

Induction of Food Tolerance Is Dependent on Intestinal Inflammatory State

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Highlights

- Hidden allergens have an important role in increasing food allergy cases.
- Introduction of food hours before the onset of gut inflammation induces tolerance.
- Food introduced next to the onset of gut inflammation induce multiple allergies.

Induction of Food Tolerance Is Dependent on Intestinal Inflammatory State

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ABSTRACT

Food allergies are usually managed by food avoidance. Hidden allergens in food, due to cross-contamination and/or allergenic additives added during production, place an important concern in today's increasing food allergy cases worldwide. Previous studies showed that the introduction of unacquainted food components, in an inflamed intestine, results in sensitization to this food. Thus, our aim was to evaluate the kinetics of multiple food allergy induction. Adult male C57BL/6 mice were divided into five groups, four of which were submitted to an intestinal inflammation induction protocol to peanuts. Egg white (OVA) diluted 1:5 v/v in distilled water was instilled by gavage 6h-before (PRIOR), concomitant (AT) and 6h-after (DURING) the onset of the peanut challenge diet. Positive control (POS CONT) and NEG CONT received saline per gavage. Finally, animals were challenged with subcutaneous injections of OVA. Results showed no changes in diet intake were observed. Anti-OVA polyisotypic IgG antibody titers significantly increased in AT. Flow cytometry revealed significant decrease in CD4⁺CD25⁺Foxp3⁺ and significant increase in TCD8⁺ in AT. Histomorphometrically, AT and DURING were classified as Infiltrative and Partial Destruction stages. PRIOR was classified as Infiltrative, while POS CONT was classified as Partial Destruction. NEG CONT was classified as Normal. Together, our results confirm that the introduction of unfamiliar food only a few hours before the initiation of a gut inflammation process is able to induce oral tolerance, however the introduction of a dietary

protein concomitant to the onset or during an ongoing gut inflammation may induce multiple allergies.

Graphical abstract XX

Keywords: Oral Tolerance, Food Allergy, Peanuts, Ovalbumin

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Introduction

Food allergy, defined as an impaired reaction to food proteins by the immune system, has become a major public health concern in recent years [1]. Symptoms vary drastically from mild to extreme (anaphylaxis), a life-threatening condition [2, 3]. Atopic patients, with genetic predisposition, activate specific immune reactions to allergens, some of which maintain or even increase their allergenicity even when cooked [2].

Several papers in the literature have shown throughout the last decade an increase in food allergy diagnosis around the globe, mostly in Western countries [4-8]. In the United States, the estimated prevalence of food allergies is 3.9% to 8% in children and 6.6% to 10% in adults [6]. The reasons for this surge are not yet fully understood, although some researches have linked the increase of food allergies with lack of microbial exposure during infancy, resulting in immunological reactions to benign proteins, such as foods [9].

Food allergies negatively impact not only patients' health, but also their families' life qualities [10], as everyday activities like grocery shopping, cooking and dining out can be affected [9]. Therefore, it is absolutely essential to develop novel methods to prevent immunological reactions altogether. Treatments often include oral immunotherapy [11], which is an attempt to nurture the immune system by progressively increasing doses of the allergen orally until reaching a target dose that is then ingested regularly [12, 13]. Nevertheless, this therapy can trigger anaphylatic reactions, hence requiring a medical facility [14].

The amount of allergen taken cannot predict the severity of the symptoms [3, 15, 16], this means that life-threatening symptoms may develop due to even traces of the allergen [17, 18]. One important source of unknown allergenic components are food additives used by the industry in processed products, derived from both vegetable and animal proteins such as soy, egg white, milk, etc [19, 20]. The golden standard for food allergy therapy is to completely avoid the offending protein [21]. However, one may have trouble avoiding unexpected contacts, especially children, to hidden allergens [22]. During processing, such as in mills, cross-contamination may occur resulting in hidden allergens that may not appear in the ingredients list [3, 23]. Consequently, it is possible that one ingests food containing not only tolerated proteins, but also food allergens that can provoke immune reactions. Thus, our aim was to evaluate the kinetics of multiple food allergies induction and progression.

1. Material and Methods

1.1. Ethics Statement

This work was approved by the Institutional Animal Care and Use Committee (official name: Animal Research Ethics Committee - permit number 733/2016) and follows the national animal welfare committee as well as the ARRIVE guidelines [24].

1.2. Animals

C57Bl/6 adult (70 days old – n=10 animals/group) inbred male mice bred at the Animal Facility of the Federal Fluminense University (Niteroi, RJ, Brazil) were given free access to food and acidified water (pH 2.5), which is largely used in animal colonies to avoid bacterial contamination and diseases [25, 26]. They were kept in polypropylene cages with stainless steel covers (temperature of 295.15 ± 2.0 K, $60 \pm 5\%$ humidity and 12h light/12h dark cycle) in a conventional environment (neither under specific pathogens free nor germ-free conditions).

To ensure animals were healthy, they were monitored for one month prior to the experiment. Body weight was assessed weekly and a daily visual inspection of disease symptoms, like labored respiration or *pilar erecti*, was performed [27, 28].

1.3. Induction of the antigen-specific inflammatory gut reaction

Commercial mouse chow (Nuvilab CR1 - NUVILAB-NUVITAL[®], Sao Paulo, Brazil) without egg white or peanut proteins was offered conforming to the animal facility routine. According to the experimental timeline animals received *ad libitum* challenge diet for 60 days either composed of peanut *in natura* (PEANUT-CD) or in-house OVA chow (OVA-CD) according to AIN-93 (report of the American Institute of Nutrition that establishes standards for nutritional studies with experimental rodents [29]). NEG CONT group continued to receive commercial mouse chow as previously described [30-32]. Food intake was measured three times a week and the mean caloric intake was calculated per gram of body weight (gbw) in each group. In order to estimate the mean antigen ingestion per animal, the following equation was used: $y = \frac{(x-z)}{a} \times p$, where “y” = the daily antigen ingestion (mg/animal) defined by the difference between the daily food offered per cage “x” and the daily food collected from the same cage “z”, which is divided by the number of animals in the cage “a” times the percentage of protein in each food “p”.

1.4. Antigenic proteins and Immunization protocols

Animals were immunized subcutaneously twice (21-day interval), with 100µg of the specific protein: peanut protein extract (PPE) [33] or OVA with 1mg of alum adjuvant [Al(OH)₃] in the primary immunization and without alum in the booster.

1.5.Gavages

According to the experimental protocol timeline (Figure 1) animals received 0.5mL of either 20mg/mL OVA solution (Sigma-Aldrich, Darmstadt, Germany) (PRIOR and DURING), OVA + PPE (20mg/mL) (0.5mL each) (AT) or physiological saline (NEG CONT and POS CONT). Therefore, each animal received a total of 10mg of antigen in a single dose. Animals in AT group received 10mg of PPE + 10mg of OVA.

1.6.Bleeding

Animals were bled 200µl from the retroorbital plexus 14 days after each immunization and at the end of both challenge diet periods. The sera were stored at -20°C until analyses and were not pooled.

1.7.Determination of Antibodies levels

In-house serial dilution enzyme-linked immunosorbent assays (ELISA) were performed to detect specific polyisotypic IgG. Briefly, 96-well microplates (low binding, Corning, Acton, USA) were coated with 4µg of antigen (PPE or OVA). A 3-fold serial dilution of each serum (starting at 1/100 v/v) was performed. Goat HRP-anti-mouse IgG (Sigma-Aldrich, Darmstadt, Germany) was used as informed by manufacturer and reactivity was revealed with OPD (Sigma-Aldrich, Darmstadt, Germany) following specifications and read at 492nm on an automated ELISA reader (Anthos 2010 by Biochrom, London, UK). Results were interpreted as ELISA indexes (EI) based on previous description [34]. Briefly, liquid optical density (OD) on optimal sera dilution (1:100 v/v) was obtained by the subtraction of the sample's average OD value and blank wells. Cut-off titers were set based on the mean liquid OD of negative controls with the addition of two standard deviations. EI were acquired by the formula sample liquid OD/cut off. EI values under one were considered negative results. At this cut-off value, the relative sensitivity estimate was 95.4% (95% confidence interval = 90.1% to 99.8%).

1.8.Determination of T and B lymphocyte profile

Mesenteric lymph nodes (MLN) and spleen of each animal were collected, and each animal was individually analyzed. Cells of each animal were separated by mechanical disruption of MLN and spleen separately by gently using the soft tip of a sterile 3mL syringe plunger onto a sterile steel sieve on top of a Petri dish containing 3mL of PBS on ice and centrifuged at 300xg for 10 min at 4°C for each sample as previously described [35]. Then,

any debris were removed, and cell pellets were re-suspended using 3mL PBS on ice. Red blood cells were lysed using hypotonic shock and then removed from the spleen samples. After another centrifugation cycle, cell pellets were re-suspended and normalized at 2.0×10^7 cells/mL using PBS on ice. Non-specific binding sites were blocked using 1% fetal bovine serum (Sigma-Aldrich, Darmstadt, Germany) for 30 minutes. After centrifugation, cells were stained with eBioscience™ Mouse Regulatory T Cell Staining Kit (catalog no. 88-8118-40): FITC anti-mouse CD4 (catalog no. 11-0040 – clone RM4-5), PE anti-mouse CD25 (catalog no. 12-0390 – clone PC61.5) and APC anti-mouse Foxp3 (catalog no. 77-5775-40 – clone FJK-16s). We also used PerCP anti-mouse CD8 (Biolegend® catalog no. 100731 – clone 53-6.7), FITC anti-mouse CD3 (Biolegend® catalog no. 100305 – clone 145-2C11) and PerCP anti-mouse CD45R/B220 (Biolegend® catalog no. 103235 – clone RA3-6B2) according to the manufacturer's (Biolegend®, San Diego, USA / eBioscience™, Waltham, USA) dilution instructions. Briefly, 10µL of each diluted antibody was incubated for 50 min in each sample (normalized for a total of 4.0×10^4 cells/µL), then centrifuged at 700xg for 10 min at 4°C and fixed with 1% buffered formaldehyde. Samples stained with anti-mouse Foxp3 were permeabilized and fixed with buffers from the kit (permeabilization buffer eBioscience™ catalog no. 00-8333-56 and fixation buffer eBioscience™ catalog no. 00-5123-43). After running the samples on BD®-C6 Flow Cytometer (Franklin Lakes, USA), all analyses were performed in the activated lymphocyte gate in FSC (Forward scattering) and SSC (side scattering) plot. We evaluated the following lymphocytes populations (up to four color stainings): T cells - CD4⁺, CD8⁺ and CD4⁺CD25⁺Foxp3⁺ and B cells - B220⁺CD3⁺CD25⁺. Results were normalized to ensure the same cell count per plot (total of 4.0×10^4 cells per plot) and are shown as percentage of cells in specific gate and data. Gating strategies for each analysis are detailed in supplemental figure 1.

1.9.Histomorphometry of Duodenum Samples and Jejunum Swiss Rolls

After OVA-CD, all animals received an overdose of anesthetics (60 mg/kg of Xylazine + 350 mg/kg of Ketamine, produced by Sespo Industries®, Paulinia, Sao Paulo, Brazil), after which a longitudinal section was performed to expose the peritoneum. The abdominal cavity was examined to detect any macroscopic alteration. Intestinal segments were collected from each animal and were immediately fixed with 10% buffered formaldehyde, processed and stained with hematoxylin-eosin (HE). Tissue sections were scanned with Aperio® Scanscope (Leica Microsystems GmbH, Wetzlar, Germany) and then

analyzed with ImageScope software (version 11.2.0.780; Leica Microsystems GmbH, Wetzlar, Germany) to quantify duodenum histological parameters. We analyzed intestinal architecture integrity, number of villi per 8000µm of intestinal tissue, mean villus area and leukocyte infiltrates. Ratios between villus height/width (H/W) and intestinal epithelial cells/intraepithelial leukocytes (IEC/IEL) were established.

We also performed Swiss Roll in the jejunum of each animal to evaluate the integrity of the intestinal layers, global architecture of the villi, crypts and the presence of intraepithelial lymphocytes as previously described [36]. Based on duodenum and jejunum parameters, animals were classified according to the intestinal inflammation index developed by our group [37]. All segments of duodenum and jejunum, as well as flow cytometry samples, were collected, processed and scanned under the same light and temperature conditions to avoid changes in cells and tissues optical properties, such as light absorption and scattering [38].

1.10. Experimental groups and timeline

Figure 1

NEG CONT: subcutaneous sham immunized twice with 0.2 mL saline → saline gavage (n=10/group) sham immunized with 0.2 mL saline. Animals received commercial mouse chow throughout the experimental protocol.

POS CONT: subcutaneous immunized with 100µg of PPE, → saline gavage (n=10/group). After 60 days of PEANUT-CD, animals were subcutaneous immunized twice with 100µg of OVA and later received OVA-CD for 60 days.

Prior Inflammation (referred to as “PRIOR” in figures): subcutaneous immunized with 100µg of PPE, →OVA gavage 6h prior to the introduction of peanut challenge diet (n=10/group). After 60 days of PEANUT-CD, animals were subcutaneous immunized twice with 100µg of OVA and later received OVA-CD for 60 days.

At Inflammation (referred to as “AT” in figures): subcutaneous immunized with 100µg of PPE, → OVA and PPE gavage concomitant to the introduction of peanut challenge diet (n=10/group). After 60 days of PEANUT-CD, animals were subcutaneous immunized twice with 100µg of OVA and later received OVA-CD for 60 days.

During Inflammation (referred to as “DURING” in figures): subcutaneous immunized with 100µg of PPE, → OVA gavage 6h after peanut challenge diet introduction (n=10/group).

After 60 days of PEANUT-CD, animals were subcutaneous immunized twice with 100µg of OVA and later received OVA-CD for 60 days.

1.11. Statistical analysis

Kolmogorov-Smirnov test was performed for normal distribution. Student T test when comparing two variables and one-way ANOVA with Bonferroni post-test for more than two variables were used to determine the significant difference. Tests were performed using Graphpad Prism 6 Software (Graphpad Software, Inc., La Jolla, California, United States). $P < 0.05$ was considered statistically significant. Results are displayed as mean±standard deviation (SD) and show data from a minimum of 10 animals for each assay. Exact P values are expressed in the figures.

2. Results

2.1. Food Intake In Kcal does not change Regardless of food type

No significant differences were observed in kcal/gbw food intake during Peanut Allergy Induction (PRIOR: 18.46 ± 2.94 ; AT: 18.17 ± 2.98 ; DURING: 19.01 ± 3.66 POS CONT: 19.50 ± 3.34 NEG CONT: 21.89 ± 3.98 Kcal/gbw). There were also no significant differences during PEANUT-CD (PRIOR: 17.59 ± 4.93 ; AT: 14.29 ± 2.10 ; DURING: 17.45 ± 3.79 ; POS CONT: 18.75 ± 4.48 ; NEG CONT: 19.87 ± 1.14 Kcal/gbw). However, during OVA-CD NEG CONT (33.44 ± 1.82 Kcal/gbw) and PRIOR (25.92 ± 1.31 Kcal/gbw) had a significant higher consumption ($p = 0.0001$) when compared to AT (14.29 ± 1.92 Kcal/gbw), DURING (15.95 ± 3.41 Kcal/gbw) and POS CONT (17.09 ± 1.68 Kcal/gbw) (Figure 2). Since animals received food *ad libitum*, each animal ingested an estimate of 250-300mg/day of antigen (protein fraction within the food).

Figure 2

2.2. High Anti-peanuts polyisotypic IgG titers correlate to allergy induction and Anti OVA polyisotypic IgG titers are associated with intestinal milieu in first contact

All groups inoculated with PPE had significantly higher anti-peanut total IgG antibody titers ($p = 0.009$) (PRIOR: 2.18 ± 0.37 ; AT: 2.20 ± 0.42 ; DURING 2.11 ± 0.15) and POS CONT: 2.47 ± 0.45) when compared to NEG CONTROL (0.63 ± 0.06). AT and DURING maintained significant higher IgG titers ($p = 0.03$) even after weeks of PPE inoculation and PEANUT-CD when compared to PRIOR and POS CONT (Figure 3A).

Analyzing anti-OVA polyisotypic IgG antibody titers, we observed significant differences in primary inoculation ($p = 0.001$). Means of EXP (PRIOR: 1.28 ± 0.51 ; AT:

2.02±0.45; DURING: 1.70±0.64) and POS CONT (1.42±0.26) are significantly higher than NEG CONT (0.32±0.11). After OVA booster inoculation, NEG CONT (0.37±0.13) had significantly lower titers ($p=0.001$) than all other groups; PRIOR (1.21±0.32) had significantly lower titers ($p=0.001$) than AT (2.51±0.33), DURING (2.23±0.19) and POS CONT (1.73±0.19). After OVA-CD, PRIOR (1.22±0.25) showed significantly lower titers ($p=0.01$) than AT (2.21±0.40), DURING (2.21±0.43) and POS CONT (1.96±0.36). NEG CONT (0.32±0.17) continued presenting significantly lower titers ($p=0.01$) than all other groups (Figure 3B).

Figure 3

2.3.Variation of effector and regulatory immune cells in MLN and Spleen

Representative plots of each analysis performed can be found on supplemental figure 2.

After gating the lymphocyte region in MLN samples, we observed a significantly higher amount of CD4⁺ T cells compared to CD8⁺ T cells in PRIOR and NEG CONT due to a significant increase ($p=0.0202$ and $p=0.0111$, respectively) in CD8⁺ T cells observed in AT (0.36±0.01), DURING (0.34±0.04) and POS CONT (0.33±0.01) compared to the PRIOR (0.21±0.01) and NEG CONT (0.19±0.01) with no significant differences in CD4⁺ T lymphocytes population among the groups (NEG CONT: 0.38±0.03; PRIOR: 0.36±0.04; AT: 0.40±0.05; DURING: 0.39±0.01; POS CONT: 0.41±0.05). (Figure 4A).

In spleen samples, NEG CONT (0.14±0.01) and PRIOR (0.18±0.02) showed significantly lower amounts of CD4⁺ T cells ($p=0.018$ and $p=0.034$, respectively) than AT (0.27±0.04), DURING (0.25±0.04) and POS CONT (0.30±0.05). Evaluating CD8⁺ T lymphocytes, AT maintained significantly higher titers ($p=0.039$) as also observed in MLN (0.13±0.04) when compared to NEG CONT (0.05±0.02) and was not statistically different from PRIOR (0.10±0.02), DURING (0.09±0.04) and POS CONT (0.15±0.04) (Figure 5A).

The percentage of CD4⁺CD25⁺Foxp3⁺ T cells in MLN was significantly higher ($p=0.0034$) in PRIOR (0.14±0.02) when compared to all other groups: AT (0.12±0.02), DURING (0.12±0.02), POS CONT (0.11±0.02) and NEG CONT (0.04±0.01) (Figure 4B). The same was observed in the spleen, where CD4⁺CD25⁺Foxp3⁺ T cells were significantly higher ($p=0.021$) in PRIOR (0.12±0.01) when compared to all other groups: AT (0.07±0.02), DURING (0.08±0.02) and POS CONT (0.09±0.02) and NEG CONT (0.02±0.01) (Figure 5B).

B220⁺CD3⁻CD25⁺ B lymphocytes percentage showed that NEG CONT (0.01±0.001) and PRIOR (0.03±0.002) had a significantly smaller population ($p=0.0005$ and $p=0.0020$, respectively) compared to DURING (0.05±0.002) and POS CONT (0.07±0.001). No significant differences between PRIOR and AT (0.05±0.001) were observed (Figure 4C). The same was observed in spleen samples, where B220⁺CD3⁻CD25⁺ B cells were significantly reduced ($p=0.040$) in NEG CONT (0.01±0.002) and PRIOR (0.02±0.002) compared to DURING (0.03±0.002) and POS CONT (0.04±0.002). No significant differences between PRIOR and AT (0.02±0.003) were observed (Figure 5C).

Figure 4

Figure 5

2.4. Intestinal parameters worsen when both antigens are given concomitantly.

POS CONT (14.60±2.14) had a significantly lower mean villus number per 8000µm ($p=0.0001$) when compared to PRIOR (18.80±3.11) and NEG CONT (23.00±2.73). AT (17.80±3.42) and DURING (17.29±2.14) showed a significant ($p=0.0001$) lower villus number when compared to NEG CONT (Figure 5A).

Regarding villus area, PRIOR ($2.2 \times 10^4 \pm 3.6 \times 10^3 \mu\text{m}^2$) and NEG CONT ($2.4 \times 10^4 \pm 3.9 \times 10^3 \mu\text{m}^2$) had a significantly larger mean villus area ($p=0.01$) when compared to AT ($1.8 \times 10^4 \pm 2.1 \times 10^3 \mu\text{m}^2$), DURING ($1.7 \times 10^4 \pm 1.7 \times 10^3 \mu\text{m}^2$) and POS CONT ($1.7 \times 10^4 \pm 1.8 \times 10^3 \mu\text{m}^2$) (Figure 5B).

When H/W ratio was verified, it showed that NEG CONT (4.71±0.74) and PRIOR (4.11±0.53) had a significantly higher ratio when compared to AT (3.54±0.40), DURING (3.57±0.54) and POS CONT (3.12±0.45) ($p=0.0036$), which were not statistically different from each other (Figure 5C).

NEG CONT (54.60±5.32) and PRIOR (42.08±7.22) IEC/IEL ratios were significantly higher ($p=0.0001$) when compared to AT (30.91±6.78), DURING (23.66±3.87) and POS CONT (20.64±3.16) (Figure 5D).

2.5. Jejunum samples presented less inflammatory parameters in all groups

No extensive morphological changes were observed among the groups. However, POS CONT and AT and DURING presented more edema in the mucosal layer than NEG CONT and PRIOR. Reduced mean jejunum IEC/IEL ratio of the animals that presented an inflamed duodenum (AT [17.72±5.30], DURING [18.30±3.88] and POS CONT [18.63±2.61]) compared to the NEG CONT (25.67±4.11) and PRIOR (23.33±2.60) was due

to a significantly larger ($p=0.04$) infiltration of intraepithelial leukocytes in the jejunum (data not shown) (Figure 5E).

Figure 6

2.6. Classification of Intestinal Inflammatory State and Summary of Intestine Analysis

AT and DURING were classified as hybrids of Infiltrative and Partial Destruction stages. PRIOR was classified in the Infiltrative stage, while POS CONT was classified in the Partial Destruction stage. Finally, NEG CONT was classified as Normal (Table 1).

The alterations in duodenum and jejunum in each group (described in items 3.4 and 3.5 above) are shown in the representative digital slides. All slides are shown in the same zoom, so it can be observed that villi in AT, DURING and POS CONT are much smaller than those in NEG CONT. Villi in PRIOR resembled those of NEG CONT (Figures 6 and 7).

Table 1

Figure 7

Figure 8

3. Discussion

In this work we further evaluated the timing of unfamiliar food protein introduction during the induction of a chronic antigen specific intestinal inflammation, therefore C57Bl/6 mice were chosen because of their predominantly Th1 driven response [39]. Retrospective studies have identified several participants as intolerant to a wide variety of proteins commonly present in food [40], although it is not known whether these foods are in fact more allergenic or simply the most widely consumed foods or whether the first intake of the food induced sensitization as a result of gut milieu [41, 42]. An investigation in the literature showed that the distribution of food allergies varies according to populations and their cultural eating habits. For example, the top three food allergies in Brazil, although understudied, are wheat, egg, and milk [43], while in Israel egg, milk, sesame and in North America shellfish, milk and peanut [44]. In clinical trials involving patients with allergic diseases, though changes in serological tests were observed, a total improvement of their clinical conditions is not always perceived [45].

Food intake showed the animals in all groups consumed equivalent amounts of commercial chow and peanuts in allergy induction and PEANUT-CD periods, respectively, supporting the results found by other studies in our group [33, 46, 47]. The exclusive diet

supply prevents animals from avoiding an allergenic food, as has already been shown for animals that have free choice between allergenic food and other food sources [30-32].

Body weight evaluation demonstrated that during allergy induction all groups presented weight gain as expected for normal development, with variations consistent with animal growth and manipulation procedures to which they were submitted. Animals which only received PPE injections, commercial chow to which they were weaned and had no contact with allergens in the diet, had no changes in their intestinal mucosa. These results agree with those shown by Paschoal [46] and by Tordesillas and Berin [48].

A significant number of studies have shown that food allergies may develop with low expression or even absence of IgE antibodies [49-52], thus leading to the current consensus in the literature that not all food allergies are caused by IgE antibodies [53, 54]. Morita et al showed that there are three types of food allergies, from the immune response standpoint: “IgE-mediated,” “combined IgE- and cell-mediated” and “non-IgE-mediated [55]. In our model, using C57BL/6 mice, no OVA-specific IgE was found after OVA gavage during a gut inflammation process (data [not shown] from previous [47] and other ongoing experiments), thus our model correlates to Non-IgE-mediated gastrointestinal food-induced allergic disorders [56, 57].

In our study, all animals were submitted to a peanut allergy induction protocol, except NEG CONT. Thus, all groups showed high anti-peanut polyisotypic IgG antibody titers, except NEG CONT. Our group has shown an increase in anti-OVA antibodies in animals that received OVA while experiencing gut inflammation because of eating an allergen-containing diet [47]. This present work agrees with our previous paper; however, we here show that animals that received OVA concomitant to the initiation of the challenge diet (AT) presented the highest overall anti-OVA IgG titers. Animals that received OVA six hours after the initiation of the challenge diet presented the same sensitization profile and both with higher antibody titers than the subcutaneous immunization induces. On the other hand, oral introduction of food only six hours before the initiation of PEANUT-CD is sufficient to induce oral tolerance, as seen by the lower IgG titers compared to the others sensitized groups. The antibody titers are higher than those of NEG CONT, but previous studies from our group and in the literature showed that a small elevation in IgG titers is an indication of oral tolerance due to an increase of tolerogenic antibodies production, such as IgG4 [58-60].

In a scenario where the interaction with an unacquainted food protein is concomitant or shortly after the introduction of a protein to which one is already allergic may lead to bystander activation and not induce tolerance, even with a local low inflammatory cytokines

profile [61, 62]. Consequently, multiple allergies may be developed [63, 64]. However, sensitization was blocked by feeding the novel food protein six hours prior to the initiation of the challenge diet. Therefore, we agree with Korte and collaborators, in which foods that share similar matrixes can cause cross responses, exacerbating immunological reactions (tolerance or sensitization) [65]. Though, it is not the case of this work, since the first sensitization was to a vegetable protein and the second to an animal protein that do not share a similar food matrix.

To better understand the characteristics of the immune response, both local and systemic, phenotypic analysis of cells in MLN and spleen were performed. We evaluated populations of effector and regulatory T cells and effector B cells, which were chosen once they are hallmarks of effector T cells ($CD4^+$ and $CD8^+$) and regulatory T cells ($CD4^+CD25^+Foxp3^+$), two major populations present in allergic reactions and tolerance induction [66, 67]. The B cell population $B220^+CD3^-CD25^+$ was chosen due to its role as antigen presenting cell and increased number in food allergy immune responses [68, 69].

We noticed a significant increase in the percentage of $CD8^+$ T lymphocytes in AT in both MLN and spleen. A possible reason for this increase compared to the other sensitized groups is the activation of both $CD4^+$ and $CD8^+$ T cells by dietary antigens in the gut, which correlates to the reduced IEC/IEL ratio in this group due to increased IEL. Since this group received two antigens concomitantly, this could increase IEL recruitment. This phenomenon has already been observed in celiac patients and in peanut-allergic individuals [70, 71].

According to Dahlman-Hoglund and others, $IFN-\gamma$ secreted by $CD8^+$ T lymphocytes acts as a suppressor of antigen-specific $CD4^+$ Th2 cells in mouse models using OVA. $CD8^+$ T lymphocytes also secrete $TGF-\beta$ and IL-10, which are non-antigen-specific immune response suppressor molecules, and that suppression can be induced in naïve animals through the transfer of $CD8^+$ T lymphocytes [72, 73]. This can explain the increased $CD8^+$ T cells titers of PRIOR compared to NEG CONT.

Analyzing the percentage of $CD4^+CD25^+Foxp3^+$ T lymphocytes in both MLN and spleen, our results showed an increase in PRIOR, which may be associated with a tolerogenic bystander suppression performed by these cells [74-76]. Several studies have shown that increased $Foxp3^+$ cells may indicate tolerance induction for different allergenic proteins [77-79]. $B220^+CD3^-CD25^+$ B cells titers were also assessed in our study. NEG CONT, PRIOR and AT presented lower titers in MLN and spleen samples than DURING and POS CONT. This can be explained by the highly effector activity of $B220^+CD3^-CD25^+$ B cell

subpopulation [80, 81], which are activated mainly by T cells [82] and act as antigen presenting cells [68, 69].

Villus number quantification aimed to demonstrate the health or fragility/destruction of the intestinal mucosa. Our results agree to previous studies, which showed that damage to global architecture and epithelial barrier, consequent to the loss of cell-cell junctional integrity, is a typical reaction caused by inflammatory processes [83, 84].

To ascertain, from the architectural point of view, that not only villus number is altered we continued our analysis by assessing villus area and H/W ratio. For both parameters, the group that received OVA six hours before PEANUT-CD (PRIOR) compares to NEG CONT but not to EXP 2, DURING and POS CONT. These findings can be explained by the changes observed in the height and width of each villus.

A fine balance between the numerous signaling pathways and transcription factors lead to proliferation and differentiation of the intestinal stem cells to epithelial cell types. As cells mature, they migrate to the villus tip where they are shed after apoptosis is triggered [85]. These are crucial for the adequate physiology and mucosal barrier that reinforces intestinal epithelial integrity and architectural maintenance [86]. Absorptive enterocytes, secretory goblet, enterochromaffin and Paneth cells all derive from the intestinal stem cells and play a central role in the different functions of the gastrointestinal tract such as nutrient absorption, avoiding the entry of both pathogenic organisms and large antigenic molecules, innate inflammatory signaling, secretion of molecules that contribute to the mucosal barrier maintenance, antigen presentation to underlying immune cells, and production of endocrine signaling molecules [87]. Inflammatory cytokines disrupt this balance and induce the flattening of the villi as observed in the EXP 2, EXP 3 and POS CONT groups. As the villi become wider and shorter the absorption diminishes as the number of villi per area also diminishes per mm². Animals in these three groups reduced food intake and presented weight loss and lower activity in the cage (data not shown), which are all symptoms of intestinal inflammation, however they did not present symptoms of anaphylaxis, such as diarrhea and labored respiration [78, 88]. Villus area is a parameter still poorly described in the literature. Studies from our group have already proposed that this is an important aspect to be assessed since the identification of the occurrence of histological adaptation may indicate a change in the absorption surface of the small intestine [36, 37, 89].

Serological and histological data correlate well in our experimental model. PRIOR that received OVA six hours prior to PEANUT-CD showed low antibody titers and normal villus morphology permitting us to conclude that there was an induction of tolerance to OVA

(the unfamiliar food protein). AT and DURING received the novel food protein, at or after the onset of the peanut challenge diet respectively and were challenged with both a subcutaneous injection of OVA and OVA-CD presented both high antibody titers and morphological alterations of the gut. These results are in line with those found by other studies carried out in our research group[33, 46]. The same was observed in the villus IEC/IEL ratio, where AT, DURING and POS CONT showed significant lower ratios than NEG CONT and PRIOR. This IEC/IEL ratio reduction occurs due to increased proliferation of leucocytes in the lamina propria and massive infiltration in epithelium as already observed [90-92].

Jejunum swiss rolls showed increased leucocyte infiltration in AT, DURING and POS CONT when compared to NEG CONT, i.e., one has a greater chance to present altered jejunum mucosa when the duodenum is also altered. This behavior was already verified by us [36] and by other authors in the literature [93].

In this study, although it was possible to better understand the timing between the introduction of an allergenic food together with an unfamiliar food protein to avoid sensitization (allergy), other studies still need to be performed to comprehend the role of the various immune cell types as well as cytokines in this process. Here we confirm again that feeding an unacquainted food protein in a physiological context of the gut mucosa induces tolerance to this protein even if shortly afterwards an inflammatory insult occurs. However, the introduction of a food protein concomitant or after the initiation of an inflammatory process tends to induce food allergy. In conclusion, multiple food allergies may be the consequence of food proteins introduction during a gut inflammation due to a prior to a food-induced allergic reaction, as frequently occurs in pediatric scenarios.

Table 1 – Overview of duodenum and Jejunum parameters and classification of inflammatory state.

Parameters Groups	Villus Number	Villus Area in μm^2	H/W	IEC/IEL	Jejunum IEC/IEL	Classification
NEG CONT	23.00 $\pm$ 2.73	2.4 $\times 10^4 \pm 3.9 \times 10^3$	4.71 $\pm$ 0.74	54.60 $\pm$ 5.32	25.67 $\pm$ 4.11	Normal
PRIOR	18.80 $\pm$ 3.11	2.2 $\times 10^4 \pm 3.6 \times 10^3$	4.11 $\pm$ 0.53	42.08 $\pm$ 7.22	23.33 $\pm$ 2.60	Infiltrative
AT	17.80 $\pm$ 3.42*	1.8 $\times 10^4 \pm 2.1 \times 10^3$ *	3.54 $\pm$ 0.40*	30.91 $\pm$ 6.78*	17.72 $\pm$ 5.30*	Infiltrative/ Partial Destruction
DURING	17.29 $\pm$ 2.14*	1.7 $\times 10^4 \pm 1.7 \times 10^3$ *	3.57 $\pm$ 0.54*	23.66 $\pm$ 3.87*	18.30 $\pm$ 3.88*	
POS CONT	14.60 $\pm$ 2.14*	1.7 $\times 10^4 \pm 1.8 \times 10^3$ *	3.12 $\pm$ 0.45*	20.64 $\pm$ 3.16*	18.63 $\pm$ 2.61*	Partial Destruction

H/W: height/width ratio; IEC/IEL: intestinal epithelial cells/intraepithelial leukocytes ratio. * indicates statistically different from NEG CONT.

Conflict of Interest

The authors declare no conflict of interest that could bias the findings of this study.

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Figure Legends

Figure 1 – Timeline of experimental protocol. PPE: Peanut Protein Extract; OVA: Ovalbumin; PEANUT-CD: Peanut challenge diet; OVA-CD: OVA challenge diet; s.c.: subcutaneous injection.

Figure 2 - Mean caloric intake/gbw during OVA challenge diet $\pm$ SD per group. NEG CONT and PRIOR showed a significant higher consumption than all other groups. Each dot represents data of one animal.

Figure 3 – Mean group polyisotypic IgG. Results were expressed as ELISA index (EI), where values of EI >1.0 (horizontal dashed line) were considered positive. No pooled sera were used, each individual mouse was analyzed. **A:** Mean group anti-peanut polyisotypic IgG titers $\pm$ SD per group. All groups immunized with PPE showed significant higher titers when compared to NEG CONT, which was immunized with saline. POS CONT and PRIOR presented lower IgG titers at the end of the experiment, while AT and DURING maintained high IgG titers throughout the protocol; **B:** Mean Anti-OVA polyisotypic IgG titers $\pm$ SD per group after: primary inoculation with OVA: NEG CONT showed significant lower titers than AT, DURING and POS CONT; post OVA booster: NEG CONT and PRIOR showed significant lower titers than AT, DURING and POS CONT; and post OVA-CD: NEG CONT and PRIOR showed significant lower titers than AT, DURING and POS CONT.

Figure 4 – **A:** Mean percentage of CD4⁺ T lymphocytes and CD8⁺ T lymphocytes in MLN in gate P3 $\pm$ SD per group. No significant differences were observed in CD4⁺ cells, but NEG CONT and PRIOR showed a significant lower percentage of CD8⁺ cells than the other groups, while AT showed the highest titers; **B:** Mean group percentage of CD4⁺CD25⁺Foxp3⁺ T lymphocytes in MLN in gate P3 $\pm$ SD. PRIOR showed significantly higher percentage when compared to AT, DURING, POS CONT and NEG CONT; **C:** Mean group percentage of effector B220⁺CD3⁺CD25⁺ cells $\pm$ SD in MLN. NEG CONT and PRIOR had a significantly smaller population compared to DURING and POS CONT. We did not observe significant differences between groups PRIOR and AT. Each dot represents data of one animal.

Figure 5 - **A:** Mean percentage of CD4⁺ T lymphocytes and CD8⁺ T lymphocytes in the spleen in gate P3 $\pm$ SD per group. NEG CONT and PRIOR showed significantly lower amounts of CD4⁺ T cells. AT the highest titers; **B:** Mean group percentage of CD4⁺CD25⁺Foxp3⁺ T lymphocytes in the spleen in gate P3 $\pm$ SD. PRIOR showed significantly higher percentages when compared to the other groups; **C:** Mean group percentage of effector B220⁺CD3⁺CD25⁺ cells $\pm$ SD in the spleen. NEG CONT and PRIOR had a significantly smaller population compared to DURING and POS CONT. We did not observe significant differences between groups PRIOR and AT. Each dot represents data of one animal.

Figure 6 – **A:** Mean group villus number per 8000 μ m in the duodenum $\pm$ SD. PRIOR showed a significant higher villi number than AT, DURING and POS CONT; **B:** Mean villus area $\pm$ SD per group. NEG CONT and

PRIOR showed a significant higher area in each villus than the other groups; **C**: Mean group villus H/W ratio + SD. NEG CONT and PRIOR showed a significant higher H/W ratio than the other groups; **D**: Mean group villus IEC/IEL ratio + SD per group. NEG CONT and PRIOR showed a significant higher ratio than the other groups; **E**: Jejunal Mean villus IEC/IEL ratio + SD per group. NEG CONT showed a significant higher ratio than the other groups, except PRIOR. Each dot represents data of one animal.

Figure 7 – Digital slide representative of each group showing general morphology of duodenum villi.

Figure 8 – Digital slide representative of each group showing general morphology of jejunum villi. The arrows show inflammatory cells in the epithelial layer, which can be an inflammatory marker.

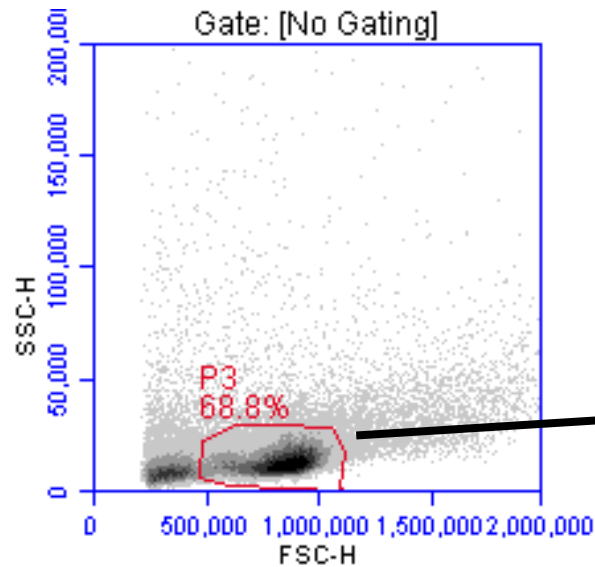
Supplemental Figure 1 – Gating strategy for the identification of T cells and B cells subsets from MLN and spleen.

Supplemental Figure 2 – Representative plots of each flow cytometry analysis in MLN and spleen.

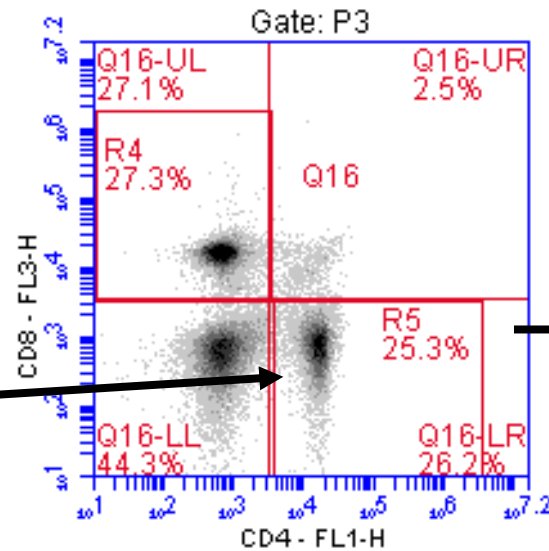
Gating Strategy CD4⁺ / CD8⁺ / CD4⁺CD25⁺Foxp3⁺ in MLN

SSCxFSC

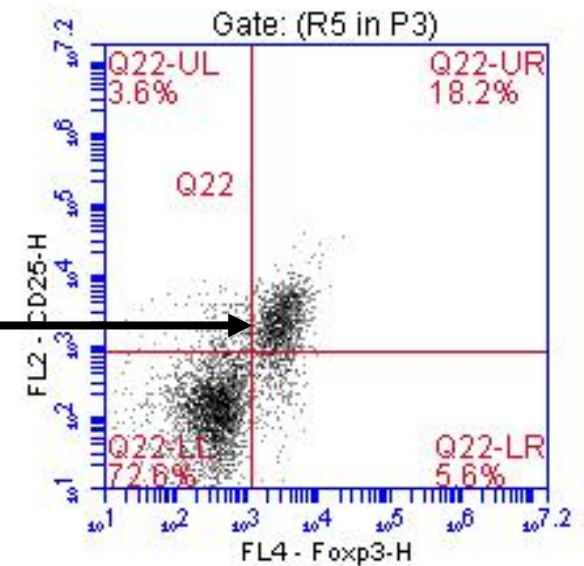
Lymphocyte gate
(P3)



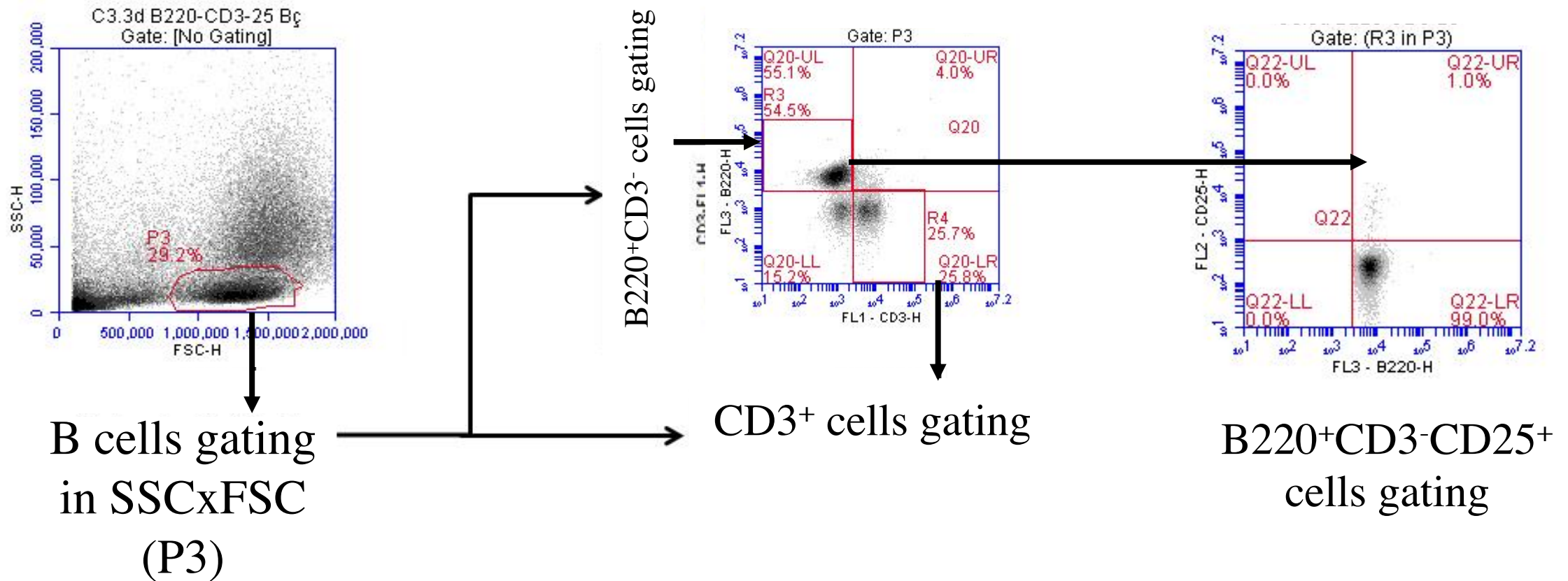
CD4⁺ and CD8⁺ cells



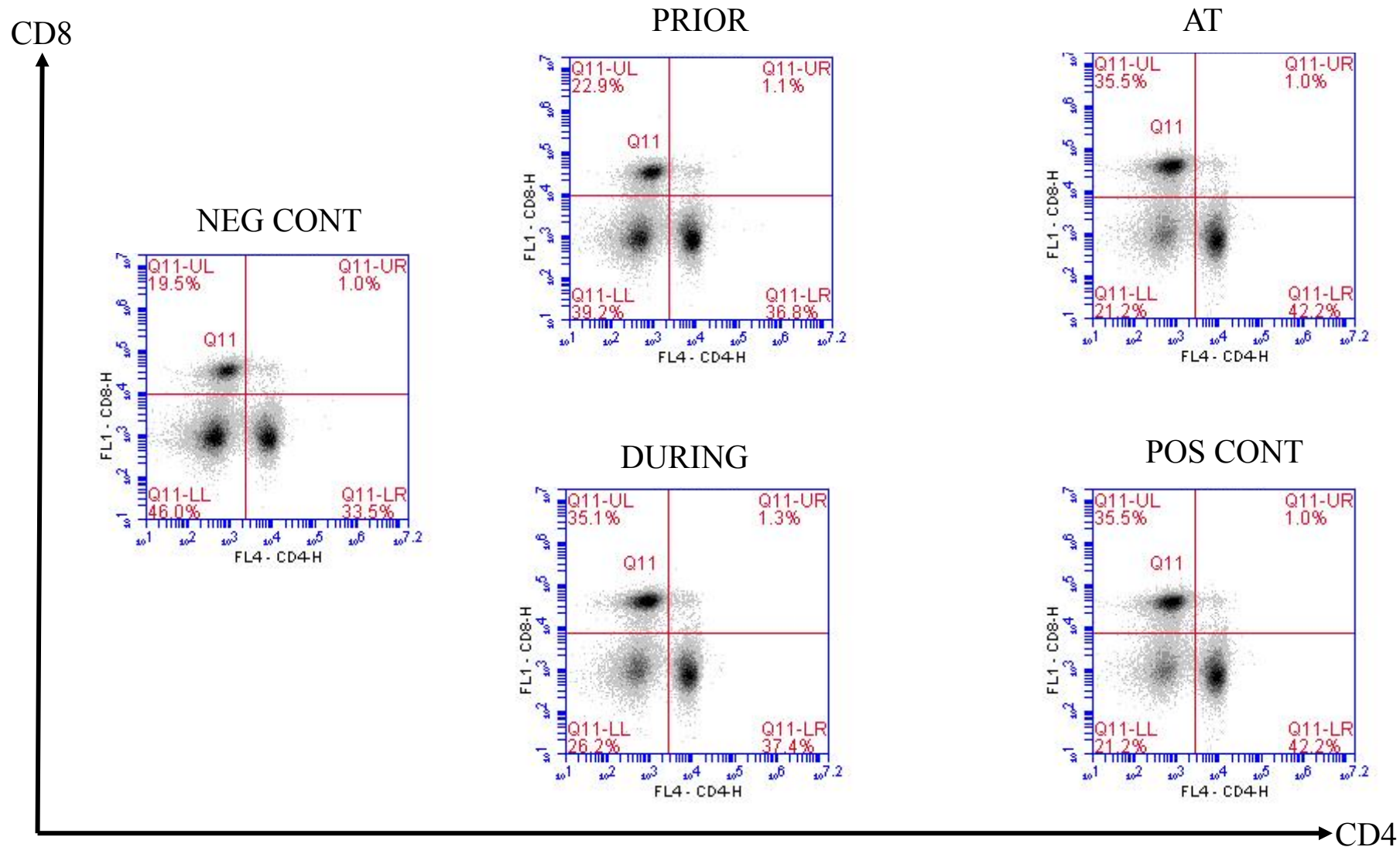
CD4⁺CD25⁺Foxp3⁺ cells



Gating Strategy B220⁺CD3⁻CD25⁺ Cells in spleen



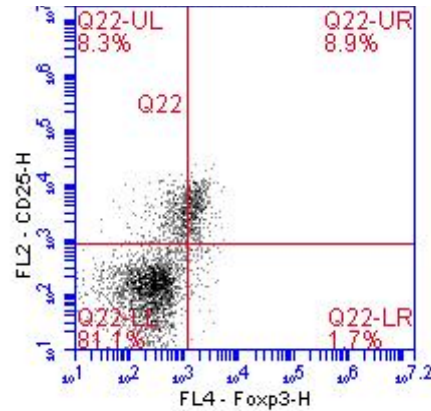
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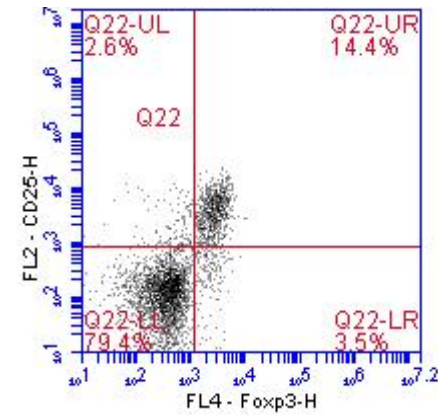
CD4⁺CD25⁺Foxp3⁺ T cells in MLN

CD4-CD25

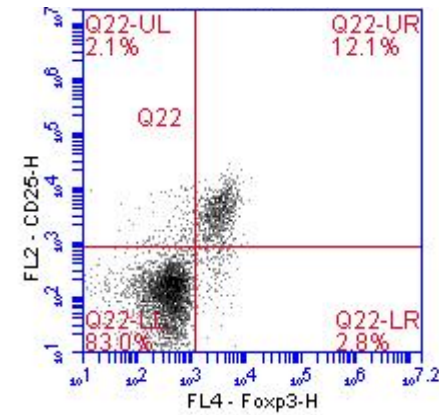
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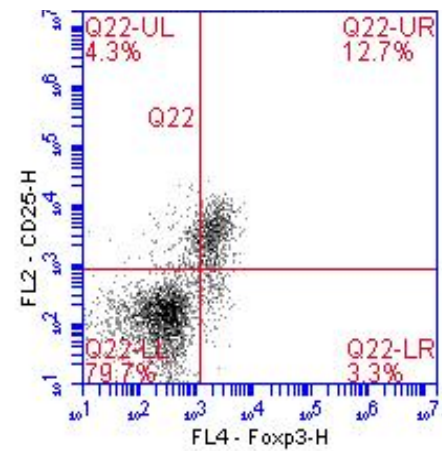
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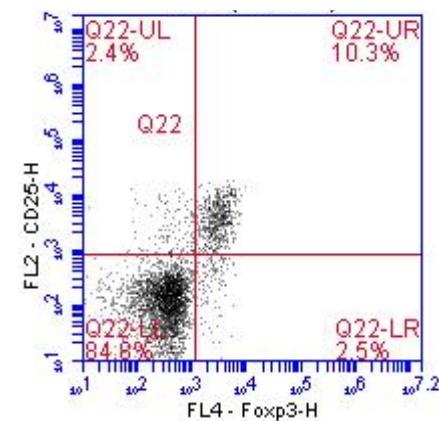
AT



DURING



POS CONT

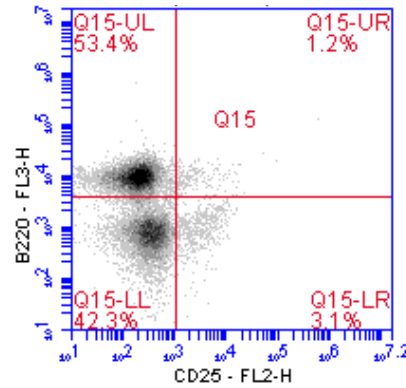


Foxp3

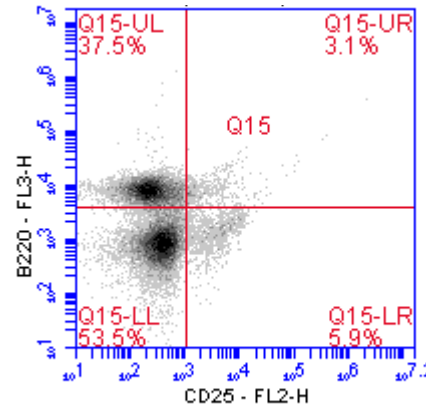
B220⁺CD3⁻CD25⁺ B cells in MLN

B220

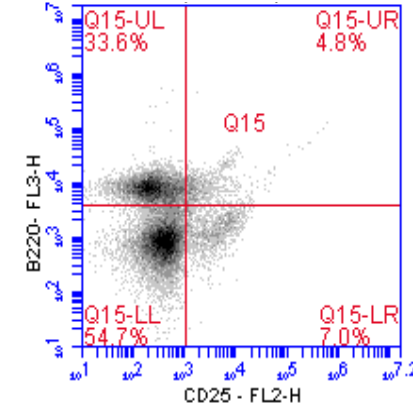
NEG CONT



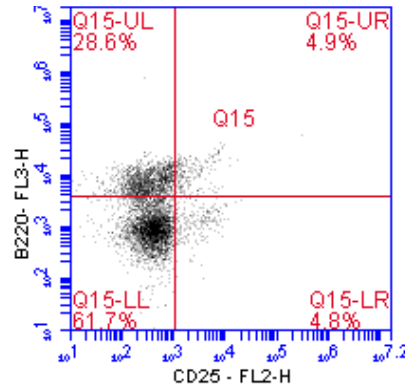
PRIOR



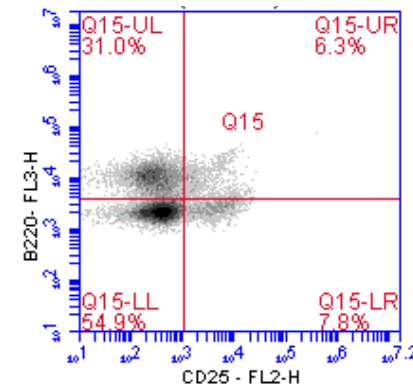
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DURING

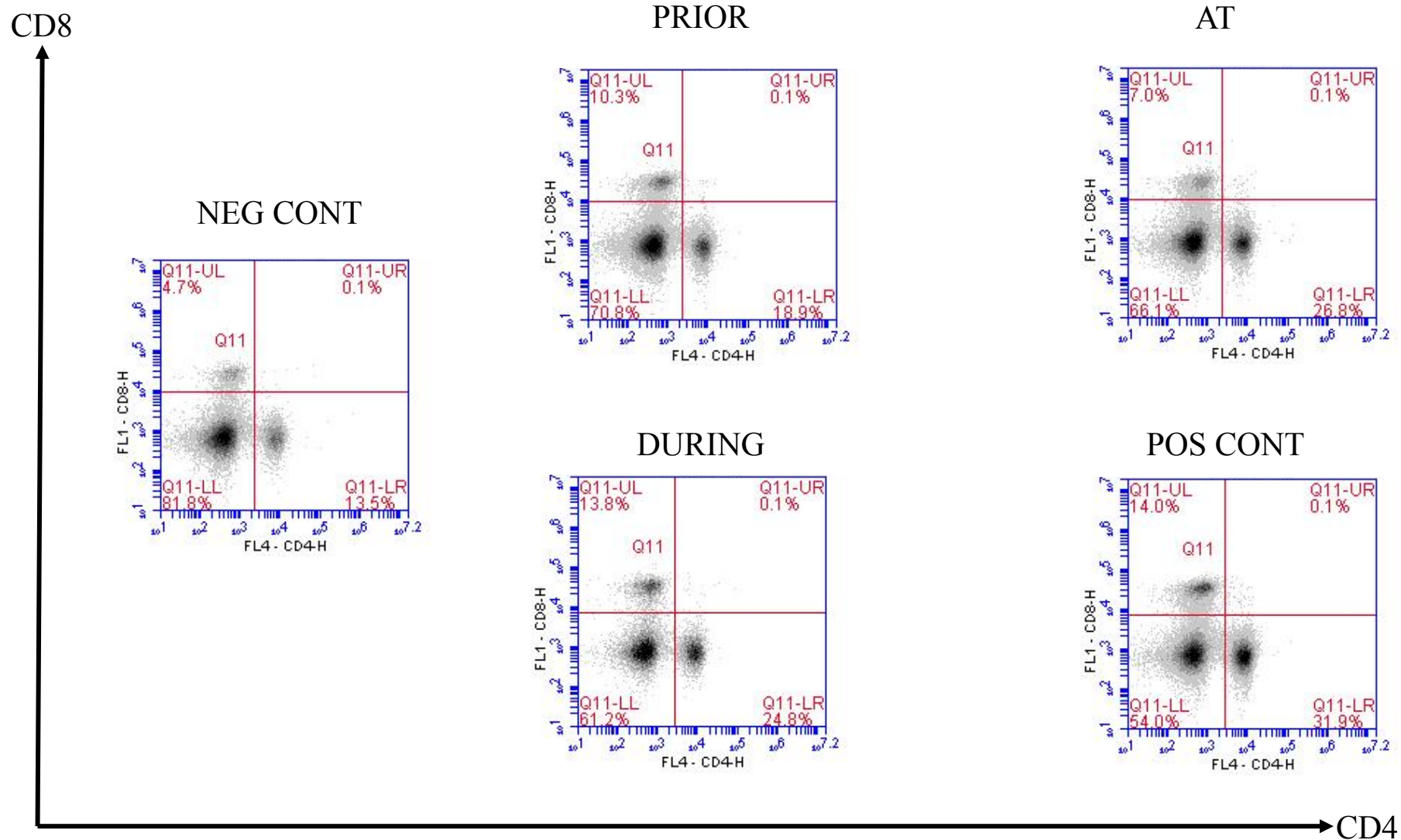


POS CONT



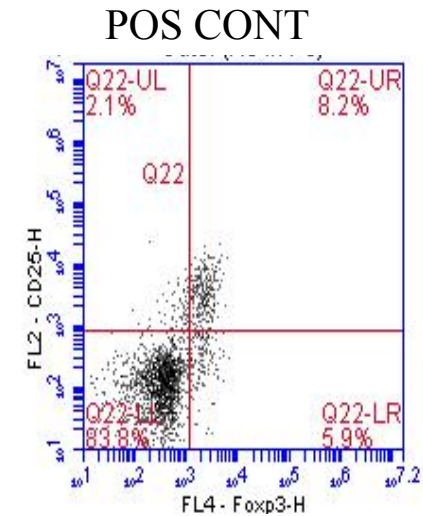
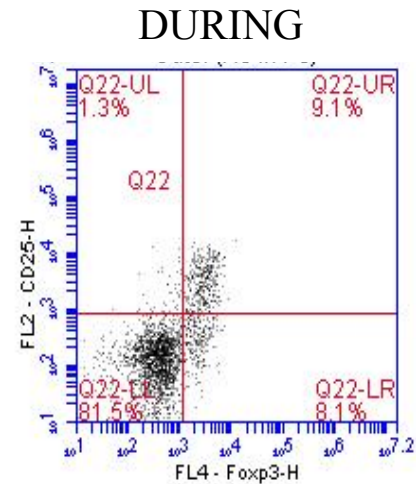
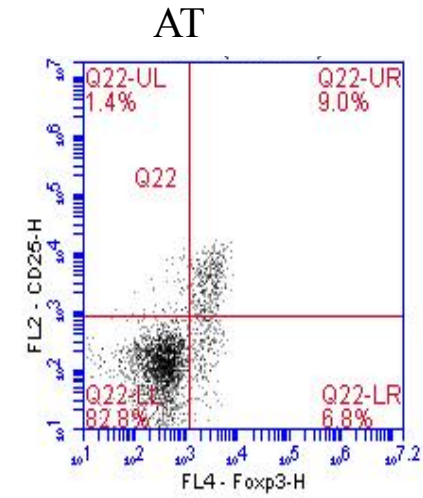
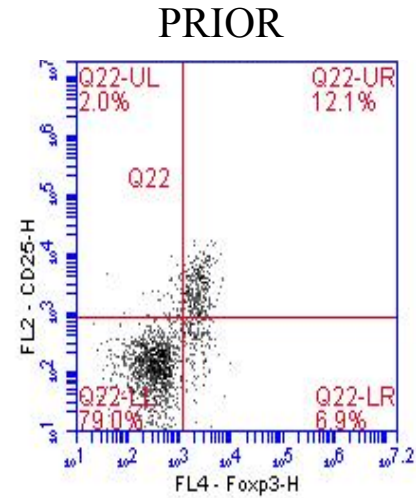
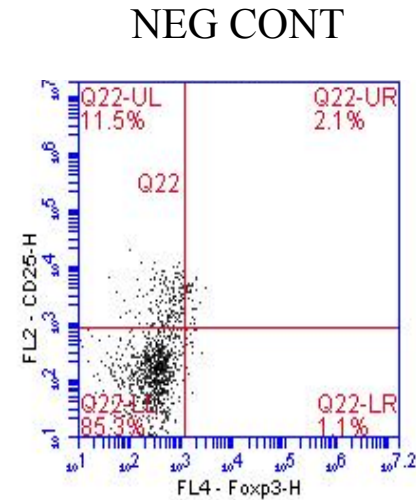
CD25

CD4⁺ T cells and CD8⁺ T cells in Spleen



CD4⁺CD25⁺Foxp3⁺ T cells in Spleen

CD4-CD25



Foxp3

B220⁺CD3⁻CD25⁺ B cells in Spleen

