moderate disease [70]. Such results need to be further confirmed in the context of large-scale-double blind placebo-controlled studies.

6. Conclusions

Sublingual vaccines based on biological extracts are being used as a safe and efficacious treatment for type I respiratory allergies. To provide consistent pharmaceutical-grade products despite the inherent variability associated with biological extracts, well-established standardisation procedures and comprehensive proteomic characterization methods are being used to guarantee the quality of allergen extracts and the robustness of manufacturing processes. Those improvements have been recognised by regulatory authorities with the registration in 2008 of sublingual grass pollen tablets as pharmaceutical specialties. New applications are being pursued, encompassing the development of sublingual tablets for mite and ragweed pollen allergies, as well as the evaluation of SLIT as a treatment of asthma. Additional frontiers to explore in the long term include the development of sublingual vaccines for food allergies and atopic dermatitis. In parallel, second-generation vaccines based on recombinant allergens are being investigated to treat birch pollen allergies. These vaccines will associate recombinant allergens in a native conformation, together with Th1/T Reg adjuvants

and/or mucoadhesive particulate vector systems. If successful, such recombinant sublingual vaccines could enhance clinical efficacy while reducing treatment duration and decreasing the dose of allergen administered.

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Review Article

Inflammation as an Animal Development Phenomenon

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Inflammation is a term that has been used throughout history in different contexts; it may represent a simple collection of clinical symptoms for which drugs are developed, a disease mechanism, or even a defense mechanism against microbes validating Pasteur's studies on bacteriology and Darwin's proposed struggle for survival. Thus, an explanation of this term must also consider the scientific questions addressed. In this study, I propose that several of the inflammatory events typically described in immunological, pathological, and pharmacological contexts can also be perceived as mechanisms of animal development. Thus, by recognizing that the generation of an animal form, its conservation, and its regeneration after tissue damage are phenomena of the same nature, inflammation can be addressed through the approach of developmental biology, thereby acquiring a much neglected physiological counterpart.

1. Introduction

The capacity to maintain and restore the integrity of tissues is crucial for the survival of all organisms, and the pathways through which the structure and organization of tissues are restored after a lesion may be as diverse as the forms of animal life. A starfish larva, for example, reacts to the insertion of a rose thorn with an intensive migration of phagocytic mesenchymal cells [1]. In contrast, an amphioxus responds to the same type of challenge through extracellular digestion promoted by the secretion of enzymes from its epithelial cells [2]. A salamander that has a limb amputated is capable of completely reconstructing a new functional limb, whereas other amphibians substitute the lost limb with fibrous tissue [3]. Even within a specific group (e.g., mammals), the pathways by which tissues are assembled and reassembled vary greatly. For example, a deep cut in the skin of an adult human generally triggers acute inflammation that is followed by a fibroproliferative process and scar formation; the same lesion inflicted on a fetus, however, may result in complete skin regeneration [4].

Irrespective of the peculiarities of the tissue repair processes in diverse groups of animals, it is rather intuitive to accept that regeneration and inflammation are related processes. Certainly, the processes that underlie the formation of a new salamander tail and the inflammation that occurs in response to a myocardial lesion in a mouse injected with high doses of isoproterenol are similar phenomena. Nonetheless, regeneration is viewed as the building of a structure, whereas inflammation is not recognized as such. These differences in our perception of these two phenomena are likely due to the history of the characterization of these phenomena, the experimental approaches used to address these topics.

2. Origins of Inflammatory Certainties

The initial framework used to study inflammation, which is widely accepted by the scientific community, is the description of the cardinal sinuses—*rubor et calor cum tumor et dolor*—performed by the Roman doctor Celsus approximately two thousand years ago [5–7]. This expression is still widely used and is representative of the general perception of inflammation. What is not usually mentioned, however, is that when Celsus proclaimed these words, he viewed inflammation simply as a collection of clinical symptoms and not as a phenomenon in itself. When Aristotle coined the term "development" to refer to the evolution of the shape of a chicken embryo, he defined it as a process of living. In contrast, inflammation put in Celsus terms was

not considered to be a process, but rather it was defined as a collection of signals resulting from process which remain unknown. For a long time, pathologists studying inflammation contented themselves with enumerating more and more details of the inflammatory process in organisms becoming ill or injured tissues, without focusing on defining the concept of inflammation [8, 9].

As a result, in the 19th century, it was common to find scientists arguing for an end to the use of the term "inflammation" [10]. Even Virchow, the German pathologist who proposed a fifth cardinal inflammatory signal, *functio laesa*, categorically affirmed that inflammation was not a real entity [5], but a term that encompassed a series of phenomena so distinct that they should be treated separately.

From Celsus until the mid-19th century, the lack of an organismal/biological context to unify the different descriptions of the reactions of damaged tissue created a gap in the concept of inflammation. Even more concerning, the examination of this phenomenon, which was strictly medical, was isolated from other very similar biological processes. Animal regeneration, for example, was discovered in the 18th century and resulted in a period of enriching debates concerning the origin of the animal form [11]; during the same period, inflammation was reexamined through the lens of cell pathology. However, there was no attempt to converge these two ideas, as will be discussed later. The comparison of these two phenomena did not occur because regeneration was conceived as a phenomenon of form construction whereas inflammation had not attained the status of a biological phenomenon even by the end of the 19th century.

This perspective was changed by the seminal study described by Julius Cohnheim on the passage of white blood cells through capillaries and into inflamed tissue, known as diapedesis. For Cohnheim, this was not a mere histological description of what occurred in the disease process but the generative mechanism of the cardinal signs of inflammation. In describing changes in capillary structure, with the consequent movement of plasma and the passage of blood cells that compose the pus corpuscles, Cohnheim proposed a mechanism demonstrating how the symptoms described by Celsus were generated [12]. Thus, this proposal by Julius Cohnheim, demonstrating that the cause of inflammation resided in the vessels, unified diverse and subsidiary problems around a singular phenomenon and defined inflammation as an organic process. Within this context, inflammation was clearly a pathological event.

There is no doubt that this was a fundamental step in inflammation research. There are, however, two important limitations to Cohnheim's proposal. First, he suggested that inflammatory processes are exclusively pathological mechanisms without a physiological counterpart. Placing the vascular lesion as the ontological precedent of the inflammatory responses precludes the possibility of explaining how these processes are involved in the physiology of a healthy organism. This situation was unusual because we usually seek to understand the physiological role of a process prior to investigating its role in pathology. For example, we first sought to understand the electrical physiology of cardiac function prior to investigating the pathology of cardiac arrhythmias; however, we do not have the same reservations when studying inflammation in an exclusively pathological context. Except for very few instances, the term physiology is not mentioned in the immune-inflammatory jargon [13, 14].

A second limitation of Cohnheim's proposal on the origin of the cardinal signs of inflammation based on vascular events is that this process only occurs in a limited group of animals; only warm-blooded birds and mammals may present all the cardinal signs of inflammation. However, if the vascular lesion was a *sine qua non* condition for the emergence of an inflammatory dynamic, what would occur when an animal lacking a circulatory system suffered a tissue injury? How do all other animals repair themselves?

Therefore, although Cohnheim's explanation was a breakthrough we still lacked a broader perspective on the construction and reorganization of the organism. It was only when this problem was addressed by other disciplines that these limitations were noticed. Metchnikoff, a Russian embryologist, was important for this transformation.

Metchnikoff was interested in the formation of new embryonic forms during animal development and was investigating the role of a group of migratory and phagocytic mesenchymal cells in these phenomena. To Metchnikoff, understanding phagocytosis was important because this event can be observed in all animals, even in the simplest and most primitive forms (except in the amphioxus). When comparing phagocytosis among several groups of animals, Metchnikoff observed that phagocytes could ingest not only food particles, but also foreign particles and invading microorganisms (reviewed in [15]).

This last observation, in particular, acquired considerable relevance because it occurred at the time when Pasteur proposed that disease was caused by specific germs and when Darwin's work proposed the struggle for survival as the central problem in biology. With the proposal that phagocytes were a defense mechanism against the challenges of the environment, Metchnikoff united the most important medical and biological theories of the 19th century (1891), and because phagocytes are common to all animals, Metchnikoff understood that this would be the *primum movens* of inflammation. Thus, inflammation was transformed from a human pathological reaction to an animal health defense response [15].

It was Metchnikoff who developed the notion of a "defensive function" for inflammatory activity, which became fundamental to the modern concept of immunity. When Cohnheim described diapedesis, the defensive aspect was not included in his description. However, it is important to emphasize that Metchnikoff's idea of defensive action should not be accepted with naiveté, as is commonly the case when considering the notion of function. He had the insight to realize that the same process that the organism was using for defense was also participating in the embryonic and physiological processes of development. For example, Metchnikoff had already described that phagocytosis was involved in the reabsorption of the tail in one genus of amphibians. Thus, Metchnikoff described a physiological role for inflammation, and for him, the building of

an organism was a problem that preceded its defense. For Metchnikoff, inflammation and immunity were subsidiary conditions of animal development; these situations were particular to the construction of metacellular harmony. Thus, he created the opportunity for studying the physiological aspects of inflammation [16].

Without discarding the important advances in pathology, Metchnikoff [1] circumvented the limitations of Cohnheim's proposal and developed an idea of great biological value. However, the only idea of Metchnikoff that was actually accepted by his peers was the defensive connotation of phagocytic activity (and generally with a naiveté that he himself lacked). All of his other considerations regarding the physiology of form construction in animals were quickly ignored. The newly founded discipline of immunology grew more concerned with understanding the pathogen-host relationship than any other generative or physiological aspect of the immune system or inflammatory activity. As a result, two main schools for addressing the inflammatory response were created: traditional pathology, which sees inflammation as a reaction to disease, and immunology, which sees it as a defense response.

A third more recent trend in inflammation research is the pharmacological approach. With the birth of the pharmacological industry, also at the end of the 19th century, the race to develop new methods to intervene in inflammatory processes became important. Thus, while pathologists described the reactions of organisms in response to disease, and the immunologists studied the detection of foreign bodies, the pharmacologists searched for methods to intervene in these events. In this context, there is one event in inflammation research that deserves to be highlighted: the invention of carrageenan-induced paw edema by researchers of the Merck pharmaceutical company [17].

During the first half of the 20th century, the methods for developing anti-inflammatory drugs were laborious and slow. In general, to characterize a potential anti-inflammatory agent, it was necessary to study inflammation as part of the repair of injured tissue. These models were tiresome, with protocols that lasted several weeks.

In 1962, a group of researchers from Merck developed a model that complied with all of the requirements needed by those interested in rapidly developing a product: carrageenan-induced paw edema in rodents. This protocol could be completed within four hours, it only needed one application of the drug to be tested, and its principle was based on the measurement of one of Celsus's cardinal signals: edema. For heuristic reasons, the industry created a model that separated the cardinal signals of inflammation from the complex processes of tissue repair; as a result, inflammation returned to being a mere clinical symptom.

The practical success of this idea was immediate, and in less than a year, Merck had developed indomethacin, a drug that still is used as a reference drug in the development of new anti-inflammatory agents. Other industries rapidly reproduced this model, and dozens of new anti-inflammatory agents successfully entered the market in that decade. It is interesting that this experimental protocol was widely accepted in the academic sphere and was widely used in basic research, as if it was an adequate model for understanding the inflammatory phenomenon. This had serious consequences because, by adopting a protocol that had been developed to simplify the research model for the pharmaceutical development of anti-inflammatory drugs, inflammation was once again no longer viewed as a physiological phenomenon; instead, it was studied as a cardinal sign of disease, as it had been two thousand years ago in Roman medicine.

3. Origin of Certainties in Regenerative Biology

The initial characterization of the regenerative process in animals is attributed to Adam Trembley for his studies on medusa polyps [11]. At that time, it was unclear if the medusa polyps were plants or animals. Thus Trembley sectioned them because only plants were thought to be capable of regeneration. Then after his experiment, it was observed that hydras had the capacity to reconstitute their lost parts, induce complete tissue repair, and rescue the *status quo ante* as if it was a plant. However, all other observations on the life cycle of these organisms led to the conclusion that they were indeed animals. Therefore, the possibility of animal regeneration was recognized with great surprise.

There is, however, something even more important in this finding by Trembley: because both halves of the hydra could promote a perfect tissue repair (regeneration), this situation was simultaneously a process of tissue reassembly and animal reproduction. Thus, the discovery of regeneration was also the discovery of a new form of asexual reproduction. This exceptional circumstance led to a conception of animal regeneration as not only a repair mechanism for lesions, but also as an event in animal development linked to the problem of reproduction and the generation of form. In addition, Trembley's finding occurred approximately at the same time as the climax of the embryological debate between preformationism and epigenesis in the 18th century. Therefore, it is not surprising that embryologists began to study regeneration [11].

Thus, contrary to inflammation, which in this period was barely treated as a phenomenon in its own right, animal regeneration emerged as part of a framework of well-defined biological ideas and occupied a central position in a world of rich debate. The theories of regeneration were placed next to those of embryonic development and metamorphosis; that is, regeneration was always perceived as a physiological phenomenon of animal development.

4. Inflammation as an Animal Development Phenomenon

Today, research in inflammation has certainly expanded its frontiers into several areas of scientific knowledge and could hardly be addressed within the limits of only one discipline. This plurality is not only desirable but also necessary. My particular interest in commenting on the emergence of the three main schools of inflammation research—pathological,

immunological, and pharmacological—is not merely for the sake of providing historical background, but also to show that it is the context in which we make observations that defines the nature of the phenomenon being studied. Because of the way it has been perceived throughout history, inflammation emerged as both a symptom and a mechanism. I cannot negate the importance of the medical or industrial perspectives on this topic, but I hope to show that it is also valid to view the topic from a physiological and biological perspective. For this reason, I will not further discuss the definition of inflammation but show the delineations that it can be acquired when visualized within the context of developmental biology.

From the outset, one must perceive that although inflammation and regeneration are phenomena that emerge from very different needs, it makes no sense to study them separately within modern biology. Albeit legitimate, the medical interest in inflammation should not obscure the fact that, beyond the symptoms and magic bullets, inflammation can be seen in its physiological processes. Thus, there is a need to reconcile this theme with the development and construction of the animal form. More than their role in defense, inflammatory processes are part of the organism construction, and this can be illustrated with innumerable examples.

The urodele amphibians are capable of regenerating their eyes, including delicate tissues such as the retina and lenses. The surgical removal of the lenses from salamander newt eye triggers changes in the pigmented epithelial cells of the pupillary margin of the iris, which are capable of activating the cell cycle and differentiating into a new lens [18]. In a recently developed experimental model [19], it was shown that events typically described as immune inflammatory participate in the process of generating a new ocular lens. When the lenses are pricked with a needle through the cornea, they degenerate by autophagy, a process mediated by dendritic cells, and the elimination of the damaged lenses allows for the regeneration of new tissue from the dorsal edge of the iris. The authors observed that the transference of dendritic cells isolated from the ocular tissue of animals in the process of autophagy/regeneration into naïve animals (with their eyes untouched) was capable of promoting the genesis of a second lens even in the absence of injury. Furthermore, if the animals receiving a transplant of these activated dendritic cells were previously splenectomized, this generative process was inhibited. Therefore, the formation of new ocular tissues depends on processes that occur in a lymphatic organ, such as the spleen. This is an example of the generation of complex tissues mediated through immune-inflammatory processes; thus, this example illustrates the generative nature of inflammatory activity.

The proposal that immune-inflammatory activity is associated with generative phenomena gained even more experimental support when framed within the context of comparative evolution [20]. The tunicates (Urochordates) form a sister group of the vertebrates and thus occupy a relevant taxonomic position for understanding the phylogenetic origin of vertebrates and the adaptive immunity as well. In this context, the species *Botrylloides leachi* is a well-studied animal model for understanding the emergence of immunological activities. This species is a very common tunicate in the Mediterranean that exhibits the unique capacity of completely regenerating the adult organism from small vascular fragments. This phenomenon has been designated as whole-body regeneration. In a recent study, Rinkevich et al. [20] analyzed the complete mRNA profile transcribed during the process of full body regeneration and compared these profiles with other developmental processes such as metamorphosis, blastogenesis, and budding (asexual reproduction). To the authors' great surprise, they observed that

> "comparison of genome-wide transcription of whole body regeneration with five other developmental processes in ascidians (including metamorphosis, budding and blastogenesis), revealed a broad conservation of immune signaling expressions, suggesting a ubiquitous route of harnessing immune-related genes within a broader range of tunicate developmental context."

It becomes intuitive, after this revelation, that the problems addressed by embryologists and pathologists are phenomena of the same nature. However, we need to go further and recognize that the consequences of this admission are not trivial. The genesis of the biological form neither ends at birth nor resumes with disease. To understand this concept, it is necessary to view life as an incessant dynamic of transformation, like a Heraclitian fire [21]. To accomplish this, one must escape the animal models of birth and disease in which the framework is too well defined. In this respect, I believe that the hydras represent an interesting model to bridge the work of embryologists and pathologists.

5. Inflammation Physiology: Genesis Does Not End at Birth and Does Not Resume with Disease

An adult hydra polyp is comprised of two layers of epithelial cells: one derived from the endoderm and one derived from the ectoderm, which arrange to form a two-layer tube around the gastric cavity. At the apical end of this tube, there is a head where a mouth opening with tentacles is formed, and at the other end, there is a disc of cells responsible for attaching the organism to the substrate. In addition to these two layers, these animals are also constituted by a simple group of interstitial cells that give rise to neurons, gonads, and secretory cells [22].

In a series of elegant experiments, Campbell [23] stained the cells in several portions of this animal and tracked the dynamic movement of tissues during the life of the hydras. His findings were impressive because, although these animals conserve their body size for many years, the movement of tissues is incessant. Campbell [23] found that, given the incessant mitosis of the epithelial cells composing the hydra, all of the cells are constantly changing their position relative to the axial axis (Figure 1). It is a dramatic dynamic, although

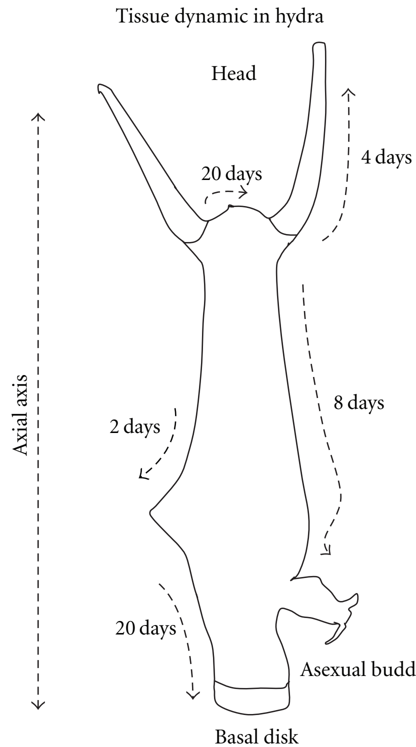

Figure 1: Tissue dynamic in a hydra polyp. Tissue movements were monitored after the insertion of tissue stained with methylene blue at different points, as described by Campbell [23]. The arrows indicate the direction and route of the tissue movements and the time elapsed. In these animals, cell identity is defined by its position relative to the axial axis, but these positions are not constant.

it is invisible to our eyes when we observe these animals *in natura*. It is also remarkable that, through these tissue movements, an epithelial cell that belongs to the central column of the organism will eventually reach the end of the body and will then differentiate into the specialized cells of the tentacles or basal disc [24]. Coordination of cell differentiation in the hydras depends on their position relative to the axial axis of the organism, but these positions are not constant. It is an incredible example of phenotypic plasticity that a cell with a given identity in a specific context can move to a new position and modify its phenotype. This same process occurs in the cells of the interstitial compartment. For example, a secretory interstitial cell found at a medial position along the anterior-posterior axis acquires a neuronal phenotype upon reaching the head [24]. The body is conserved, but all of its components continue changing and moving throughout its life.

The form of the animal is conserved throughout its life, but all of the mechanisms of generation and change are incessant. This example blurs the lines between embryology and pathology because, in this case, it is difficult to determine at which point "regeneration" has begun given that "generation" never ceased.

When we escape from animal models centered on the adult organism, we realize a second point that will be strongly defended in this paper: the genesis of form is never constrained to a particular moment in the animal's life cycle. The adult form is not finished, and it is not merely a product of our uterine past; instead, it is altered daily. Currently, this conserved generative process of daily life is neglected; it has not been studied by embryologists, whose study ends at birth, or by pathologists, who begin their study with a perturbation of the conserved shape. I believe that both disciplines have much to gain by extending their territories to study the condition of a healthy living adult. I believe this would have serious consequences because it would allow us to visualize a physiological counterpart of the studied processes that occur with disease—the physiology of inflammation.

A difficulty in accepting this proposal of the similarities between animal development and inflammation, which I call inflammation physiology, is our poor understanding of the living. An adequate view of the physiology of a healthy organism must include the tissue dynamic and the explicit notion that health, or physiological normalcy, is actively built. However, it is not just the medical realm that lacks a clear view of the organism; biology is also missing this view. Since Claude Bernard, we have greatly praised the notion of homeostasis, which is defined by a state of equilibrium. But this idea of a constant state is derived from an adultocentric premise that harmony between the parts needs no explanation.

In contrast, Metchnikoff, who came from the embryological world, perceived the complexity of the construction of form and profoundly disagreed with Bernard [16, 25]. For him, at the beginning of ontogeny there was disharmony; as a result, harmony was finely built and constantly woven. He was therefore interested in the construction processes. Moreover, because Metchnikoff studied phagocytes not only in their defensive context, but also in physiological situations, such as the metamorphosis of the tadpole's tail, he attributed the genesis of harmony to a "physiological inflammation" [15]; pathological inflammation was secondary to this incessant physiological genesis. In addition to Metchnikoff [1, 15], rare exceptions to a disease-biased view of the immune-inflammatory phenomena may also be found in the work of Vaz et al. ("conservative physiology of the immune system" [14]) and Cohen (body maintenance and "corrective inflammation" [26]).

Based on what has been described previously, I maintain that the corollary of this essay can be summarized as follows: *the generation, conservation, and regeneration of form* are all related problems, dealing with a more central question in biology, which is the construction of an organism.

Currently, the term "inflammation physiology" has also been mentioned in the work of Medzhitov [27]. However, it is important to stress that the physiological aspects of inflammatory activity mentioned in the present paper and those mentioned by Medzhitov are concepts which arise from different questions; they are not different answers to the same question. Medzhitov's initiative is not meant to be a conciliation between inflammation and developmental biology nor a contemplation of the healthy living dynamics of self-construction. Thus, in his work the physiological

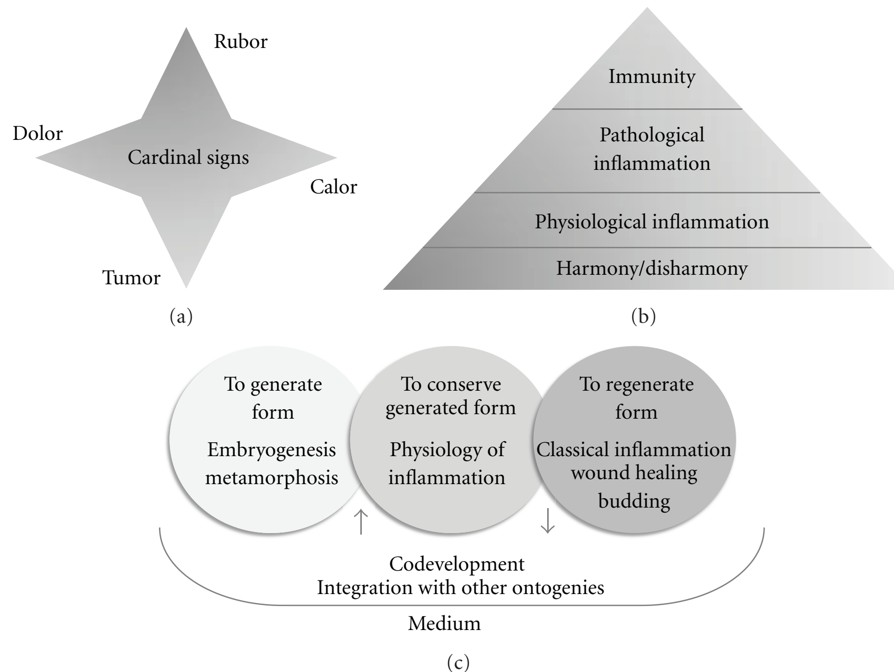

Figure 2: Inflammation in the eye of the observer. Since its origins, inflammation has frequently been viewed as a collection of *signals* (a). In this context, what is studied is not a biological process but the results of a process that remains ignored. According to Metchnikoff, however, inflammation and immunity are particular instances of a broader *process* of animal harmony generation ((b), adapted from Tauber [37]). Because the same mechanisms involved in pathology were also involved in embryogenesis, Metchnikoff coined the term physiological inflammation, which he claimed preceded the problem of pathological inflammation. A modern reinterpretation of this developmental contextualization of inflammation could be represented as in (c). Thus, the genesis of form, its conservation, and its regeneration are problems of the same nature, which deal with the construction of an organism even when examining the animal/microbiota interaction.

inflammation is still perceived as a "response" to a stimulus. A schematic representation of the argument herein put forward, including a comparison to other schools of thought on inflammation, is presented in Figure 2.

6. Codevelopment: The Relationship with the Microbial World Revisited

Initially, an attempt to contextualize the study of inflammation within a developmental biology perspective seems to negate the main concern of this discipline, which is the defense against microbes. Clearly, we cannot neglect the importance of microorganisms in our lives or the fact that serious microbial infections exist. However, it is important to perceive that our current microbiological knowledge is much different from that which led Pasteur to propose the idea that germs cause disease. With the advent of molecular techniques for gene amplification, it was discovered, for example, that less than 1% of marine microbes grow on conventional culture media. Therefore, it was not possible to discover these microbes until very recently [28]. Thus, our understanding of microbial diversity has expanded at least 100-fold in recent years. In addition, it is worth highlighting the findings from a recent genomic characterization of the microbiota associated with humans, which discovered the existence of more than 2000 species of commensal microorganisms, of which less than 100 species are characteristically pathogenic [29].

Despite the importance of understanding the pathogenesis that emerges from the relationships among organisms, is it critical to recognize that microbial colonization is not a synonym of infectious disease (we are all healthy carriers of an immense diversity of microorganisms) and that a whole discipline exclusively dedicated to explaining infectious disease is a discipline that is focused on an exception [29]. A modern treatment of the relationship between microorganisms and their hosts should be capable of explaining both, medical bacteriology and also the recent explosion in our knowledge of microbial ecology.

In this context, one of the most renowned developmental biologist of our times, Scott Gilbert, has studied the integration of animal development within an evolutionary and ecological context (Eco-Evo-Devo), attributing great importance to the microorganism immune system interface in the process of constructing the animal form. Today, this is one of the most important and accepted perspectives in modern developmental biology, and it has as its main premise the idea that "all development is codevelopment." Thus, in embryology, it is currently understood that it is not enough to reveal the details concerning the gastrulation of an animal, but it is also necessary to understand how its ontogeny is integrated with the ontogenies of its surrounding organisms [30]. In this codevelopmental context the host-microbial relationship and the immune-inflammatory phenomena has been revisited. According to some authors, such

as Gilbert and Epel [30], Hooper and Gordon [31], and McFall-Ngai [29], who are important references in this field, immune-inflammatory activity can be viewed as a relevant phenomenon in the integration of the ontogenies of different organisms and not merely as a military defense system.

The host associations with bacteria, which codevelop with other organisms, have very curious nuances. The McFall-Ngai group studies the symbiotic association of certain squid species with bioluminescent bacteria (*Vibrio fischeri*) [32, 33]. These squids, which are nocturnal predators, are born without the bacteria and develop a rudimentary organ that hosts them and is an important step in their predatory behavior. This organ fully develops only in the presence of the bacteria, and these bacteria change their phenotype within the organ. It is also interesting to note that the components involved in the establishment of the relationships between these two organisms are exactly the same as those that participate in the reactions that we would consider to be inflammatory; peptidoglycans, Toll-type receptors, phagocytes, and nitric oxide synthase are involved in this process within a codevelopmental context.

This example has been frequently referenced as representative of this relationship. When we consider the mice with which we work, we return to the same warfare metaphors—defensive mechanisms—to describe inflammation. However, this is unnecessary. For example, the development of the vascular network in the intestinal villi of mice raised in the absence of bacteria (germfree) is severely damaged [34]. Thus, this certainly represents a codevelopmental issue.

The point is not to negate the occurrence of pathologies in relationships with intestinal bacteria, but rather to view it in terms of developmental deviations. We are not simply organisms that live to defend ourselves from pathogenic germs, as it was originally conceived when the concept of bacteriology was emerging with Pasteur. Today, we know that germ-free rats that receive an active transfer of adoptive microbiota from zebrafish, for example, assemble their native microbiota with the profile of their own species and not with the profile initially transferred from the zebrafish [35]. Similarly, the transfer of microbiota from rats into germ-free zebrafish generates the standard microbiota in the receiving fish species. Therefore, the relationships between the organism and its microbiota are actively built, and the microorganisms that we carry within us are not merely temporary passengers but carefully cultivated coinhabitants. Furthermore, in a series of elegant experiments comparing the gut colonization dynamic in gnotobiotic/ $Rag1^{-}/^{-}$ and gnotobiotic/ $Rag1^{-}/^{-}$ mice which received adoptive transfer with igA-producing hybridoma cells, Peterson et al. [36] have demonstrated that gut IgA promotes symbiosis homeostasis rather than immunological defense.

The formation of n bacteria biofilm in a particular region of the mammals' digestive tract is as sophisticated an event as the formation of a neuronal network in the encephalic region. Thus, it is possible to envision how immune-inflammatory activity plays a role in the construction of life even if it is involved in the establishment of relationships with other organisms.

7. Coda

The term inflammation has many different meanings depending on the context in which it is studied. Sometimes, it is defined as a salutary defense phenomenon; occasionally it is described as a pathological phenomenon, and on some occasions it does not even acquire the status of a phenomenon, being merely designated as a group of signals resulting from an otherwise ignored process. However, when the context of birth, which encompasses the discipline of embryology, and the context of tissue damage, which belongs to the discipline of pathology, are transcended, it can be seen that these disciplines are actually dealing with very similar problems. Curiously, this approach has been widely recognized when dealing with the regeneration of a limb in amphibians or an arm in starfish, but not when dealing with repair of injured tissues in mammals.

Therefore, in this work, I argue in favor of a rediscovery of inflammation as a phenomenon that also involves the genesis of form, through the example of the study of animal regeneration. This initiative does not negate the important pathological findings contributed to date, but embraces them within a broader context that is more in agreement with modern biology. Finally, this approximation between pathology and embryology is responsible for creating a physiological basis for describing inflammatory phenomena, which is currently neglected. Thus, by becoming a physiological process, *inflammation* becomes *formation*.

Acknowledgments

The author is grateful to Dr. Nelson Vaz—the major reference in his attempt to develop an organism-centered approach to inflammation, to Dr. Henrique Lenzi and Archimedes Barbosa for their suggestions and contributions, to Dr. Jamil Assreuy, for creating the proper conditions for this work to be done, and to the financial support of CNPq.

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Research Article

Indirect Effects of Oral Tolerance Inhibit Pulmonary Granulomas to *Schistosoma mansoni* Eggs

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Parenteral injection of tolerated proteins into orally tolerant mice inhibits the initiation of immunological responses to unrelated proteins and blocks severe chronic inflammatory reactions of immunological origin, such as autoimmune reactions. This inhibitory effect which we have called "indirect effects of oral tolerance" is also known as "bystander suppression." Herein, we show that i.p. injection of OVA + $Al(OH)_3$ minutes before i.v. injection of *Schistosoma mansoni* eggs into OVA tolerant mice blocked the increase of pulmonary granulomas. In addition, the expression of ICAM-1 in lung parenchyma in areas outside the granulomas of OVA-orally tolerant mice was significantly reduced. However, at day 18 after granuloma induction there was no difference in immunofluorescency intensity to CD3, CD4, F4/80, and α-SMA per granuloma area of tolerant and control groups. Reduction of granulomas by reexposure to orally tolerated proteins was not correlated with a shift in Th-1/Th-2 cytokines in serum or lung tissue extract.

1. Introduction

Oral tolerance is a T-cell-mediated phenomenon described as the inhibition of immune responsiveness to a protein previously contacted by the oral route [1, 2]. Oral tolerance may prevent autoimmune and allergic diseases by mechanisms that are still controversial [3–5]. Two aspects of oral tolerance are of special interest to us because they may reflect its broad and systemic character, suggesting that more insights into these issues may improve our knowledge of the mechanisms of oral tolerance. First, oral tolerance is never absolute (complete), that is, parenteral immunization of tolerant animals with the tolerated antigen may induce antibody formation at levels inversely proportional to the ingested (tolerizing) dose of the antigen, but, this antibody formation can no longer be boostered by further parenteral immunizations [6]. Second, parenteral reexposure to a tolerated antigen blocks the initiation of immune responses to a second unrelated antigen—the effect we have named "indirect effect of oral tolerance" [7, 8] and is also known as "bystander suppression" [3, 9]. We have shown that such inhibitory effect occurs with different orally tolerated antigens and even when the tolerated antigen and the second unrelated antigen are injected into separated sites [7, 8]. The inhibitory indirect effects of oral tolerance does not require the simultaneous injection of the tolerated protein and the second antigen: it is still present 72 h after an injection of the tolerated antigen, but does not occur if the tolerated protein is injected after the second antigen [10]. Furthermore, parenteral re-exposure to a tolerated antigen has systemic effects on the migration of leucocytes and bone-marrow eosinopoiesis [11], blocks delayed-type hypersensitivity (DTH) reactions triggered by keyhole limpet haemocyanin (KLH) and paw oedema triggered by carragenan [12]. Amazingly, the indirect effects

of oral tolerance to OVA also hinder the inflammation after an incisional skin lesion and improve wound healing in skin reducing fibrosis [13].

Granulomatous inflammation is involved in a number of diseases, and chronic granulomatous inflammation can cause damage and fibrosis to surrounding tissue [14, 15]. In schistosomiasis mansoni, the chronic egg-induced granulomatous response in the liver and intestines may eventually cause extensive tissue scarring and development of portal hypertension [16]. Immune responses to products secreted by the eggs (soluble egg antigens, SEA) result in the formation of granulomas that are composed of macrophages, eosinophils, lymphocytes, and fibroblasts [17]. Similarly to other inflammatory reactions, one critical aspect of granuloma formation is leukocyte migration dependent on the expression of adhesion molecules and cytokines [14, 18, 19]. Granulomatous inflammation triggered by *S. mansoni* eggs and the subsequent fibrosis has been considered a Th2-cytokine-driven inflammation [20]. However, different cytokines including IL-4, TNF-α, IL-10, and IFN-γ are produced during the course of granuloma formation [21]. *Schistosoma mansoni* eggs injected into the tail vein of mice are transported into the lung tissue via the pulmonary arteries where they become trapped within the lung parenchyma [22, 23]. The injection of *S. mansoni* eggs into normal mice allows the study of granulomatous reaction to the eggs without interference of additional factors triggered by the presence of the worms and reduces the variability in the size of granulomas otherwise produced by natural oviposition [21, 22]. Using the pulmonary granuloma model we have previously shown that indirect effects of oral tolerance triggered by i.p. injection of dinitrophenylated conjugates of OVA (DNP-OVA) emulsified in complete Freund's adjuvant (CFA) inhibit the formation of pulmonary granulomas [24].

To further characterize the indirect effects of oral tolerance upon inflammatory reactions, we tested if re-exposure of OVA orally tolerant mice to OVA + $Al(OH)_3$ block the concomitant formation of pulmonary granuloma. Mice orally tolerant to OVA and controls not tolerant were i.p. injected with OVA concomitant with i.v. injection of *S. mansoni* live eggs. We compared granulomas size from day 1 to day 18 after i.v. eggs, granuloma cellular composition, spleen, lung and serum cytokines levels, and the expression of intercellular adhesion molecule-1 (ICAM-1) in the lung.

2. Materials and Methods

2.1. Animals. 8-week-old female C57BL/6 mice were bred and maintained in the animal breeding unit at the Institute of Biological Sciences, Universidade Federal de Minas Gerais (UFMG), Brazil. The animals were fed, housed, and treated according to the guidelines of the Ethics Committee of Animal Experimentation (CETEA) of the UFMG. Experimental groups contained at least five mice per each time point.

2.2. Feeding Regimens for Oral Tolerance Induction. Oral tolerance to ovalbumin (OVA) was induced by requiring mice to drink, *ad libitum*, a 1 : 5 solution of chicken egg white in drinking water for 3 consecutive days. The egg white solution was prepared in our laboratory from commercially available eggs and contained an average of 4 mg OVA/mL. Daily estimated average consumption was 20 mg OVA/mouse, and this resulted in significant levels of tolerance [25]. Bottles were changed every day to avoid contamination. Control groups received filtered tap water. Oral treatment was discontinued 7 days before granuloma induction.

2.3. Pulmonary Granuloma. 7 days after oral tolerance induction, control and experimental animals were injected i.v. with 2,000 eggs from *S. mansoni* through a tail vein. Live *S. mansoni* eggs were purified from the livers of *S. mansoni* cercariae-infected Swiss mice, which were kindly provided by Dr. Débora Negrão Correa, from Universidade Federal de Minas Gerais, Brasil.

2.4. Parenteral Immunizations. Purified OVA was obtained commercially (grade V, Sigma, St. Louis, MO). Mice which had been pretreated orally with egg white (tolerant group) and control mice (immune group) received one intraperitoneal (i.p.) injection of 0.25 mL of a suspension containing 10 μg OVA plus 1.6 mg $Al(OH)_3$ immediately before the i.v. egg injection. The other control group (granuloma group) was not i.p. immunized.

2.5. Bleeding. Blood samples were collected in the absence of anticoagulant, and serum samples were obtained and stored at -20°C until used in a serum antibody assay to test for tolerance induction or cytometric bead array (CBA) for quantitative analysis of cytokines.

2.6. Sacrifice. Mice were sacrificed by cervical dislocation 1, 5, 11, 14, and 18 days after inoculation of *S. mansoni* eggs; lungs were collected and fixed for either histology or immunostaining. In one experiment the spleens were also collected.

2.7. Histology. For histology lungs were fixed immediately in Carson's modified Millonig's phosphate buffered formalin (pH = 7,0 for 24 h) and embedded in paraffin. Serial sections of 4 μm were stained with hematoxylin and eosin (HE) or Gomori's trichrome for bright field microscopy. Digital images of tissues were obtained using a BX50 Olympus microscope (Olympus, Japan) and an Olympus Q Colour 3 Camera, which was connected to a computer running the Q-Capture Pro software program (Q Imaging, Canada).

2.8. Morphometry. The areas of the granulomas were measured in a blinded fashion on digitalized photomicrographs of HE-stained sections with Image Tool 3.0 software (UTHSCSA, San Antonio, Tex, USA http://ddsdx.uthscsaedu/dig/itdesc.html).

2.9. Immunostaining and Confocal Microscopy. Immunofluorescence labeling and quantitative confocal microscopy

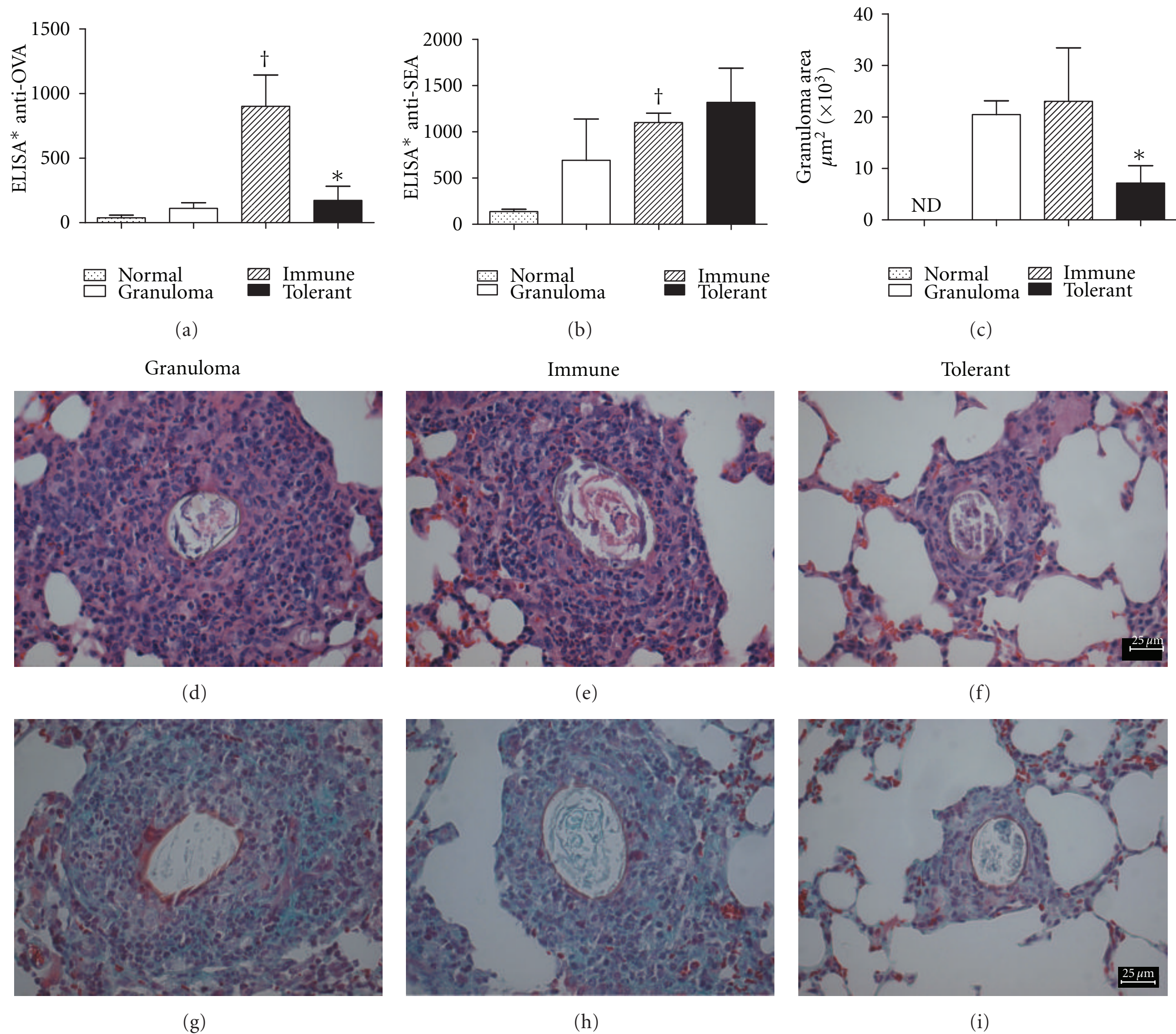


Figure 1: Reduction of granuloma by re-exposure of orally tolerant animals to the tolerated antigen. Serum levels of (a) anti-OVA antibodies and (b) anti-SEA antibodies and (c) pulmonary granuloma area and (d–i) histological aspect of pulmonary granuloma 18 days after i.v. injection of *S. mansoni* eggs in nonimmunized mice (granuloma group, open bars), OVA immune controls (hatched bars), and OVA-orally tolerant (black bars). Normal mice (doted bars) were not immunized with OVA neither injected with eggs. Data represent mean ± SEM. $^{*}P \leq 0.05$ tolerant versus immune $^{\dagger}P \leq 0.05$ immune versus normal. nd: not detected. Original magnification of HE (d–f) and Gomori's trichrome (g–i) photomicrographs 400X; scale bars = 25 μm.

were used to investigate the distribution and quantity of macrophages (F4/80), lymphocytes (CD3+), CD4+ cells, myofibroblast (α-SMA), and ICAM-1. Briefly, lungs were immediately fixed and cryosubstituted in a -80°C solution containing 80% methanol and 20% dimethyl sulfoxide for 5–7 days, transferred to -20°C for 1-2 days, and then brought to room temperature as described elsewhere [26]. Samples were rinsed 3X in absolute ethanol, 2X in xylene and embedded in paraplast following standard protocols. Five μm longitudinal sections from the middle of the lung were dewaxed with xylene and rehydrated through a graded series of ethanol into PBS. Blocking was achieved using 2% BSA in PBS at room temperature for 1 h followed by an overnight incubation at 4°C with primary antibodies diluted in PBS containing 0.1% BSA and 0.01% Tween-20. The following antibodies were used: rat anti-F4/80 (eBioscience San Diego, CA), rat anti-CD3 (Pharmigen, San Diego, CA), rat anti-CD4 (Pharmigen, San Diego, CA), mouse anti-α-SMA (Sigma St. Louis, MO) and mouse anti-ICAM-1 (R&D Systems, San Diego, CA). After 4-5 rinses in PBS, sections were incubated for 1 h at room temperature in the dark with Alexa 488-conjugated goat antimouse IgG secondary antibodies (Molecular Probes, Eugene, OR) or FITC-conjugated goat antirat IgG-polyclonal secondary antibody (eBioscience San Diego, CA). Nuclear counterstainig was made with 4′6-diamidino-2-phenylindol (DAPI). After several rinses in PBS, sections were mounted in a mixture of 10% 1.0 M Tris-HCl, pH 9.0, and 90% glycerol and analyzed using a laser scanning confocal microscope (Zeiss 510META; Carl Zeiss AG, Oberkochen, Germany). Optimal confocal settings

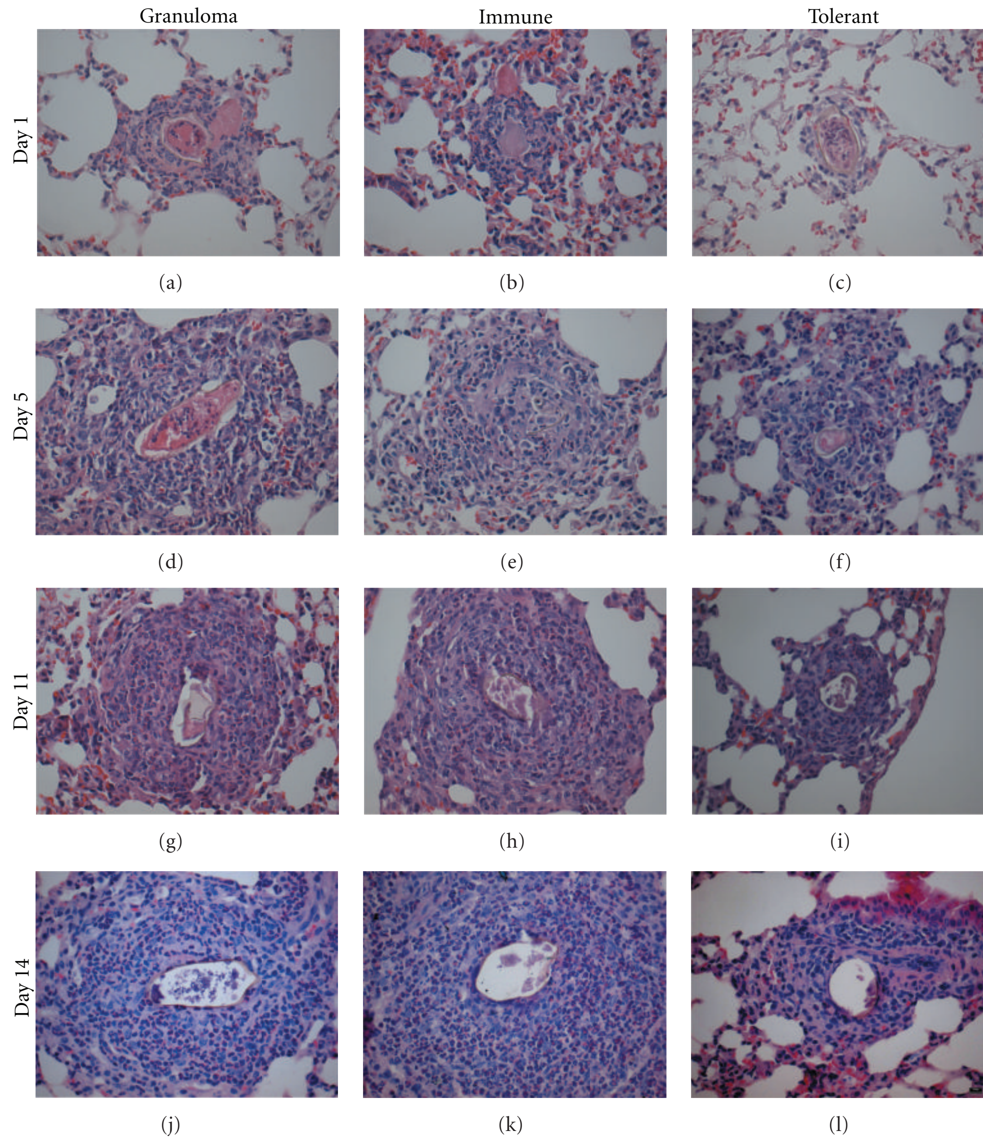


FIGURE 2: Granuloma at different times after egg injection. Lung HE staining 1, 5, 11, and 14 days after i.v. injection of *S. mansoni* eggs. (a–c) At day 1, an inflammatory infiltrate with predominance of neutrophils and macrophages can be detected around eggs in all groups, but it is less intense in the tolerant group. (d–f) At day 5, macrophages, eosinophils, and some lymphocytes can be detected. (g–l) At days 11 and 14 granulomas are more organized and some fibroblasts can be detected. Granulomas in tolerant mice follow the same pattern of organization but do not reach the same size of granulomas in controls group. Scale bars = 25 μm.

(aperture, gain, and laser power) for each antibody used were determined at the beginning of each imaging session and then held constant during the analysis of all the samples.

The distribution patterns and levels of expression of F4/80, CD3, CD4, α-SMA, and ICAM-1 were analyzed on digitalized photomicrographs with Image Tool 3.0 software (UTHSCSA, San Antonio, Tex, USA, http://ddsdx.uthscsa.edu/dig/itdesc.html). Images were captured at 12 bit and analyzed in the gray scale range of 0 to 255. Green fluorescence intensity was recorded as the sum of gray values of all pixels divided by the area (in μm^2) $\times 10^{-3}$. Background fluorescence was measured in each sample and subtracted from the values obtained for the fluorescence intensity.

2.10. Spleen Cell Cultures and Cytokine Assay. Spleen cells were counted and adjusted to concentrations of 1×10^7 cells/mL in RPMI 1640 supplemented with 2% heat inactivated FCS, 2 mM L-glutamine (Sigma-Aldrich, Inc.), and antibiotics (100 U/mL penicillin, 100 lg/mL streptomycin) (Sigma-Aldrich, Inc.). Cells were cultured in 96-well flat-bottom plates at 125 μL/well in a humidified atmosphere with 5% CO_2 with or without soluble schistosome egg antigens at 50 μg/mL culture fluid, ovalbumin at 1 mg/mL, or concanavalin A at 2 μg/mL. After 72 h, supernatant fluids were harvested and frozen -20°C for subsequent cytokine analysis. The production of IL-10 and IFN-γ by spleen cells was measured by cytokine capture ELISA.

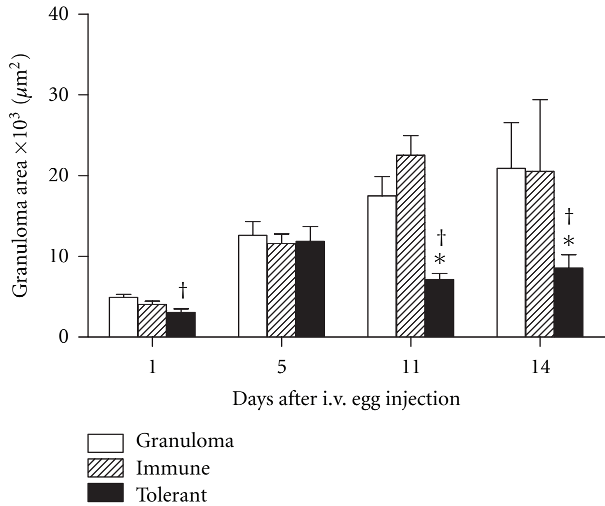


Figure 3: Re-exposure of orally tolerant animals to the tolerated antigen block enlargement of granuloma area. The area of granulomas at days 1, 5, 11 and 14 after i.v. injection of eggs in nonimmunized mice (granuloma group, open bars), OVA immune controls (hatched bars), and OVA-orally tolerant (black bars). Data represent mean ± SEM (five mice/group). $^{*}P \leq 0.05$ tolerant versus immune and $^{\dagger}P \leq 0.05$ tolerant versus granuloma.

2.11. Quantitative Analysis of Serum and Lung Cytokines. Serum samples were collected as previously described and stored at −20°C until used. One hundred milligrams of lung tissue samples from animals of each experimental groups were homogenized in 1 mL of PBS (0.4 M NaCl and 10 mM de NaPO4) containing proteases inhibitors (0.1 mM phenylmethylsulfonyl fluoride, 0.1 mM benzethonium chloride, 10 mM EDTA, and 20 KI aprotinin A) and 0.05% Tween 20. The samples were then centrifuged for 10 minutes at 3,000 ×g and the supernatant immediately used for quantitative analysis of cytokines. The cytokines (IL-2, IL-4, IL-5, IFN-γ, and TNF-α) in serum and lung samples were measured with Cytometric Bead Array (CBA) Mouse Th1/Th2 kit according to the manufacturer's specifications (BD Biosciences, CA, USA).

2.12. Antibody Assay. Anti-OVA and antisoluble egg antigen (SEA) antibody titres were determined by standard enzyme-linked immunosorbent assay (ELISA) using an automatic ELISA reader (BioRad, Hercules, CA). ELISA scores were computed by calculating the sums of the optical densities obtained from the six serum dilutions between 1 : 50 and 1 : 1600 of individual mice. The details of the assay method have been described previously [11, 24, 27]. Each score shown represents the mean ± SEM of the 5 animals in the group.

Statistical Analysis was performed using GraphPad Prism 4 (GraphPad Software, CA, USA), and the statistical significance of differences between groups was determined using one-way ANOVA followed by Student-Newman-Keuls test. Values of $P \leq 0.05$ were considered significant. The results are expressed as the mean ± SEM.

3. Results

3.1. Reexposure of OVA-Orally Tolerant Animals to the Tolerated Antigen in Al(OH)$_3$ Blocks Granuloma but Not Anti-SEA Antibody Formation. To induce oral tolerance to OVA C57BL/6 mice were offered an egg white solution for three days as their only liquid source (called "tolerant"), and control mice (called "immune") drank tap water. Seven days after interrupting the oral treatment, mice were immunized i.p. with OVA in Al(OH)$_3$ immediately before the i.v. injection of live *S. mansoni* eggs. Another control group (called "granuloma") received i.v. injection of eggs without any other previous treatment. Eighteen days thereafter, mice were sacrificed and blood and lung were removed for serum antibodies and pulmonary granuloma evaluation. Figure 1(a) shows that the oral pretreatment with egg white resulted in tolerance to OVA, that is, anti-OVA antibodies were significantly inhibited as compared with immune mice not orally pretreated. In contrast, anti-SEA antibodies were augmented in all groups injected with live eggs, irrespective of other treatments (Figure 1(b)). Noteworthy, granuloma area was significantly smaller in OVA-tolerant mice (Figure 1(c)).

We also performed histological analyses of Gomori's trichrome (Figures 1(g)–1(i)) and HE-stained lung sections (Figures 1(d)–1(f)). Eighteen days after i.v. injection of eggs, pulmonary granulomas were well organized with concentric arrangement and composed of macrophages, eosinophils, lymphocytes and some fibroblasts and epithelioid cells (Figures 1(d)–1(f)). Granulomas were observed around small branches of pulmonary arteries. Initial collagen deposition could be better observed after staining with Gomori's trichrome in all groups (Figures 1(g)–1(i)). Inflammatory infiltrates in lung parenchyma and alveolar macrophages were characteristic of all groups, but more prominently in immune group. Of note, in OVA-tolerant mice the majority of eggs were surrounded by typical, although small, granulomas (Figure 1(f)) and their lung parenchyma presented less inflammatory infiltrates (not shown).

3.2. Re-Exposure of OVA Orally Tolerant Animals to the Tolerated Antigen Blocks Initial Phases of Pulmonary Granuloma Formation. As described in the literature, the granuloma formed around *S. mansoni* eggs has a defined maturational stage followed by a stage of involution, and, from a morphological point of view, these stages may be classified as pregranulomatous and granulomatous stages [28]. The pregranulomatous, exudative stage is characterized by accumulation of neutrophils, eosinophils, and macrophages around the egg. The granulomatous stage can be divided into three phases: exudative-productive, productive, and involutional. In order to compare the kinetics of granuloma formation in OVA tolerant and not tolerant mice we performed histological analyses of Gomori's trichrome (not shown) and HE-stained lung sections. Figure 2 shows HE-stained sections of the pregranulomatous stage at days 1 and 5 after egg inoculation (Figures 2(a)–2(f)) and granulomas in the exudative-productive phase of the granulomatous stage at days 11 and 14 (Figures 2(g)–2(l)). At day 14, scarce deposition of collagen fibres could be observed in Gomori's

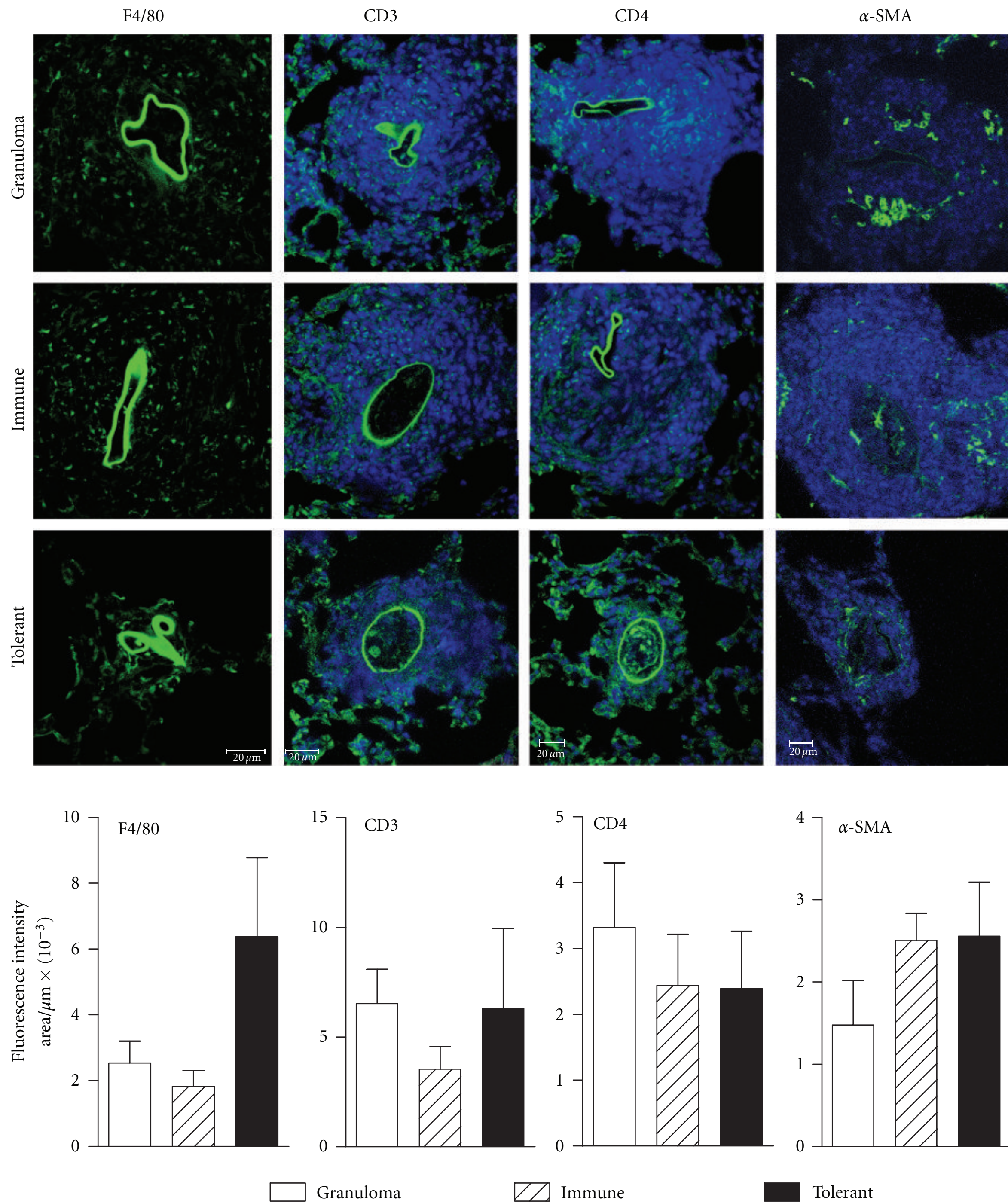


FIGURE 4: Cell subsets in pulmonary granulomas 18 days after i.v. egg injection. Immunolocalization using specific antibodies followed by secondary antibodies coupled with fluorescein (green) and nuclear counterstainig with 4′6-diamidino-2-phenylindol (blue), 18 days after i.v. eggs injection. Confocal microscope images were captured with a 63X objective, and the graphs represent the green fluorescence intensity (the sum of gray values of all pixels divided by the area (in μm^2) $\times 10^{-3}$) of expression of F4/80 (macrophages), CD3 (T-lymphocytes), CD4+ cells, and α-SMA (myofibroblasts) in nonimmunized mice (granuloma group, open bars), OVA immune controls (hatched bars), and OVA-orally tolerant (black bars). Data represent mean ± SEM of fluorescence intensity of duplicate slides ($n = 5$ mice/group). The green autofluorescency of eggs was excluded from all analyses. No significant difference was found between groups.

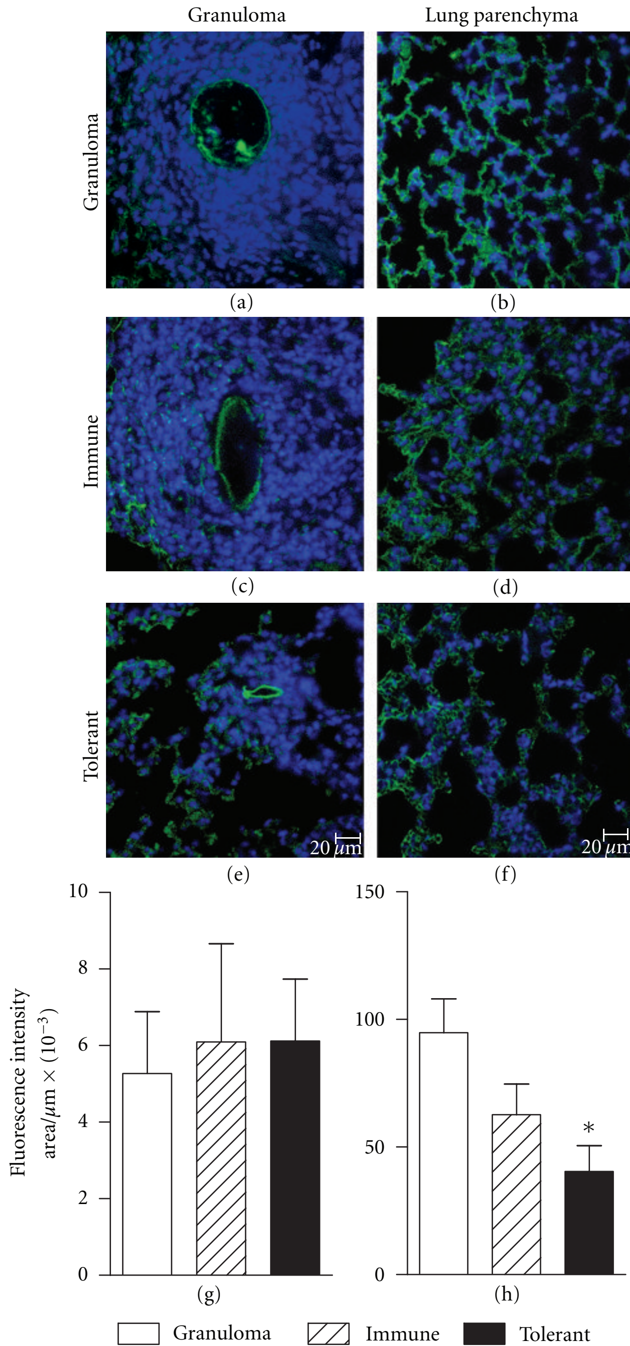


FIGURE 5: Re-exposure of orally tolerant animals to the tolerated antigen block the rise of ICAM-1 expression in lung parenchyma. Immunolocalization of ICAM-1 in granulomas and lung parenchyma using specific antibody coupled with fluorescein (green) and nuclear counterstainig with 4′6-diamidino-2-phenylindol (blue), 18 days after i.v. eggs injection. Confocal microscope images were captured with a 63X objective, and the graphs represent the green fluorescence intensity (the sum of gray values of all pixels divided by the area (in μm^2) $\times 10^{-3}$) of expression of ICAM-1 in the granuloma area (a–d) and in lung parenchyma (e–h) in nonimmunized mice (granuloma group, open bars), OVA immune controls (hatched bars), and OVA-orally tolerant mice (black bars). Data represent mean ± SEM of fluorescence intensity of duplicate slides ($n = 5$ mice/group).

trichrome-stained sections (not shown). Granulomas in the tolerant group followed similar kinetics as that from controls group, but less intense.

Morphometric analysis (Figure 3) showed that at day 1 the area of granulomas from OVA-tolerant mice is reduced as compared to control mice, but this difference disappears at day 5. However, after day 5, the area of granulomas increased in controls group and became significantly higher than the area of granulomas in OVA-tolerant mice at days 11 and 14 (Figure 3).

3.3. Re-Exposure of Orally Tolerant Mice to the Tolerated Antigen Do Not Change Granuloma Cell Composition. To further characterize granuloma cell composition macrophages, T lymphocytes and myofibroblasts were identified and quantified by immunostaining followed by confocal microscopy. Despite their smaller area, granulomas from tolerant mice present the same cell subsets as the large granulomas of controls groups (Figure 4). Even myofibroblasts (α-SMA+) were present in the smaller granulomas of the Ova orally-tolerant mice (Figure 4). For technical reasons we could not perform double immunostaining with anti-CD3 and anti-CD4 antibodies. To quantify fluorescency, images were captured at 12 bit and analyzed in the gray scale range of 0 to 255. Green fluorescence intensity was recorded as the sum of gray values of all pixels divided by the area (in μm^2) $\times 10^{-3}$ as described in Section 2. The green auto-fluorescency of eggs was excluded from all analyses. No significant difference in fluorescency intensity was found between groups. Then we can conclude that the reduction in the area of granulomas is due to proportional reduction of the inflammatory cells.

3.4. Re-Exposure of Orally Tolerant Mice to the Tolerated Antigen Reduces ICAM-1 Expression. Adhesion molecules enable circulating leukocytes to accumulate in areas of lung inflammation, and adhesion is the initial phase of a process whereby activated endothelial cells induce leukocyte migration into tissues. As ICAM-1 has been described as a predominant adhesion molecule after egg deposition in the liver of *S. mansoni* infected mice [19] we compared its expression in lungs after i.v. injection of eggs. Our results show that the majority of *S. mansoni* egg-induced ICAM-1 expression 18 days after pulmonary granuloma induction was restricted to the lung parenchyma outside the granulomas (Figure 5). The intensity of ICAM-1 expression in the granuloma of tolerant and controls group was not different. However, in the lung parenchyma outside granulomas, the expression of ICAM-1 was significantly inhibited in the tolerant mice (Figure 5).

3.5. Reduction of Granulomas by Re-Exposure to Orally Tolerant Proteins Was Not Correlated with a Shift in Th-1/Th-2 Cytokines. Using a commercial kit to detect typical Th1/Th2 cytokines, we compared the levels of IFN-γ, TNF-α, IL-2, IL-4, and IL-5 in lung homogenates 14 days after granuloma induction (Figure 6) and in serum samples (Figure 7) collected 1, 5, 14 and 18 days after granuloma induction. IFN-γ could be detected in lung homogenates

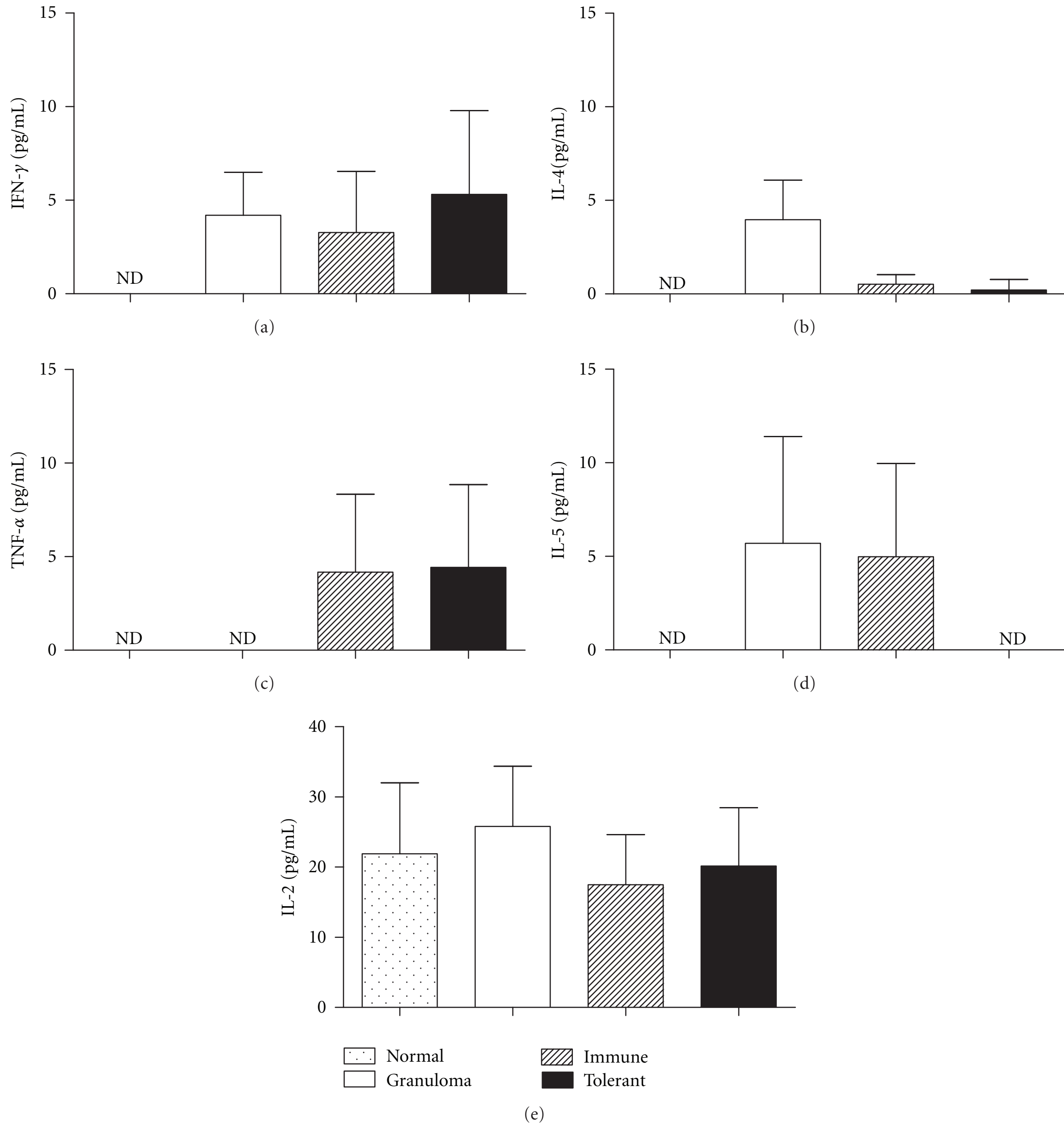
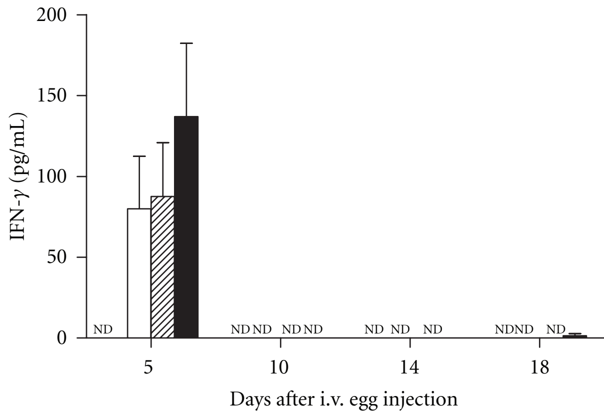
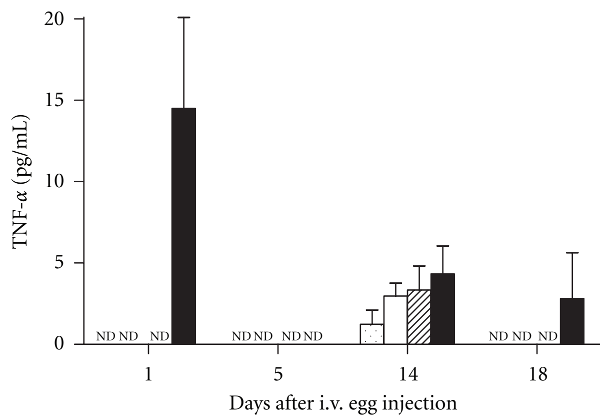


Figure 6: Cytokines production of lungs. Fourteen days after i.v. injection of *S. mansoni* eggs in nonimmunized mice (granuloma group, open bars), OVA immune controls (hatched bars), and OVA-orally tolerant mice (black bars), lungs were removed and homogenized in extract buffer. Normal mice (doted bars) were not immunized with OVA and neither injected with eggs. Extract supernatant was collected for cytokine assay. IFN-γ, TNF-α, IL-2, IL-4, and IL-5 were measured using a Cytometric Bead Array (CBA) kit. The results are shown as mean concentrations ± SEM. nd: not detected.

(Figure 6) and serum at day 1 (Figure 7) in some mice injected with *S. mansoni* eggs and not in normal (naïve) mice, but no difference was found between the experimental groups. IL-2 and IL-4 were not detected in serum samples from any group. IL-2 was detected in the same level in lung homogenates of normal and experimental groups, and the low levels of IL-4 detected in lung homogenates of immune and tolerant mice did not correlate with the size of their granulomas. TNF-α was detected in the serum at the same level in all groups, 14 days after egg injection. At day 1, TNF-α, and IL-5 were detected in the serum only in the tolerant group, but not in all mice from this group. In conclusion, reduction of granulomas in tolerant mice does not correlate with a shift in Th-1/Th-2 cytokines.

We also compared the production of IFN-γ and IL-10 by spleen cell restimulated "in vitro" with OVA or SEA. The





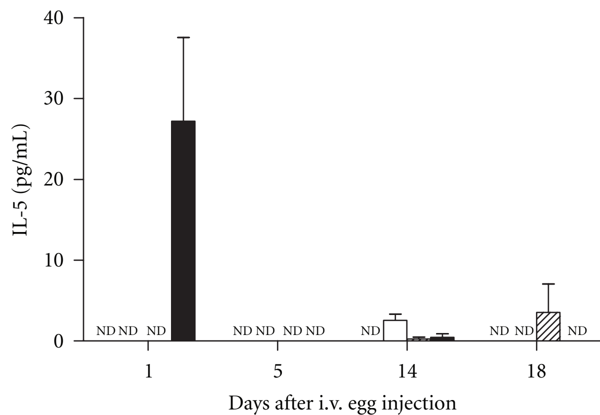
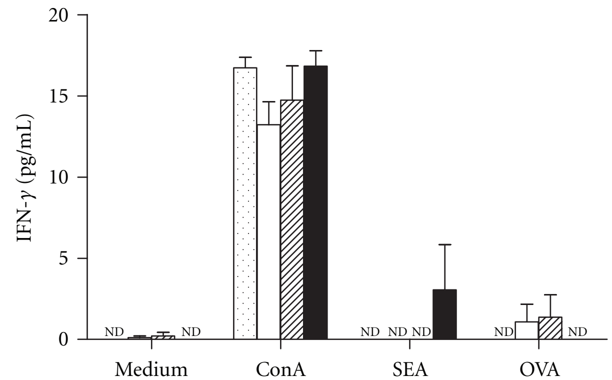


Figure 7: Time course of serum cytokines after granuloma induction. IFN-γ, TNF-α, IL-2, IL-4, and IL-5 were measured using a Cytometric Bead Array (CBA) kit in serum samples collected from nonimmunized mice (granuloma group, open bars), OVA immune controls (hatched bars), and OVA-orally tolerant mice (black bars). Normal mice (doted bars) were not immunized with OVA and neither injected with eggs. The results are shown as mean concentrations ± SEM. nd: not detected. IL-2 and IL-4 were not detected.



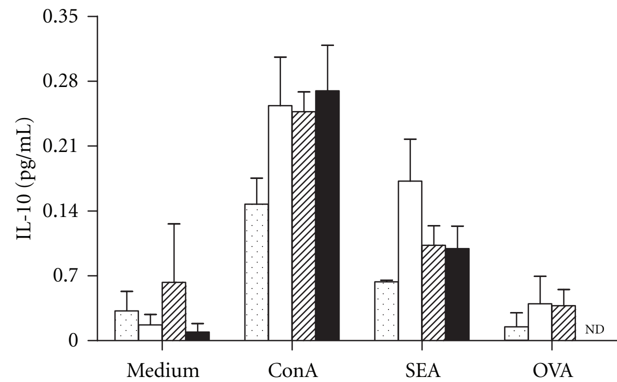


Figure 8: IFN-γ and IL-10 production of spleen cells stimulated with SEA or OVA. Eighteen days after i.v. injection of *S. mansoni* eggs in nonimmunized mice (granuloma group, open bars), OVA immune controls (hatched bars), and OVA-orally tolerant mice (black bars) spleen cells were cultured with medium, ConA, SEA or OVA for 3 days. Normal mice (doted bars) were not immunized with OVA neither injected with eggs. The culture supernatant fluids were harvested and IFN-γ and IL-10 measured by sandwich ELISA. The results are shown as mean concentrations ± SEM. nd: not detected.

results in Figure 8 do not make us confident to attribute the reduction of granulomas in tolerant mice to systemic alteration in the production of these cytokines.

4. Discussion

The standard protocols used to demonstrate tolerance in orally pre-treated animals involve challenge with the antigen in adjuvant, and there is evidence that adjuvants play a significant role in tolerogenesis during the triggering/parenteral phase affecting the kind of Ig isotype that is suppressed or maintained for long periods after oral feeding [29, 30]. Tobagus et al. [30] suggested that when a Th1-selective adjuvant (such as CFA) is used the resulting response displayed selective inhibition of the Th1 component (IFN-γ) of the immune response while orally pre-treated animals

challenged with the antigen in a Th2-selective adjuvant (alum) displayed a selective inhibition of Th2 responses. While oral tolerance is specific to the antigen contacted by the oral route, it is noteworthy that the parenteral injection of small doses (e.g., 10 μg) of proteins to which the animal is orally tolerant triggers a strong inhibition of primary responses to unrelated antigens [3, 8, 10].

In previous work we have shown that in mice orally-tolerant to ovalbumin (OVA), anti-SEA and pulmonary granulomas triggered by i.v. injection of eggs from *S. mansoni* were inhibited by i.p. injection of dinitrophenylated conjugates of OVA (DNP-OVA) emulsified in complete Freund's adjuvant (CFA) [24]. In that work we analysed granulomas only at day 18 after i.v. egg injection and found that the more prominent granulomas occurred in nontolerant mice concomitantly injected with DNP-OVA + CFA and small granulomas were found in orally tolerant mice injected with DNP-OVA + CFA. In the tolerant group eggs were predominant in intravascular locations with initial periovular reactions containing monocytes, eosinophils, and collagen fibers derived from the vascular wall [24]. Herein, we have shown that i.p. injection of OVA plus $Al(OH)_3$ into OVA-tolerant mice also inhibited pulmonary granuloma but not anti-SEA antibodies production (Figure 1). Our previous report and the present one as well show that reduction of granuloma in orally-tolerant mice is independent of the kind of adjuvant used. On the other hand, inhibition of anti-SEA antibody formation only occurred when the tolerated antigens were injected with CFA [24]. Nevertheless, we have shown that inhibition of antibodies to other proteins such as KLH and haemoglobin occurs with injection of OVA + $Al(OH)_3$ in OVA orally-tolerant mice [10, 12]. So, unknown factors associated with the eggs make it more difficult to inhibit the anti-SEA antibody response.

This and already published work [3, 9, 11–13] show that the re-exposure of orally tolerant animals to the tolerated antigen blocks inflammatory reactions. One hallmark of inflammatory processes is the migration of leukocytes to local areas. Herein we have shown that the injection of tolerated antigen into orally tolerant mice weakens the influx of leucocytes into the lung and reduces the size of granuloma (Figures 1, 2, and 3). However, the inhibitory effect of oral tolerance hindered the intensity of migration of cells into the lung, but not its kinetics, since granulomas followed the same pattern of formation in tolerant and not tolerant mice (Figure 2). Furthermore granulomas in tolerant mice have the same cell composition although in low numbers as compared to not tolerant mice (Figure 4).

Changes in the expression of cell adhesion molecules initiate leukocyte trafficking, and ICAM-1 is the predominant adhesion molecule in schistosome egg granuloma formation [19]. The reduction in the expression of ICAM-1 in tolerant mice, as shown herein (Figure 5), is certainly involved in the demonstrated inhibitory effect. We could not find significant changes in cytokine secretion, neither in the blood, nor in lung extracts (Figures 6 and 7). IL-10 was detected after spleen cell cultures with SEA, but no difference was found between tolerant and not tolerant group, and IL-10 concentrations in supernatants of spleen cells cultured with OVA were not different from basal production (Figure 8). This detection may require proper timing, but the present results argue against major changes in the Th1/Th2 axis.

It is important to pursue these findings with additional experiments. Antibody formation may be involved in the reduction of granulomas in orally-tolerant mice, since B cells and anti-idyotipic antibodies are involved in the regulation of granulomas [31, 32] and oral tolerance also affects B cell and antibody production [29]. In searching for possible mechanisms involved in inhibitory indirect effects triggered by parenteral injection of tolerated antigens we must keep in mind that they affect the initial phases of the inflammatory response which are thought to be primarily innate, as shown herein and in previous work [13]. This may be taken as indication that, in addition to specific immunological (clonal) events, the exposure to tolerated antigens triggers other phenomena, for example, of neuroendocrine nature.

5. Conclusion

Parenteral injection of tolerated proteins into orally tolerant mice blocked the increase of pulmonary granulomas and the expression of ICAM-1 in lung parenchyma in areas outside the granulomas. The reduction in the area of granulomas in tolerant mice is due to proportional reduction of the inflammatory cells and was not correlated with a shift in Th-1/Th-2 cytokines in serum or lung tissue extract.

Conflict of Interests

The authors have no conflict of interests to be disclosed.

Acknowledgments

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Review Article

The Role of the Intestinal Context in the Generation of Tolerance and Inflammation

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The mucosal surface of the intestine alone forms the largest area exposed to exogenous antigens as well as the largest collection of lymphoid tissue in the body. The enormous amount of nonpathogenic and pathogenic bacteria and food-derived antigens that we are daily exposed sets an interesting challenge to the immune system: a protective immune activity must coexist with efficient regulatory mechanisms in order to maintain a health status of these organisms. This paper discusses how the immune system assimilates the perturbations from the environment without generating tissue damage.

1. Interface between the Outside and Inside Environments

The intestinal mucosa forms the largest area of the body in direct contact with the exterior environment. If expanded, the surface of the small intestine alone can reach roughly the size of a tennis court, or 100 times the area of the skin [1]. In the skin, several layers of cells, including stratified epidermis, and dermis, generate a physical barrier that separates the internal components of the body from the outside. On the other hand, in the intestine, a single layer of absorptive epithelial cells forms an interface between the lumen (outside environment) and the lamina propria (inside environment). If one sees our body as a target for attack from infectious and pathogenic organisms, the structure of intestinal epithelia is counterintuitive, since the intestine is exposed to constant colonization by bacteria and is a host to an enormous quantity and diversity of microbes, including commensals and potential pathogens. More than 100 trillion microbial cells colonize the human gut, which amounts to ten bacteria for every one human cell. The vast majority of these bacteria are not pathogenic, but rather perform a variety of beneficial functions to the host [2]. A recent study, using extensive Illumina sequencing of fecal DNA samples, estimated that the human microbiome contains more than 1000 bacterial species, with more than 160 different species generally present in each person [3]. These results highlight a high degree of person-to-person variation, possibly influenced by a distinct host genetic landscape and environmental conditions.

Other mucosal surfaces also harbor a diverse microbiota. For instance, over 200 genera of bacteria were identified in a human skin microbiome study [4]. However, the intestinal mucosa is peculiar since it has to deal with intense bacterial colonization and at the same time absorption and digestion of nutrients. In that regard, it should be noted that the large intestine contains most of the microbiota while the small intestine is the main place for absorption and digestion of nutrients.

In addition to the exposure to innocuous antigens, the intestine is also a place where many different types of infections can occur, including infection by viruses, bacteria, parasites, and fungi. Commensal bacteria, generally involved in symbiotic interactions with the host, have also been correlated with the development of inflammatory bowel diseases such as ulcerative colitis and Crohn's disease. Similarly, dietary proteins can trigger food allergies and celiac disease. Therefore, it is reasonable to argue that the great majority of processes in the gut are not generated towards "defense" against invading organisms, but are rather a consequence of

chronic exposure to large amounts of harmless and often beneficial antigens. This scenario poses an interesting challenge to the immune system, since most of the "nonself" interactions should probably be tolerized as "self". How does the immune system associated with the intestine influence and assimilate the perturbations from the environment without generating pathology?

2. The Immune System at the Intestinal Interface

As expected, the intestinal mucosa is filled with a diverse and large number of immune cells. The gut-associated-lymphoid-tissue (GALT) includes the Peyer's patches (PP) and isolated lymphoid follicles (ILFs). However, most of the immune cells in the intestine are associated with the intestinal villi, either in the intraepithelial or lamina propria compartments, which are the focus of this paper. Estimations based on histological sections indicate that there are more T cells in the intraepithelial compartment alone than in the spleen [5]. Moreover, the cells in the intestinal mucosa consist of mainly activated or antigen experienced T cells ($CD45RB^{lo}$, $CD44^{hi}$, $CD69^{hi}$, $CD62L^{lo}$) that are capable of producing several proinflammatory cytokines such as IL-4, IFN-γ, IL-17A/F, IL-22, and TNF-α [6–15].

The intraepithelial compartment of the intestine is unique in regards to its lymphocyte populations. Most of intraepithelial lymphocytes (IELs) are CD8$\alpha\alpha$-expressing TCR$\alpha\beta$ and TCR$\gamma\delta$, with CD8$\alpha\alpha$-CD4 and -CD8$\alpha\beta$ TCR$\alpha\beta$ cells and double-negative T cells contributing in lower numbers. While CD8$\alpha\beta$ and CD4 IELs are rare early in life, these populations steadily increase with age likely as a consequence of exposure to exogenous antigens [16–19]. IELs also express natural killer (NK) cell receptors, both activating and inhibitory, which allow these cells to change their resting state to a cytotoxic and potentially inflammatory state [7, 9, 20–23]. The development and function of IELs have been recently reviewed elsewhere [24] and will not be the focus of this paper.

Contrary to the IELs, lamina propria lymphocytes (LPLs) contain T and B cell populations with similar frequency to peripheral lymphoid tissues. Additionally, lamina propria B cells produce large amounts of immunoglobulin (Ig) molecules, mostly belonging to the IgA isotype, which is the most abundant antibody isotype in the body. Furthermore, LPL cells reside among several types of antigen presenting cells (APCs) and other types of innate immune cells, so-called innate lymphoid cells, that can function to either promote or suppress inflammation [25–28]. IELs, LPL, dendritic cells (DCs), and intestinal epithelial cells are in constant interaction and their cross-talk is reinforced by cell surface receptor-ligand interactions, including $\alpha 4\beta 7$/MadCAM, $\alpha_E\beta 7$(CD103)/E-cadherin and CD8$\alpha\alpha$/TL contact [29–31]. Through the expression of tight junctions, epithelial-associated $CX_3CR_1{}^+$ APCs are able to establish contacts with the neighboring epithelial cells, while extending their dendrites to sample luminal antigens, including whole bacteria [32]. Both APCs and epithelial cells express toll-like receptors (TLRs) that induce cellular activation and lead to the migration of DCs to regional lymph nodes, where they can present processed antigens to the naïve T cells. The diversity of functions exerted by innate immune cells in the intestinal lamina propria is also achieved through the production of several different cytokines and other soluble factors such as IL-22, IL-23, and retinoic acid [25–28]. Consistently, high frequency of proinflammatory Th17 cells and regulatory T cells can be found in the lamina propria of the small and large intestine, respectively [33–35].

Recent advances in mucosal immunology research contributed to our understanding of how the intestinal context [36] plays that critical role in the balance between protective immune responses and tolerance to harmless antigens.

3. The Intestinal Context: Microbiota

Commensal microorganisms actively interact with the absorptive intestinal mucosa and influence the basal activity of the immune system as well as the amplitude of the immune response. The importance of the microbiota to the development of the host immune system is evident in germ-free animals (born and raised in completely aseptic conditions). The development of the local or systemic immune system is defective in germ-free mice. For example, germ-free mice show reduced germinal-centers in the spleen and reduced systemic IgG and IgA antibodies [2, 37, 38]. These mice have fewer and smaller Peyer's patches, reduced mesenteric lymph nodes, decreased cell numbers, and virtually no IgA production in the lamina propria relative to conventional animals [37, 39]. IELs are also compromised in germ-free animals, particularly TCR$\alpha\beta$ IELs, with a drastic decrease in cell number and cytotoxic capacity throughout the intestine [36, 40].

Similarly, the microbiota is able to modulate the activity of innate immune cells, including APCs and innate lymphoid cells, in the lamina propria [28, 41]. Commensal bacteria-derived ATP has been shown to directly activate lamina propria $CD11c^{low}CD70^{high}$ cells to produce IL-6, IL-23, and TGF-β and induce local differentiation of Th17 cells [42]. The reduced amount of ATP in germ-free animals was proposed [42] as an explanation to the depletion of Th17 cells in the lamina propria of these mice [33, 34, 42]. Conversely, recent studies found that the commensal *segmented filamentous bacteria* (SFB) is present in mouse colonies with a high frequency of IL-17-producing cells in the intestine [33, 34]. While germ-free mice lack Th17 cells in the lamina propria of the small intestine, the mono-colonization of germ-free mice with SFB restores the number of Th17 cells to conventional levels. In addition, no change in Th1 cells was observed indicating that SFB induces differentiation of CD4 T-cells into Th17 cells. However, no association between SFB colonization and ATP levels was reported [33, 34]. The IL-23/IL-17 axis of the CD4 LPLs exert a protective function against extracellular pathogens while being detrimental in different models of inflammatory bowel diseases [43]. Interestingly, the SFB-induced Th17 responses also enhance susceptibility to systemic autoimmune disorders such

as arthritis and experimental autoimmune encephalomyelitis (EAE) [44, 45].

A contrasting example of a bacterial metabolite that contributes to the mucosal immunity is the short-chain fatty acids (SCFAs), which is produced by fermentation of dietary fiber by *Bifidobacterium* [46]. SCFAs bind to G-protein-coupled receptor 43 (GPR43, also known as FFAR2) and inhibit inflammatory responses during DSS-induced colitis by suppressing the differentiation of IL-17 producing cells in the lamina propria of conventional mice, suggesting that germ-free mice are more susceptible to this model of colitis due to reduced SCFA in the intestinal environment [46].

The commensal bacteria *Bacteroides fragilis* is also associated with suppression of Th17 and other inflammatory responses in the intestine by expression of polysaccharide A (PSA) via IL-10 production [47, 48]. A recent study showed that PSA is recognized by TLR2-expresing T cells and promotes their production of IL-10 [49]. Furthermore, PSA-treated Tregs are more efficient at suppressing activated T-cells *in vitro*, and *Bacteroides fragilis* mono-colonized recipient-mice induce higher numbers of Treg cells and show reduced Th17 responses after naïve CD4 adoptive transfer [49]. In addition to *Bacteroides fragilis*, a recent report by Atarashi and coworkers identified *Clostridium* spp., a genus of gram-positive bacteria, belonging to the Firmicutes phylum, as a major inducer of Tregs in the colon of conventional mice [35]. The authors showed a specific depletion of the induced-Treg (iTreg) population in the colon of germ-free animals, and mono-association with 46 different species of *Clostridium*, but no other classes of bacteria, completely restored this population [35]. These results show that microbial-derived mechanisms can affect both innate and adaptive immunities and promote immune-regulation in the intestinal surface.

In the same vein, several studies have documented alterations of gut microbiota (dysbiosis) in patients with IBD [50–54]. Frank et al. described a case control study of the intestinal microbial ecology in IBD and non-IBD controls where they found a marked decrease in the representation of two prominent constituents of the gut microflora, Bacteroides and Lachnospiraceae, in the IBD-specific group compared to controls [55]. However, there remains to be established a cause-effect relationship between dysbiosis and IBD. Furthermore, it is not clear how immune activity affects the composition of the commensal bacteria in health and disease. These results highlight the crucial role that the microbiota play in the homeostasis of intestinal immunity.

4. The Intestinal Context: Diet

Although much of the focus in mucosal immunology in recent years was given to the microbiota, most of the immune system antigen interaction in the gut is associated with the small intestine, where the nutrients are absorbed. The majority of these antigens readily get access to the immune system through the intestinal epithelia, M cells, or direct sampling by CX_3CR_1-expressing lamina propria macrophages and probably additional APCs.

Beside the microbiota, the exposure to food proteins has also been shown to play a crucial role in the development and maintenance of the intestinal immune system [56] as well as in susceptibility to systemic infection [57]. The importance of food proteins to systemic immunity can also be appreciated by the fact that we ingest around 100 grams of protein daily, and up to 0.5% (500 mg/day) of ingested proteins can be found intact in blood circulation a few hours after ingestion [58].

The relevance of dietary proteins in the maturation of the immune system was demonstrated by elegant studies in "antigen-free" mice (germ-free mice fed an elementary diet). The studies reported a marked reduction in the lymphocyte populations in the gut in such an antigen-deprived environment, with further reduction in systemic immunoglobulins (IgG and IgA) when compared to germ-free animals [59–61]. However, both the repertoire and total production of IgM is maintained in antigen-free animals, suggesting that the immune system has a basal or natural level of activity independent of exogenous antigens [62].

Similarly to the microbiota, food proteins are potentially immunogenic and help to maintain the "immunological tonus". Nevertheless, in general, the exposure to dietary antigens does not generate pathological responses. Indeed, mucosal exposure to antigens efficiently inhibits the development of immune responses to subsequent challenges with the same antigen, a phenomenon described as oral tolerance [63, 64]. It was demonstrated that peripheral generation of Foxp3-expressing Treg cells by TGF-β [65] is a crucial event in oral tolerance induction in mice harboring monoclonal repertoire by both B and T cells (TBmc). Moreover, using the same experimental model, Curotto de Lafaille et al. showed that lack of functional Foxp3 results in abrogation of oral tolerance induction [66].

Recent studies have further elucidated induction and effector phases of oral tolerance. It is thought that antigen-sampling by lamina propria APCs (including CX_3CR_1 resident macrophages) followed by antigen-transporting and presentation by migratory $CD103^+CCR7^+$ in the MLN is crucial for generation Foxp3-expressing Treg cells oral tolerance induction [27, 65–69]. Hadis and coworkers have also shown that after Treg cell induction in the MLN, their migration to the lamina propria and expansion mediated by CX_3CR_1 resident macrophages is essential for the effector phase of oral tolerance [68].

In addition to their regulatory role, it was also demonstrated that mucosal DCs from mesenteric lymph nodes (MLNs) and Peyer's Patches (PPs) are unique in their capacity of degrading vitamin A to generate retinoic acid (RA) [70]. RA, in a TGF-β-dependent process, was proposed to play a crucial role in iTreg induction [25, 27, 69], demonstrating that diet-derived factors are also part of immune regulatory mechanisms involved in the prevention of aberrant immune responses towards the diet itself and other environmental antigens.

When oral tolerance is abolished, inflammatory processes generally arise resulting in the development of food

allergies and other diseases. An example of food-related gut disorder is celiac (or coeliac) disease (CD), a condition that damages the lining of the small intestine and prevents it from absorbing nutrients. The damage is due to a lack of tolerance to gluten, a group of proteins found in wheat, barley, rye, and possibly oats. The break in tolerance leads to an exacerbated (mostly) Th1 immune response to specific gluten antigens (gliadin) in the small intestine after ingestion of gluten (reviewed by [71]). The pathogenic immune response in celiac disease is dependent on antigen (gluten peptides) presentation via major histocompatibility complex (MHC) class II molecules to CD4 positive T-cells. Celiac disease is strongly linked to genetic predisposition found in individuals expressing the MHC- or human leukocyte antigen- (HLA-) DQ2, DQ8, or DQ2/8 and the absence of these human HLA alleles rules out the diagnosis of celiac disease in suspected patients. These MHC class II molecules can be induced in intestinal epithelium under inflammatory conditions and are able to efficiently bind and present gluten peptides, activating proinflammatory $CD4^+$ effector T cells [71]. The genetic background together with the intestinal context, in which the gluten protein is presented to T-cells, are the main factors in the balance between tolerance and inflammation in CD development. IFN-γ and IL-21 double-producing $CD4^+$ T cells [72] and also Th17 cells [73] have been implicated in celiac disease as well. The differentiation of pathogenic, rather than regulatory, $CD4^+$ T cells is thought to be induced by proinflammatory cytokines, including IL-15 and IFN-α, that are present in the intestinal mucosa from celiac disease patients [71]. One of the mechanisms proposed for IL-15 function in CD is the disruption of TGF-β-mediated signaling through SMAD3 [74]. Additionally, an elegant study by DePaolo and coworkers reported that IL-15 may also synergize with retinoic acid to enhance inflammatory responses and CD-like inflammation [75]. It should be noted that this is in sharp contrast to the anti-inflammatory effects of retinoic acid in conjunction with TGF-β reported above, which include induction of Treg cells and suppression of inflammatory Th17 differentiation [25]. These results demonstrate how one metabolite may have strikingly different effects depending on microenvironment milieu and cell target of this metabolite. They also point out how deleterious are the consequences when the robust mechanisms of tolerance induction in the mucosal surfaces are broken.

5. Concluding Remarks

The dilemma faced by the mucosal immune system to induce tolerance to antigens (rule) or to engage an inflammatory immune response (exception) is daily dealt with through multidirectional interactions between the immune cells and environmental factors that permeate the mucosal surfaces. The identification of cellular and molecular mechanisms involved in this process will likely contribute to new approaches for prevention and treatment of inflammatory bowel diseases (IBD) and also other systemic inflammatory diseases.

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Research Article

Lactococcus lactis NCC 2287 Alleviates Food Allergic Manifestations in Sensitized Mice by Reducing IL-13 Expression Specifically in the Ileum

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Objective. Utilizing a food allergy murine model, we have investigated the intrinsic antiallergic potential of the *Lactococcus lactis* NCC 2287 strain. *Methods*. BALB/c mice were sensitized at weekly intervals with ovalbumin (OVA) plus cholera toxin (CT) by the oral route for 7 weeks. In this model, an oral challenge with a high dose of OVA at the end of the sensitization period leads to clinical symptoms. *Lactococcus lactis* NCC 2287 was given to mice via the drinking water during sensitization (prevention phase) or after sensitization (management phase). *Results*. *Lactococcus lactis* NCC 2287 administration to sensitized mice strikingly reduced allergic manifestations in the management phase upon challenge, when compared to control mice. No preventive effect was observed with the strain. *Lactococcus lactis* NCC 2287 significantly decreased relative expression levels of the Th-2 cytokine, IL-13, and associated chemokines CCL11 (eotaxin-1) and CCL17 (TARC) in the ileum. No effect was observed in the jejunum. *Conclusion/Significance*. These results taken together designate *Lactococcus lactis* NCC 2287 as a candidate probiotic strain appropriate in the management of allergic symptoms.

1. Introduction

The prevalence of allergic diseases has been increasing dramatically in the past decades [1, 2]. Allergic sensitization starts in early childhood mainly to common food allergens encountered in everyday food products such as cow's milk, eggs, and wheat. Subsequent exposure to the allergen involves an intricate interplay of cellular components of the adaptive immune system in which CD4+ T cells are activated to secrete cytokines such as IL-4, IL-5, and IL-13 [3, 4]. The development of allergic manifestations can be altered via two approaches, one in which sensitization to new allergens is prevented, thereby inhibiting the development of the Th-2 conditioning [5]. We refer to this approach as "prevention" in the context of our study. Once sensitization to the allergen has occurred, subsequent exposure can trigger allergic symptoms; the effective management of these allergic manifestations then becomes the primary goal. We have named this approach "management" in relation to our study.

Probiotics are defined by the WHO as "living micro-organisms that when administered in adequate amounts confer a health benefit to the host" [6]. Among potential health promoting attributes, the capacity of probiotic strains to modulate the host immune system, either by direct signal-ing or by modulating the intestinal microbiota, is currently an area of intense research. The beneficial role of probiotics,

especially *Lactobacillus* and *Bifidobacterium* strains in atopic diseases, has been investigated with increasing interest over the past few years with both animal studies [7, 8] and human clinical trials [9–12]. These studies have yielded conflicting results that in part reflect the diversity of clinical settings studied as well as the different probiotic strains that have been investigated. The importance of intervening at the appropriate time window in relation to allergies, that is, either by preventing sensitization or in management of allergic symptoms, has remained under investigated.

Based on extensive *in vitro* immune profiling of different candidate probiotic strains using both murine and human cell-based assays, we selected for the current study a lactic acid bacterial strain *Lactococcus lactis* (*L. lactis*) NCC 2287. We sought to evaluate the benefits of intervening at the two phases, that is, prevention and management, in a murine model of food allergy via *L. lactis* NCC 2287. *Lactococcus* strains have been used to deliver therapeutic molecules [13] but have rarely been individually studied in disease models for their probiotic effects. We report that while no preventive effect with the strain was observed, *L. lactis* NCC 2287 administration to sensitized mice strikingly reduced allergic scores induced upon oral challenge in the management phase when compared to control mice. In addition, we investigated the different mechanisms via which *L. lactis* NCC 2287 may exert its therapeutic effect. *L. lactis* NCC 2287 administration during the management phase leads to a decrease in IL-13 production from restimulated mesenteric lymph node (MLN) cells along with a significant decrease in the relative expression levels of IL-13 and Th-2 associated chemokines CCL11 (eotaxin-1) and CCL17 (TARC) in the ileum but not in the jejunum.

2. Materials and Methods

2.1. Reagents and Bacterial Biomass. Lactococcus lactis (*L. lactis*) strain NCC 2287 is a dairy starter strain from the Nestlé culture collection (NCC) that was deposited at Collection Nationale de Cultures de Microorganismes at Institut Pasteur, Paris, France (CNCM I-4154). Bacterial biomass was produced by culture of NCC 2287 under standard conditions. Growth curve was determined for the strain, and according to this, bacterial cells were harvested by centrifugation 3 h after entering in the stationary phase. The biomass was washed 2x in cold PBS and frozen in PBS 20% glycerol at -80°C.

2.2. OVA Food Allergy Murine Model. All animal studies were approved by a Nestec internal Ethics Committee and the Service Vétérinaire of the Canton of Vaud, Switzerland (Authorization no. 1970). This model has been described in detail before [14]. Briefly, six-week old conventional female BALB/c mice (Harlan Laboratories, France) were sensitized ($n = 10$ per group; negative control $n = 5$) orally via gavage at weekly intervals by 20 mg of ovalbumin (OVA) (Fluka, Buchs, Switzerland) and 10 μg/mouse of Cholera toxin (CT) (List Biologicals, purchased from LuBioscience, Lucerne, Switzerland) for 7 consecutive weeks. Animals were challenged orally via gavage with 100 mg of OVA one week after the last sensitization (Figure 1(a)). Starting 30 minutes after challenge, mice were individually observed for 30 min. Clinical symptoms were recorded and quantified as follows (allergic score): 0: no symptoms, less than 4 episodes of scratching; 1: 4–10 episodes of scratching around the nose and head, no diarrhoea; 2: more than 10 episodes of scratching or bristled fur and immobility or soft stool; 3: diarrhoea or laboured respiration or cyanosis; 4: diarrhoea in combination with immobility after prodding, bristled fur, laboured respiration or cyanosis; 5: anaphylaxis. Mice demonstrating a symptom severity of ≥ 4 were sacrificed immediately. Four hours after challenge, mice were sacrificed after isoflurane anaesthesia and terminal bleeding. Blood and the last centimetre of ileum and jejunum were taken and frozen in liquid nitrogen. *L. lactis* strain NCC 2287 (5×10^8 CFU/mL in drinking water) was administered at different phases of the experiment and its effect was compared to the positive (OVA + CT) control groups. To evaluate the efficacy during the prevention phase, we administered the probiotic starting 5 days before the first oral sensitization. Administration was then continued during the entire experimental period. To assess the effect of administering the probiotic in sensitized mice, *L. lactis* NCC 2287 was provided in drinking water starting after the last sensitization up to the challenge with OVA for a total duration of 8 days (management phase).

2.3. Quantification of Serum Levels of Mouse Mast-Cell Protease 1 (MMCP-1). MMCP-1 was analyzed in mouse serum by ELISA, purchased from Moredun Scientific (Penicuik, Scotland) according to the manufacturer's instructions. The MMCP-1 concentration was obtained by converting OD values in pg/mL using a polynomial standard curve.

2.4. Quantification of Serum Levels of OVA-Specific IgE, IgG1, and IgG2a. OVA-specific immunoglobulin-E (IgE), immunoglobulin-G1 (IgG1), and immunoglobulin-G2 (IgG2a) concentrations were measured by ELISA as described previously [14, 15]. For IgE measurement, plates (NUNC Maxisorp; VWR, Nyon, Switzerland) were coated overnight at 4°C with rat antimouse IgE (2 μg/mL; BD Pharmingen, Allschwil, Switzerland). After washing, wells were blocked with PBS-1% BSA for 1 h at RT. Serially diluted sera and standard (monoclonal mouse anti-OVA; ABD Serotec, Düsseldorf, Germany) were incubated for 2 h at 37°C. Then, biotinylated-OVA (1 μg/mL) was added to the plate for 1 h at 37°C, followed by incubation with HRP-labeled streptavidin (1 : 1000; KPL; Socochim, Lausanne, Switzerland) for 30 min at 37°C. Plates were developed with tetramethylbenzidine (TMB) substrate (KPL). The reaction was stopped with 1 M HCl (Merck, Darmstadt, Germany). Optical densities were measured at 450 nm. Concentrations were calculated by converting OD values in pg/mL using a polynomial standard curve. For IgG1 and IgG2a, microtiter plates were coated with OVA (Sigma, Buchs, Switzerland) (100 μg/mL) overnight at 4°C. Wells were washed with PBS 0.05% Tween (Biorad, Reinach, Switzerland) and then blocked with PBS-1% BSA for 1 h at room temperature.

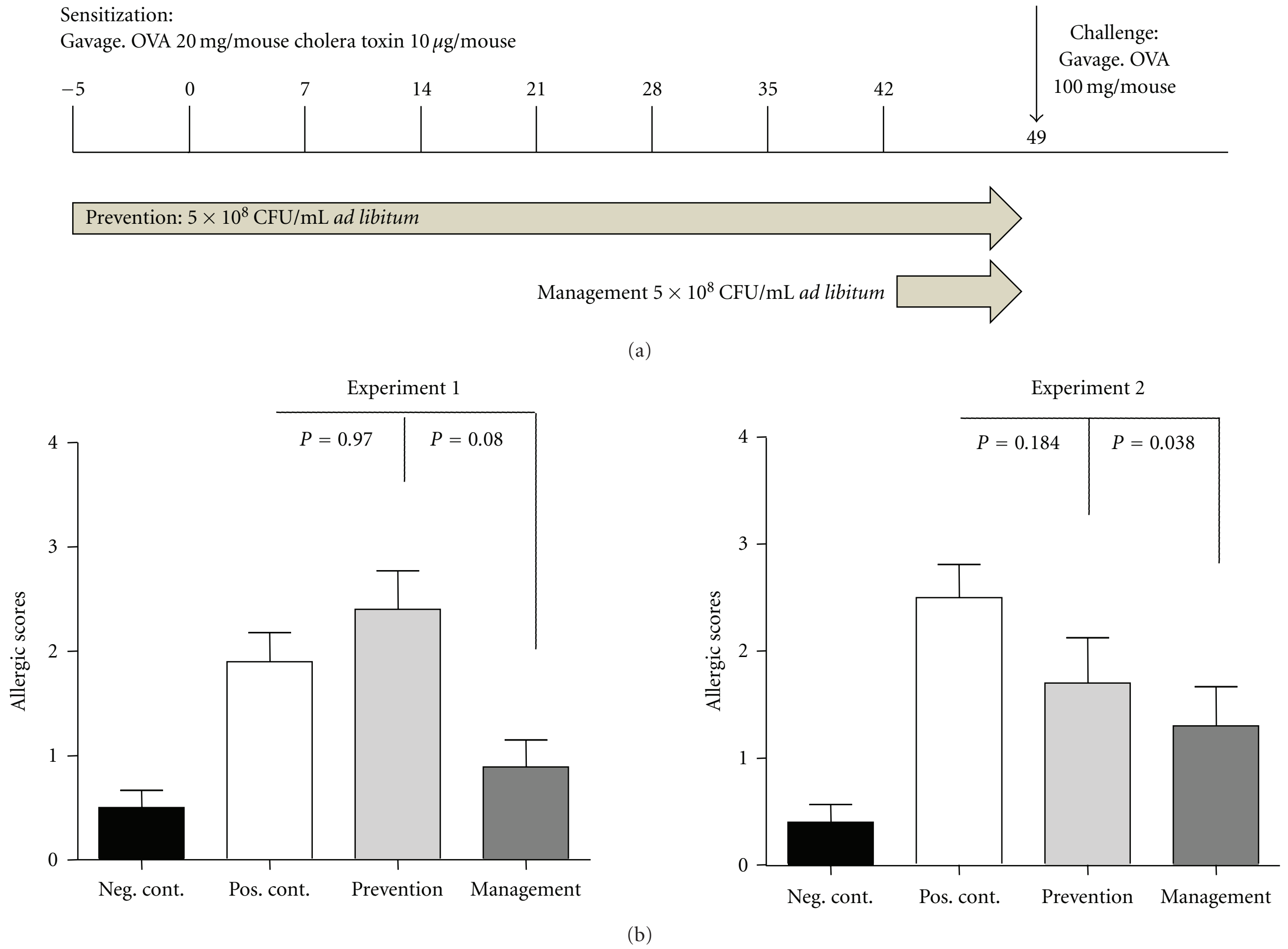

Figure 1: *L. lactis* NCC 2287 alleviates allergic symptoms in sensitized mice. *L. lactis* NCC 2287 (5×10^8 CFU/mL) was given *ad libitum* to mice ($n = 10$, $n = 5$ in Neg. control) orally via drinking water (a). Administration was either before the first sensitization and given throughout the experiment (prevention phase) or in the one week after the last sensitization (management phase). After challenge, mice treated with *L. lactis* NCC 2287 in the management phase (dark grey bar graph) developed significantly reduced clinical scores than sensitized, untreated animals in the positive control group (white bar graph). Mice consuming *L. lactis* NCC 2287 during the prevention phase of the experiment (grey bar graph) did not exhibit reduced symptoms. An exploratory experiment 1 (left panel) and a confirmatory experiment 2 (right panel) are shown. 3 experiments were performed in total (b).

Serially diluted standard (monoclonal mouse anti-OVA IgG1 and anti-OVA IgG2a from Antibody Shop; LucernaChem, Lucerne, Switzerland) and serum samples were incubated for 2 h at 37°C, followed by incubation for 2 h with a HRP-labelled goat antimouse IgG1 or IgG2a antibody (1 : 5000; Southern Biotech; Bioconcept, Allschwil, Switzerland). Plates were then developed, read, and analyzed.

2.5. Isolation and Culture of MLN Cells. Mesenteric lymph nodes (MLN) were homogenized with a syringe plunger in a cell strainer (BD Falcon; Milian, Meyrin, Switzerland). Cells were centrifuged and washed 2x in RPMI medium (Sigma) complemented with 10% fetal bovine serum (FBS; Bioconcept, Paris, France), 1% L-glutamine (Sigma), 1% Penicillin/Streptomycin (Sigma), 0.1% Gentamycin (Sigma), 50 μM β-mercaptoethanol (Sigma). Cells (3×10^5 cells/well) were cultured in 96 well flat bottom plates (Corning, Milian, Meyrin, Switzerland) in the absence or presence of OVA (1 mg/mL). After 72 hrs of culture, plates (including supernatant and cells) were frozen at −20°C.

2.6. Quantification of Cytokines in Culture Supernatant Fluid. Mouse IL-4, IL-5, and IL-10 were measured using the mouse Th-1/Th-2 multiplex kit (Meso Scale Discovery, Gaithersburg, Md, USA) according to the manufacturer's instructions. IL-13 was measured using a Mouse IL-13 (DY413E) ELISA kit from R&D Systems (Abington, England).

2.7. Quantitative Gene Expression Levels by Low-Density Array (LDA). Total ribonucleic acids (RNAs) from ileum and jejunum were extracted according to the manufacturer's protocol using the SV Total RNA Isolation System kit (Promega, Dübendorf, Switzerland). RNA was quantified with quant-IT Ribogreen Reagent kit purchased from Promega according to

the manufacturer's protocol. Reverse transcription was performed on 1 μg of total RNA by using the Multiscribe Reverse Transcriptase kit (Applied Biosystems, Foster City, Calif, USA). Total RNA was mixed with 50 μM of random hexamers, 0.5 mM of dNTPs, 20 U of RNase inhibitor (Applied Biosystems), 62.5 U of Multiscribe reverse transcriptase, 1X RT buffer, and 5.5 mM of $MgCl_2$ in a final volume of 50 μL. Reverse transcription was performed on a T3 thermocycler (Biometra, Göttingen, Germany) with the following cycle program: 10 min at 25°C, 30 min at 48°C, 5 min at 95°C to finish at 4°C. Low-density arrays were designed online on the Applied Biosystems website. The load and the run were performed according to the manufacturer's protocol on a quantitative ABI-Prism 7900HT. The quantification was normalized with the mean of 3 house-keeping genes: β-actin, GAPDH, and HPRT. The Ct value for each gene was corrected by the Ct mean of these three house-keeping genes. Based on the cycle threshold (Ct) values obtained, a relative and normalized mRNA expression was determined for each gene using the ΔCt. The results were calculated as a relative expression using the formula $2^{-\Delta Ct} \times K$, where K is a 10^6 factor. Fold increase results expression was normalized to expression levels in the negative control group.

2.8. Statistical Analyses. The software R 2.2.1 was used for the analyses. Clinical scores were evaluated using the Kruskal-Wallis tests, followed by Wilcoxon test. All other outcomes were treated with Kruskal-Wallis followed by Wilcoxon test. Corrections were applied following the Bonferroni-Sidak procedure. Statistical test to compute P values are calculated for median ± SEMedian values. Results were considered as significant with a P value ≤ 0.05.

3. Results

3.1. L. lactis NCC 2287 Oral Administration Is Effective in the Management of Food Allergy Symptoms but has no Effect on the Prevention of Sensitization. The *in vivo* effect of *L. lactis* NCC 2287 was tested in a murine model of food allergy in the prevention of allergic sensitization as well as in the management of allergic symptoms in sensitized mice (Figure 1(a)). For this purpose, BALB/c mice were sensitized to OVA via the oral route and challenged as described in Section 2. *L. lactis* NCC 2287 was given to mice via drinking water (5×10^8 CFU/mL; *ad libitum*) during the prevention phase (5 days before the first sensitization until the end of the experiment) or in the last week of the experiment following the last sensitization (day 43–49; management phase). Figure 1(b) illustrates the clinical symptoms observed in different groups of mice in two representative experiments. After challenge, animals in the positive control group developed statistically significant clinical scores of food allergy compared to the negative control group (clinical score of 1.9 ± 0.3 versus 0.5 ± 0.17; $P < 0.008$ in exploratory experiment 1 and clinical score of 2.5 ± 0.31 versus 0.4 ± 0.16; $P < 0.001$ in confirmatory experiment 2). Sensitized mice treated with *L. lactis* NCC 2287 in the management phase developed less severe clinical symptoms than the positive control group (0.9 ± 0.26; $P =$ 0.08 in exploratory experiment 1 and 1.3 ± 0.37; $P = 0.038$ in confirmatory experiment 2). However, mice consuming *L. lactis* NCC 2287 strain during the prevention phase of the experiment did not exhibit significantly reduced symptoms upon OVA challenge (2.4 ± 0.37; $P = 0.97$ in exploratory experiment 1 and 1.7 ± 0.42; $P = 0.18$ in confirmatory experiment 2). These findings suggest that *L. lactis* NCC 2287 strain acts more likely during the management phase following challenge and not during the prevention phase.

3.2. L. lactis NCC 2287 Administration Does Not Influence Levels of OVA-Specific IgE, IgG1, or Mouse Mast-Cell Protease-1 (MMCP-1) in Sensitized Mice. In order to determine which immunological parameters contributed to the observed beneficial effect of *L. lactis* NCC 2287 on clinical scores, we investigated the impact on serum levels of mouse mast-cell protease 1 (MMCP-1) and OVA-specific antibodies (IgE, IgG1, and IgG2a). MMCP-1 levels were increased significantly in the positive control group of animals (1107 ± 322 pg/mL) when compared to negative control, nonsensitized mice (3.8 ± 2.2 pg/mL; $P \leq 0.001$). Administration of *L. lactis* NCC 2287 did not statistically decrease MMCP-1 in either the preventive (2113 ± 521 pg/mL; $P = 0.17$) or the management (773 ± 298 pg/mL; $P = 0.48$) phases of the model (Figure 2(a)). OVA-specific IgE (Figure 2(b)), IgG1 (Figure 2(c)), and IgG2a (Figure 2(d)) levels increased significantly in the positive control group (1674 ± 551 pg/mL for IgE, 535 ± 192 pg/mL for IgG1 and 6058 + 3474 pg/mL for IgG2a) when compared to the negative control group (19 ± 11 pg/mL for IgE and below detection level for IgG1 and IgG2a; $P \leq 0.001$). However, the administration of *L. lactis* NCC 2287 did not significantly impact the levels of the immunoglobulin subtypes, neither in prevention (2986 ± 661 pg/mL for IgE, 857 ± 221 pg/mL for IgG1 and 5603 + 1306 pg/mL for IgG2a; $P \geq 0.1$) or in symptom management (2054 ± 1281 pg/mL for IgE, 558 ± 238 pg/mL for IgG1 and 8045 ± 5951 pg/mL for IgG2a; $P \geq 0.3$) phases of the model (Figures 2(b)–2(d)). These data suggest that *L. lactis* NCC 2287 is unlikely to lead to a reduction in allergic scores via its effect on two pathways commonly involved in the allergic cascade, namely, mast cell activation and humoral immunity.

3.3. Impact of Administering L. lactis NCC 2287 during Management Phase on IL-13 Production by MLN Cells. To further identify potential mechanisms via which *L. lactis* NCC 2287 exerted its beneficial effect, we next evaluated the *ex vivo* cytokine profile of antigen-restimulated lymphocytes isolated from the MLN. As shown in Figure 3, MLN cells from the negative control group secreted low levels of Th-2 cytokines (IL-4, IL-5, IL-10, and IL-13). In comparison, MLN cells from the positive control group secreted increased levels of IL-4 (Figure 3(a)), IL-5 (Figure 3(b)), IL-10 (Figure 3(c)), and IL-13 (Figure 3(d)). MLN cells from mice administered *L. lactis* NCC 2287 during the prevention phase exhibited similar levels of Th-2 cytokines as the positive control group. Of interest were the MLN cells of mice given *L. lactis* NCC 2287 during the management

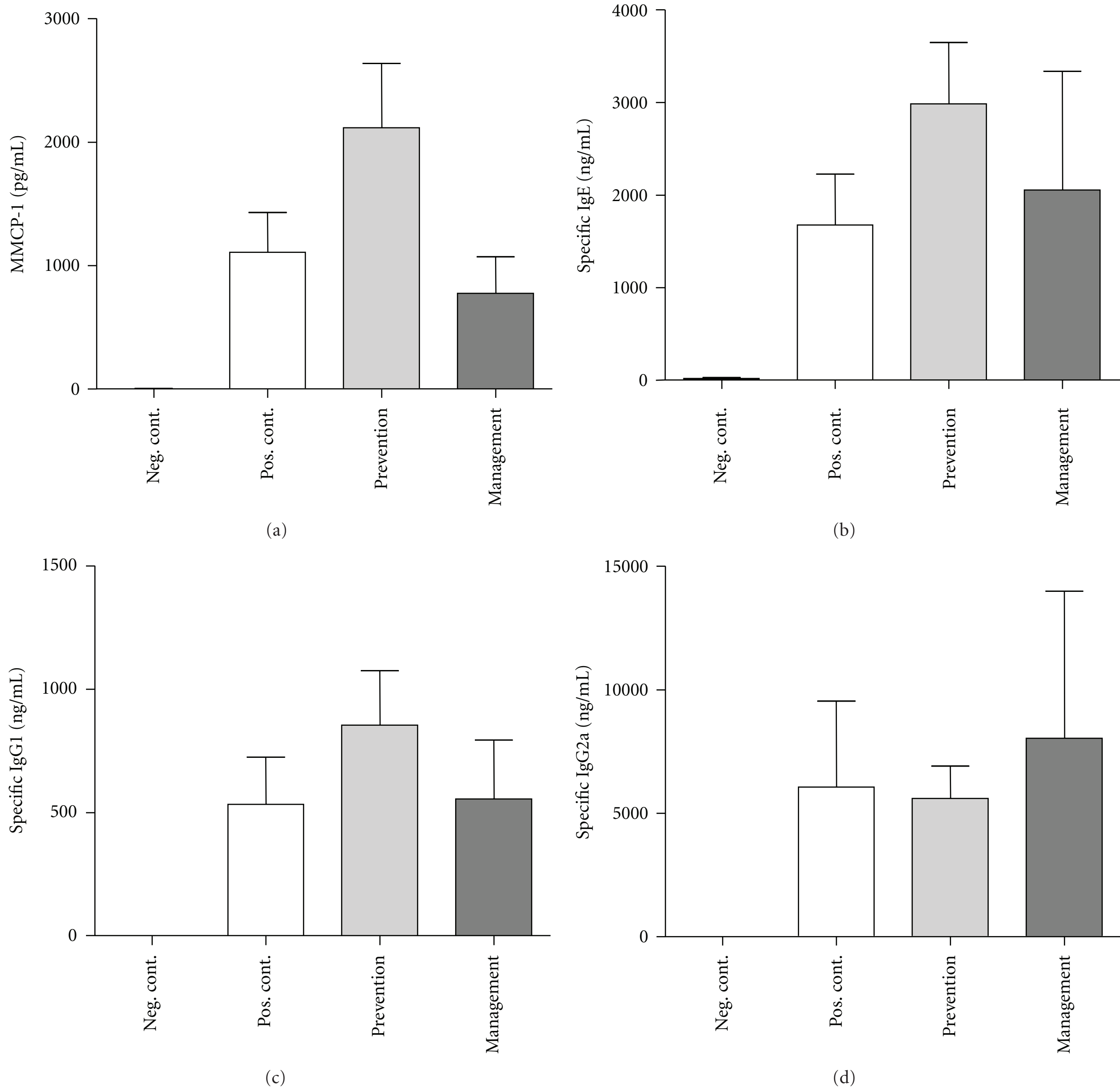

Figure 2: MMCP-1 (a), OVA-specific IgE (b), IgG1 (c), and IgG2a (d) levels in the serum 4 hours after challenge. Results from the confirmatory experiment are shown. OVA-specific levels of IgE, IgG1, and IgG2a were increased significantly in positive control mice compared to the negative control group. Administration of NCC 2287 both in the prevention and management phases did not significantly reduce levels of MMCP-1 and OVA-specific IgE, IgG1, and IgG2a. No effect of *L. lactis* NCC 2287 on mast cells and immunoglobulins.

phase that demonstrated a trend to decreased IL-13 cytokine production ($P = 0.08$) but not IL-4, IL-5, and IL-10 levels compared to the positive control.

3.4. L. lactis NCC 2287 Administration during Management Phase Reduced Relative Gene Expression Levels of the Th-2 Cytokine IL-13 in the Ileum but not in the Jejunum of Sensitized Mice. To correlate the results obtained *ex vivo* with OVA restimulation on IL-13, we investigated gene expression levels of IL-13, locally in the gastrointestinal tract. In addition, we also examined the expression of the IL-5 encoding gene. For this purpose, we isolated tissue samples from ileum and jejunum and examined relative gene expression levels by RT-PCR. IL-5 gene expression showed an increased trend in the ileum of positive control mice when compared to the negative control group (726 ± 403 in positive control versus 55 ± 47 in negative control; $P < 0.08$). The administration of *L. lactis* NCC 2287 in sensitized mice resulted in lower relative expression levels of IL-5 in the ileum (177 ± 95; $P = 0.1$ when compared to the positive control group); however, this effect was not statistically significant. There was no impact on IL-5 gene expression

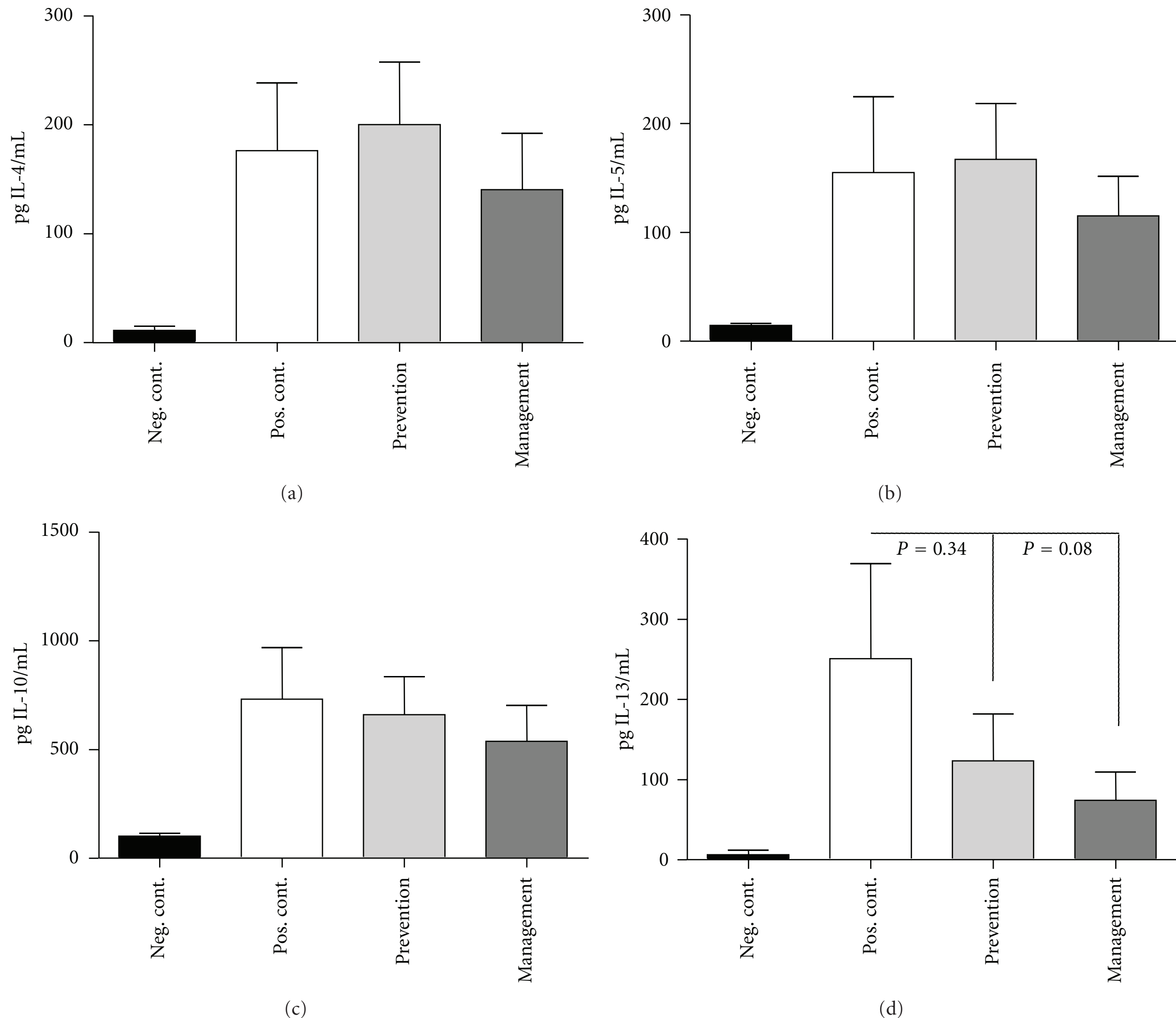

FIGURE 3: Decreased IL-13 production from restimulated MLN cells in mice administered *L. lactis* NCC 2287 in the management phase. IL-4 (a), IL-5 (b), IL-10 (c), and IL-13 (d) levels in the supernatants of MLN cells restimulated *ex vivo* with OVA. Positive control group secreted increased levels of Th-2 cytokines. MLN cells from mice administered *L. lactis* NCC 2287 during the prevention phase exhibited similar levels of Th-2 cytokines as the positive control group. MLN cells of mice administered *L. lactis* NCC 2287 during the management phase produced decreased levels of IL-13 cytokine levels but not IL-4, IL-5, or IL-10.

levels in the jejunum (Figure 4(a)). IL-13 gene expression levels on the other hand were remarkably increased in the ileum (992.6 ± 360.2 in positive control versus 7.3 ± 1.2 in negative control; $P \leq 0.01$). Interestingly, the administration of *L. lactis* NCC 2287 strongly mitigated the increase in the relative gene expression level for IL-13 (124 ± 62; $P = 0.015$ when compared to the positive control group) in the ileum of sensitized mice following challenge. There was no effect on IL-13 relative gene expression in the jejunum of mice administered *L. lactis* NCC 2287 during the management phase (Figure 4(b)).

3.5. Decreased Th-2 Chemokines CCL11 (Eotaxin-1) and CCL17 (TARC) Gene Expression Levels in the Ileum Following L. lactis NCC 2287 Administration during the Management Phase. Chemokines associated with allergic disorders and upregulated in the presence of elevated levels of IL-13 such as CCL11 (eotaxin-1) and CCL17 (TARC) were analyzed. Sensitization and subsequent challenge resulted in a significant increase in the relative gene expression levels of the chemokine CCL11 (eotaxin-1) in the ileum of positive control mice in comparison to the negative control group (24904 ± 6797 versus 7809 ± 3286 in negative control; $P = 0.006$; Figure 5(a)). A similar statistically significant upregulation was also observed on the relative gene expression levels of CCL17 in the positive control group (3943 ± 888 versus 400 ± 177 in negative control; $P = 0.001$; Figure 5(b)). The administration of *L. lactis* NCC 2287 in the management phase significantly reduced the relative gene expression levels of both CCL11 (5172 ± 1341; $P = 0.002$ versus positive

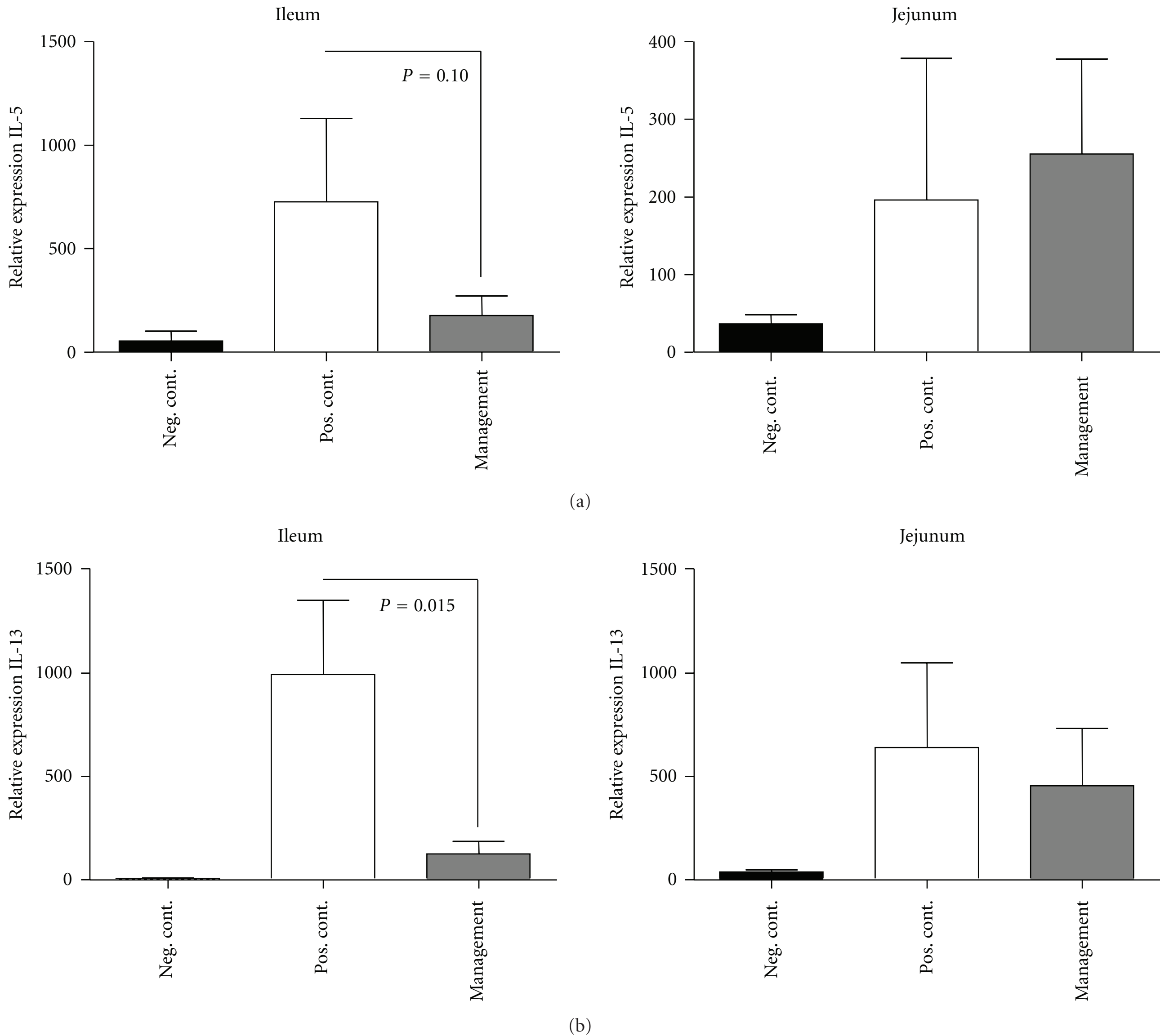

Figure 4: *L. lactis* NCC 2287 downregulates IL-13 expression in the ileum but not in the jejunum of sensitized mice. Relative gene expression of IL-5 (a) and IL-13 (b) in samples from ileum and jejunum analyzed by low-density arrays. Sensitization followed by challenge led to upregulation of IL-5 ($P = 0.08$) and IL-13 ($P = 0.001$) mRNA in the positive control group compared to the negative control in the ileum. Administration of NCC 2287 in the management phase led to a marked downregulation of IL-13 expression in the ileum ($P = 0.015$) but not in the jejunum.

control group) and CCL17 (1716 ± 521; $P = 0.04$ versus positive control group). Similarly, as observed for IL-13, no effect on CCL11 or CCL17 gene expression levels was observed in other intestinal sites such as jejunum.

4. Discussion

The current study explored the potential efficacy and mechanism of action of *L. lactis* NCC 2287 against the development of food allergy manifestations. Specifically, we delineated the efficacy of this strain in two critical phases of a food allergy mouse model, namely, by intervention during the sensitization phase (prevention phase) and in the symptomatic phase by intervention in sensitized mice shortly before challenge with the objective to manage allergic symptoms (management phase). When administered *in vivo, L. lactis* NCC 2287 significantly reduced food allergic symptoms in sensitized mice. Interestingly, the administration of the strain during sensitization was without any effect (Figure 1(b)). This underlines that certain probiotic strains may be "specialized" in modulating different phases of the allergic response. This is one of the most important findings of the current study and underlines that the efficacy of probiotic interventions is strongly dependent on the specific strain(s) used, of the clinical indication for which the strain is applied and the appropriate timing of the intervention.

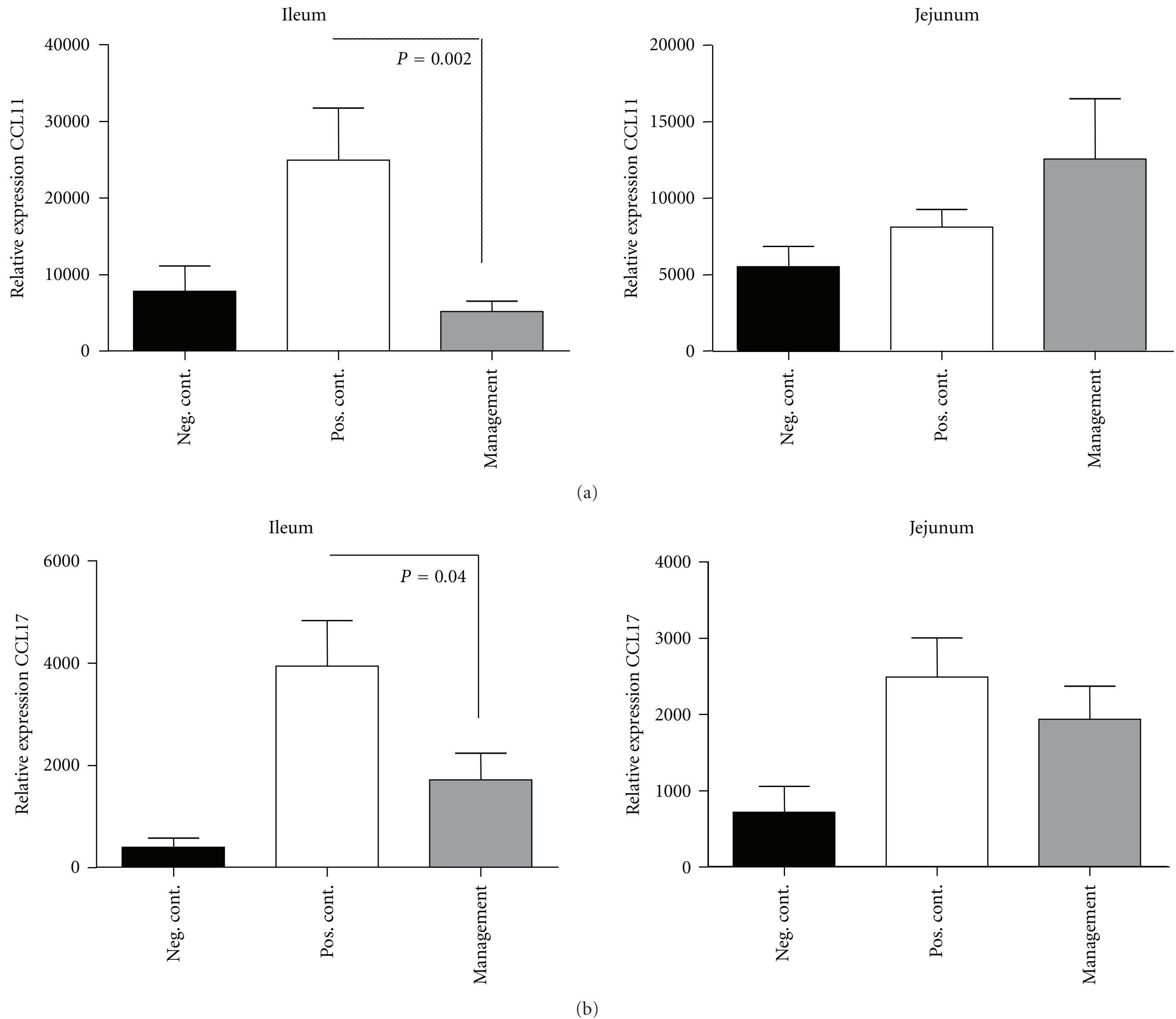

Figure 5: *L. lactis* NCC 2287 administration to sensitized mice downregulates Th-2 chemokines CCL11 (eotaxin-1) and CCL17 (TARC) in the ileum but not in the jejunum. Relative gene expression for CCL11 (a) and CCL17 (b) in samples from ileum and jejunum analyzed by low-density arrays. Sensitization followed by challenge led to upregulation of CCL11 ($P = 0.006$) and IL-13 ($P = 0.001$) mRNA in the positive control group compared to the negative control in the ileum. Administration of NCC 2287 in the management phase led to a marked downregulation of both CCL11 ($P = 0.002$) and CCL17 expression ($P = 0.04$) in the ileum but not in the jejunum.

The reduction of symptoms following *L. lactis* NCC 2287 administration in the management phase was not associated in the studied animal model with a reduction in levels of OVA-specific antibodies associated with a Th-2 phenotype, that is, IgE, IgG1, and IgG2a (Figures 2(b)–2(d)). These results are not completely surprising, since the levels of antigen specific immunoglobulins build up over time with repeated sensitizations, leading to a long-term persistence of memory B cells. Previous studies have also reported similar results upon the administration of probiotics in different allergy models [16, 17]. As we had previously observed an association between decreased MMCP-1 levels, a marker for mast cell activation and reduced allergic scores with a nutritional intervention in the same animal model [15], we also evaluated this parameter in the present study; the strain did not impact the levels of MMCP-1. It is then likely that mechanistically the antiallergic effect of this strain is not mediated by direct inhibition of mast-cell degranulation. Indeed, confirmatory *in vitro* experiments with IgE-sensitized RBL cells also did not support an inhibitory effect of *L. lactis* NCC 2287 on mediator release (data not shown).

In our model, the reduction in clinical scores in the management phase was paralleled by a decrease in the secretion of IL-13 by OVA restimulated MLN cells (Figure 3(d)) but not in IL-4, IL-5, or IL-10 (Figures 3(a)–3(c)). IL-13 is a vital Th-2 cytokine that dominates the chronic phase of allergic

sensitization and is primarily involved in the recruitment of eosinophils to sites of allergic inflammation and their subsequent activation and survival at these inflammatory sites [18, 19]. IL-13 is secreted by a variety of immune cells such as Th-2 cells, mast cells, dendritic cells, and eosinophils [20, 21]. IL-13 has been closely linked to the pathogenesis of a food allergic disorder, eosinophilic esophagitis both in animal studies and in clinical samples obtained from individuals with allergic disorder [22, 23]. IL-13 is also closely related to another well-studied Th-2 cytokine, IL-4 with respect to structure and function as both cytokines share a common receptor (IL-4Rα) [24, 25]. While relative gene expression levels of IL-4 were undetectable, we examined expression patterns of IL-5 and IL-13 following *L. lactis* NCC 2287 administration (Figure 4). There was nearly a 10-fold reduction in the relative gene expression of IL-13 in the ileum. This dramatic reduction was not observed elsewhere in the gastrointestinal tract, that is, jejunum (Figure 4). These results are also in line with our previous observations *in vitro* with *L. lactis* NCC 2287 in assays including monocyte derived DCs-resting CD4+ T cell cocultures and Th-2 skewed PBMC stimulation. *L. lactis* NCC 2287 was selected for its potential Th1/Treg immunomodulatory profile and its ability to inhibit the levels of Th2 cytokines in particular IL-5 and IL-13 (unpublished). These are intriguing results and suggest a mechanism whereby *L. lactis* NCC 2287 exerts its probiotic effect at a specific site of localized inflammation.

Supporting the local reduction of IL-13, we observed a statistically significant decrease in relative gene expression levels of Th-2 associated chemokines mainly CCL11 and CCL17 (Figures 5(a) and 5(b)). These chemokines and particularly CCL11 (eotaxin-1), are critically linked downstream to IL-13 signaling [26–28]. CCL17 (TARC) has previously been reported to be associated with allergic disorders [29] and potentially altered by probiotics in *in vitro* systems mimicking allergic pathogenesis [30]. The reduction of CCL11 and CCL17 expression was also observed only in the ileum further providing support to this site being an "active" site of inflammation in a gastrointestinal food allergy immune response as well as a site of action of *L. lactis* NCC 2287.

Multiple biological pathways exist that can contribute to the regulation of an aberrant immune response as is the case with food allergies. We have identified in this study one of the many mechanisms that could be responsible for the initiation of food allergic symptoms, mainly increased levels of IL-13 locally in the ileum and its downregulation upon administration of a probiotic *L. lactis* NCC 2287 in the management phase. The fact that the probiotic effect was observed for the strain only in sensitized mice suggests that *L. lactis* NCC 2287 acts specifically at sites of allergic inflammation. The survival rate and physiology of *L. lactis* in the digestive tract have previously been studied. In these observations, the *L. lactis* strain MG 1363 was reported to have a higher survival rate in the ileum in rodents [31]. These studies raise the possibility that in the mouse model used in this study, a similar preferential localization of the strain can happen in the ileum upon oral administration. Given such a scenario, the strain would then indeed have the maximum impact during the challenge phase of the model in the ileum. It would be interesting to study the effect of *L. lactis* NCC 2287 in either different models of allergy (skin and respiratory) and to compare the effect to other *L. lactis* strains. This approach of studying a bacterial strain for its probiotic effect in different phases of a disease model is the "way forward" for a more rational and practical approach to the selection of different strains for a particular health benefit.

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