Immunology Letters 260 (2023) 73–80

Contents lists available at ScienceDirect

Immunology Letters

journal homepage: www.elsevier.com/locate/immlet

A new experimental model to study shrimp allergy

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ARTICLE INFO

Keywords:
Food allergy
Shrimp allergy
IgE
Eosinophils
Intestinal mucosa

ABSTRACT

Shrimp is among the most sensitizing food allergens and has been associated with many anaphylaxis reactions. However, there is still a shortage of studies that enable a systematic understanding of this disease and the investigation of new therapeutic approaches. This study aimed to develop a new experimental model of shrimp allergy that could enable the evaluation of new prophylactic treatments. BALB/c mice were subcutaneously sensitized with 100 μg of shrimp proteins of *Litopenaeus vannamei* adsorbed in 1 mg of aluminum hydroxide on day 0, and a booster (100 μg of shrimp proteins only) on day 14. The oral challenge protocol was based on the addition of 5 mg/ml of shrimp proteins to water from day 21 to day 35. Analysis of shrimp extract content detected at least 4 of the major allergens reported to *L. vannamei*. In response to the sensitization, allergic mice showed significantly enhanced IL-4 and IL-10 production in restimulated cervical draining lymph node cells. High detection of serum anti-shrimp IgE and IgG1 suggested the development of allergies to shrimp while Passive Cutaneous Anaphylaxis assay revealed an IgE-mediated response. Immunoblotting analysis revealed that Allergic mice developed antibodies to multiple antigens present in the shrimp extract. These observations were supported by the detection of anti-shrimp IgA production in intestinal lavage samples and morphometric intestinal mucosal changes. Therefore, this experimental protocol can be a tool to evaluate prophylactic and therapeutic approaches.

1. Introduction

Yearly, 130–190 gs of dietary proteins are absorbed by the gut mucosa. Due to natural immunological hyperresponsiveness, and so-called oral tolerance, most individuals show no triggering of immune reactions [1]. Despite this, a failure in the maintenance of oral tolerance may lead to adverse immune responses toward dietary proteins.

IgE-mediated immunological mechanisms drive hypersensitivity reactions. Initially, Antigen Presenting Cells (APCs) present captured food allergens to T CD4+ Th0 cells which in turn trigger the release of IL-4, IL-5, and IL-13. Therefore, IgE and IgG1 titers are augmented in response to these cytokines, and mast cells and basophils become activated by the binding of IgE to FCƐRI receptors onto their membrane surface. High rates of proinflammatory mediators are released as a result of subsequent exposures, which induce a range of clinical manifestations initiated by vasodilation and higher vascular permeability [2–4]. Erythema, urticaria, itching, swelling of the pharynx, rhinorrhea, nasal congestion, diarrhea, and tachycardia are examples of localized reactions [5]. Still, severe cases of sensitization may result in anaphylaxis, a severe and potentially life-threatening reaction.

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https://doi.org/10.1016/j.imlet.2023.06.007
Received 29 April 2022; Received in revised form 29 May 2023; Accepted 10 June 2023
Available online 13 June 2023

Hypersensitivity to shellfish (crustacea and mollusks) has been associated with the highest prevalence worldwide, for instance, in the Brazilian adult population where shrimp is one of the most reported food allergens [6]. Shrimp allergy can cause severe and potentially life-threatening allergic reactions (such as anaphylaxis). These reactions can be unpredictable, and even very small amounts of shellfish can be a trigger. Approximately 130 sensitizing shrimp species-specific allergens have been characterized, predominantly muscle contractile proteins (tropomyosin), e.g., *Pen a 1, Lit v 1*, and *Met-e* (Allergome.org). Furthermore, cross-reactions are reported as their molecular structure is highly conserved among other invertebrates [7,8].

Herein, we developed a new mouse model of shrimp allergy using *Litopenaeus vannamei* proteins, the most relevant and consumed shrimp specie. The present model could be a useful tool to further investigate immunopathological aspects of shrimp food allergy and be applied to evaluate immunomodulation approaches.

2. Materials and methods

2.1. Ethics statement

Female BALB/c mice aged 4–8 weeks, weighing approximately 20 grams, were housed at the animal facility of the Gonçalo Moniz Institute or the Butantan Institute of Sao Paulo under controlled room temperature, a 12-hour light/dark cycle, and free access to food and water. All procedures were approved by the local Ethics Committees (CEUA license 021/2018 IGM/FIOCRUZ-BA and CEUA license 8,687,060,323 Butantan Institute of Sao Paulo).

2.2. Shrimp extract preparation

Total protein extract was obtained from peeled and precooked industrialized *Litopenaeus vannamei* shrimps (Maris, Brazil) following the protocol proposed by Ayuso and colleagues [9] with some adaptations. To generate a uniform homogenate, shrimp samples were crunched, resuspended in 1X PBS solution (0.05% WV), and incubated overnight. This suspension was primarily centrifuged at 3000 rpm for 10 min at 4 °C. The supernatant was centrifuged for 5 min at 15,000 rpm 4 °C. Finally, the supernatant was collected and stored at - 20 °C until used for experiments. Total protein concentration was measured by Pierce using a Micro BCA kit (ThermoFisher Scientific).

2.3. Sensitization and challenge of mice

Following the protocol suggested by Saldanha and colleagues [10], animals were sensitized by a subcutaneous administration of shrimp proteins (100 μg) adsorbed onto aluminum hydroxide (1 mg) at day 0. After 14 days, shrimp proteins (100 μg) were subcutaneously administrated again as a booster. Control groups were injected only with saline.

Throughout the oral challenge stage of this experimental design, animals continuously ingested water containing shrimp proteins (5 mg/ml) from day 21 to day 35. Control animals ingested only water. Thus, the following experimental groups were formed: Control, Challenged, Sensitized, and Allergic. Blood samples were collected before every intervention and euthanasia. Animals were euthanized on days 18, 23, or 35. The experimental design is illustrated in Fig. 1.

2.4. Protein profile and antigenicity of the shrimp extract

To profile the shrimp extract 10 μg were separated on 12% SDS-PAGE polyacrylamide gels and stained with Coomassie R-250. The western blot analysis was performed as described by Farias and colleagues (2012) [11]. Briefly, the gel was transferred to polyvinylidene difluoride (PVDF) membranes, and after blocking the membrane was incubated with 1:10.000 sera from allergic mice post-challenge (pool of 5 mice). Next, an incubation with 1:3.000 anti-IgG mouse conjugated to horseradish peroxidase (Seracare, MA, USA) was performed and revealed with ECL reagent (GE Healthcare). Images were captured using an Image Quant LAS 4000 photo documentation system (GE Healthcare, Uppsala, Sweden).

2.5. Intestinal lavage

The small intestine section was washed by the injection of cold saline (10 ml) through one of the sections. The collected suspension was homogenized and centrifuged at 1200 rpm for 20 min at 4 °C to obtain a clean supernatant which was freshly used for the detection of anti-shrimp IgA by ELISA.

2.6. In vitro restimulation of cervical draining and mesenteric lymph node cells

Cervical draining lymph nodes (CDLN) from the sensitization site and mesenteric lymph nodes (MLN) cells were seeded at 1×10^6 cells/well in RPMI 1640 (Gibco, pH 7.4) supplemented with 10% fetal calf serum and 1% penicillin/streptomycin only as a negative control. Positive controls consisted of cells stimulated by Concanavalin A (Sigma, 2.5 μg/well). For means of cytokine release evaluation after oral challenge, cells were restimulated with shrimp proteins (5 μg/well). Followed by a 24-hour (IL-4 and IL-5 measurement) or 48-hour (IL-10 and IFN-γ) incubation at 37 °C, cell-free supernatant was collected for cytokine measures.

2.7. Antibodies and cytokines detection

Titers of serum antibodies were measured by ELISA at 1:100 (anti-shrimp IgE, total IgE) or 1:2000 (anti-shrimp IgG1) serum dilution. Briefly, a solution of shrimp proteins (10 μg/ml for anti-shrimp IgE or 5 μg/ml for anti-shrimp IgG1) in 0.1 M sodium carbonate (pH 9,5) was loaded onto 96-well Nunc Maxisorp plates (Nunc, Roskilde, Denmark) overnight. Plates were washed with 1X PBS+0,05% Tween 20 between each of the following steps. After blocking with 1% BSA in 1X PBS, serum samples were incubated overnight at 4 °C (anti-shrimp IgE) for 2 h at 37 °C (anti-shrimp IgG1). For colorimetric detection, anti-mouse HRP-conjugated anti-IgE and anti-IgG1.

Similarly, anti-shrimp IgA was measured in intestinal lavage samples. Regardless of sample dilution (1:2), the protocol followed the same described for anti-shrimp IgE. Anti-mouse HRP-conjugated IgA (Life Technologies, 1:1000) was used for colorimetric detection.

Total serum IgE was quantified using an anti-mouse IgE BD OptEIA

Fig. 1. Experimental design. BALB/c female mice were subcutaneously injected with shrimp proteins (100 μg) adsorbed onto aluminum hydroxide (1 mg) at day 0, following a second administration on day 14 of shrimp proteins only (100 μg). Shrimp proteins (5 mg/ml) were added to the water from day 21 to 35 as an oral challenge protocol. Control animals were injected with saline or exposed to water. Animals were euthanized on day 18, or 35 for sample collection.

kit (BD Biosciences) as well as IL-4, and IL-10. Measurements followed manufacturer instructions.

2.8. Passive cutaneous anaphylaxis assay

The anaphylactic activity of IgE antibodies was evaluated by passive cutaneous anaphylactic reaction (PCA) in mice as described by Mota [12]. Previously shaved mice were injected intradermally in the back with 50 μL of serial dilutions of a pooled serum from each group of immunized/sensitized mice ($n = 10$) starting at 1/5. After 72 hours, the mice were challenged intravenously with 500 μL of the shrimp extract (1 mg/mL) diluted in 0.25% of Evans blue solution. The tests were made in triplicate and the IgE titers were considered as the reciprocal of the highest dilution that induced a lesion of >5 mm in diameter. Only a difference above 2-fold or less in the titers of IgE was considered significant as previously described [13,14].

2.9. Histological and morphometric analysis

Proximal jejunum samples were collected for histomorphometry evaluation. Fragments were fixed in 10% neutral buffered formalin, embedded in paraffin, and cut into 5 μm thick sections. Subsequently, sections were stained either by hematoxylin-eosin (HE) for histological analysis or by periodic acid Schiff (PAS) for mucus mensuration.

Images were obtained by a microscope coupled to a camera (Olympus). Randomly, five fields were chosen at 10X or 40X magnification from HE-stained sections for villus height and crypt depth assessment, and eosinophil counting, respectively. Accordingly, five fields were captured at 10X magnification from PAS staining sections which were converted to binary images using ImageJ® software to determine μm^2PAS/field. A millimeter rule supported villus height and crypt depth measurement using the same software.

2.10. Statistical analysis

Following the normality distribution, data sets were analyzed by one-way analysis of variance (ANOVA) followed by Turkey's post-test or *t*-test as accordingly stated. Data were evaluated using GraphPad Prism® 8 (GraphPad, San Diego, CA, United States) considering $p <$ 0,05 as a significance level.

3. Results

3.1. Sensitized mice developed responsive serum antibodies to major allergens in Litopenaeus vannamei extract

The protein content in L. *vannamei* was characterized by SDS-PAGE (Fig. 2A) followed by immunoblotting with serum from sensitized and challenged mice (Fig. 2B). Three major bands were identified: ~75 kDa, ~40-36 kDa, and ~20 kDa.

Analysis based on the allergen database Allergome (allergome.org) and previous studies revealed that these bands correspond to the most reported allergens in L. *vannamei*: Lit v HC (Hemocyanin, 72 or 75 kDa), Lit v 2 (Arginine kinase, 40 kDa), Lit v 1 (Tropomyosin, 36 kDa). Interestingly, 3 of the major muscle shrimp allergens share the same range of molecular weight: Lit v 3 (Myosin, 18~20 kDa), Lit v 4 (Sarcoplasmic calcium-binding protein, 20 kDa), and Lit v 6 (Troponin, 20 kDa). Therefore, the major band observed at ~20 kDa could correspond to one of the just-mentioned allergens or a combination of those.

These findings are a shred of evidence that the present model can efficiently mimic immunopathogenesis for the development of shrimp allergy in humans.

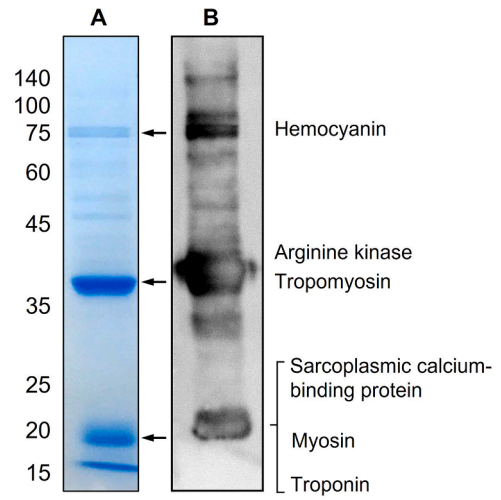

Fig. 2. Protein profile and antigenicity of shrimp extract proteins. (A) Coomassie-stained gel revealing the major constituents of the shrimp extract. (B) Immunoblotting of the extract presented in (A) after incubation with 1:10,000 of (sera from shrimp-allergic mice post-challenge) was performed. For more details, see materials and methods item 2.4.

3.2. Systemic and intestinal-specific humoral response is enhanced upon oral challenge with shrimp proteins

IgE binds onto mast cell surface receptors during sensitization triggering the release of essential proinflammatory mediators for allergic reactions. Therefore, serum IgE titers are a crucial parameter to be analyzed throughout allergy development. Our results show that the subcutaneous sensitization protocol induced a significant total IgE production in serum from Sensitized and Allergic mice collected pre-challenge. Additionally, serum anti-shrimp IgE was progressively increased in Allergic mice as a response to oral challenge (post-challenge), but not in Sensitized mice (Fig. 3A). Similarly, anti-shrimp IgE detection pre- and post-challenge correlated to total serum IgE observations confirming the development of systemic humoral response in Allergic mice (Fig. 3B).

There is evidence that IgG1 is also involved in the triggering of allergic reactions. Therefore, serum anti-shrimp IgG1 production was also evaluated. While Control animals showed no detectable specific IgG1, high titers of anti-shrimp IgG1 were detected in Sensitized and Allergic animals pre-challenge. However, only Allergic mice sustained and expanded this production post-challenge (Fig. 3C). As IgG1 shows a longer half-life than IgE [15], it could explain the high titer found in Sensitized mice even post-challenge.

Besides a systemic humoral response, the production of specific secretory IgA (SIgA) is observed in intestinal mucosa as a neutralizing antibody that actively mediates the local response against food allergens. Herein, we show that Allergic mice, but not Challenged or Sensitized mice, produced anti-shrimp SIgA, therefore, revealing the induction of a specific humoral intestinal response and the mucosal effect (Fig. 3D). Collectively, these results confirm the effectiveness of our experimental protocol in inducing a systemic and intestinal humoral response.

Considering the eosinophil migration, the presence of IL-5 in the GALT, and the high levels of systemic IgE, we evaluated the titers of shrimp extract-specific IgE in the serum of mice groups by Passive Cutaneous Anaphylactic (PCA) reaction. Our results demonstrated that IgE antibodies produced by the Allergic mice group exhibited strong PCA activity with a very high titer of 1:80, whereas the other groups, including Challenged and Sensitized groups, demonstrated low levels of IgE antibodies (Fig. 3E).

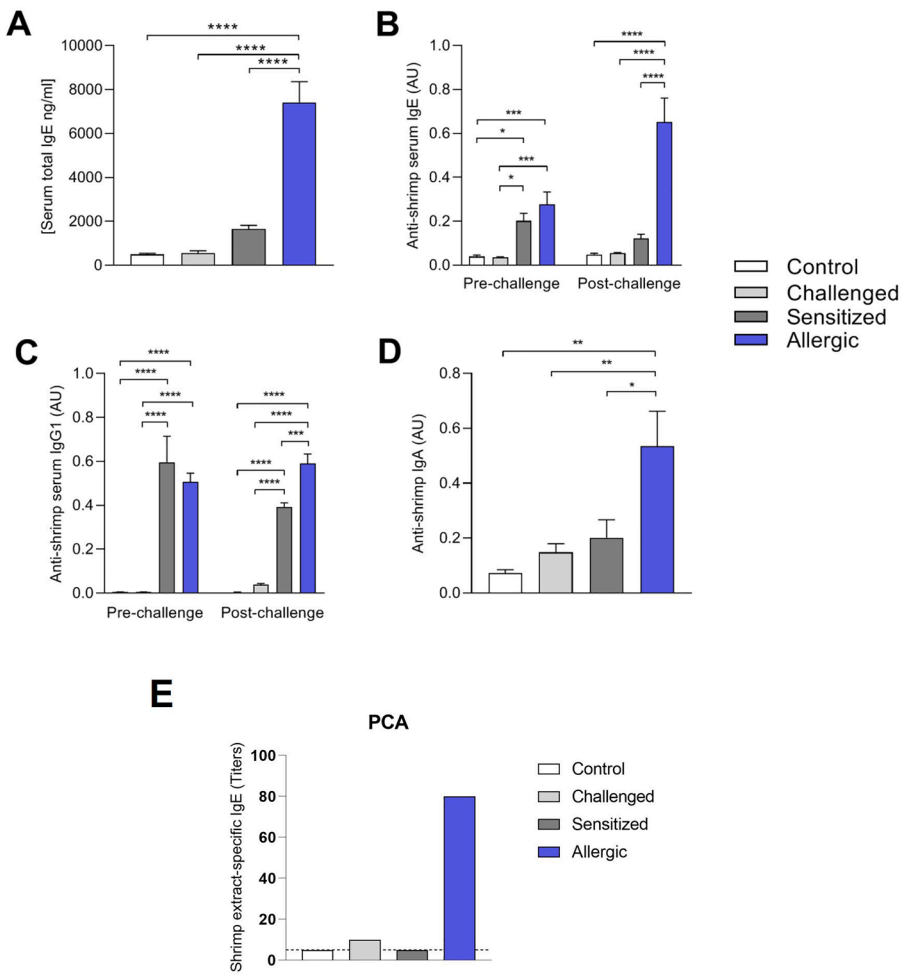

Fig. 3. Oral exposure to shrimp proteins induces a systemic and intestinal mucosa production of anti-shrimp antibodies. Blood was withdrawn before interventions and euthanasia for serum collection and detection of serum total IgE (A), anti-shrimp IgE (B), and IgG1 (C) detection. Intestinal lavage from the entire jejunum section was collected for anti-shrimp IgA (D) measurement. Data are expressed in arbitrary units (AU) in B, C, and D, and shown in Mean ± SEM (n=6). Analysis was performed by One-Way ANOVA. (E) The production of IgE anti-shrimp extract was measured by PCA. IgE titers represent the reciprocal of the highest dilution of serum pool of each mice group ($n = 10$) that gave a lesion of >5 mm in diameter. The dashed line represents the detection threshold (serum dilution at 1/5). Data are representative of two independent experiments.

3.3. Sensitization with shrimp proteins induces T helper 2 cytokines in CDLNs and MLNs

T helper 2 cytokines directly induce a switching on immunoglobulins' heavy chain class towards IgE production. After a booster, IL-4, and IL-5 (Fig. 4A-B) production were notably increased in CDLNs from Allergic mice on day 18 while there was no enhancement in IFN-γ production (Fig. 4D) indicating a Th2-driven immune response triggered by shrimp proteins after sensitization. Also, IL-10 release was significantly enhanced as this cytokine counterbalance inflammatory reactions

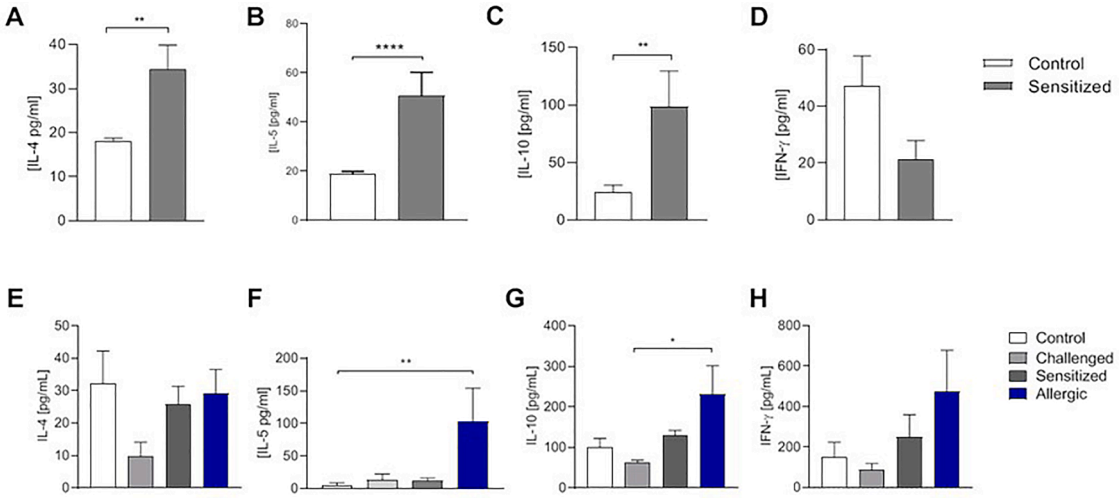

Fig. 4. Th2-driven cytokine response is induced in cervical draining and mesenteric lymph nodes after in vitro restimulation. Cervical draining lymph nodes were collected on day 18 and restimulated in vitro while mesenteric lymph nodes were collected on day 23. Cytokines were measured in cell-free supernatant. IL-4 (A/E), IL-5 (B/F), IL-10 (C/G), and IFN-γ (D/H) production are shown (n = 8–10). Data expressed in Mean ± SEM and analyzed by unpaired *t*-test (A-D) or Kruskal-Wallis followed by Dunn's post-test (E-H).

(Fig. 4C). A corresponding response was observed in MLNs from Allergic mice where IL-5 and IL-10 production were augmented on the first days of the oral challenge (Fig. 4E-H). These results suggest that the sensitization protocol was effective in inducing an allergic response.

3.4. Oral challenge-induced morphometric changes in intestinal mucosa in response to sensitization

As a result of chemotactic factors released during hypersensitivity reactions, inflammatory infiltrates, mainly composed of eosinophils, are observed at the intestinal lamina propria. After 14 days of oral exposure to shrimp extract, Allergic mice showed higher counting of eosinophils in the mucosa (Fig. 5A-B).

Morphometric changes in villi extension and crypt depth may also be observed as a consequence of allergic inflammation. To identify whether Allergic animals show mucosal morphometric alterations, histological sections of the proximal jejunum were analyzed by optical microscopy (Fig. 5C). This analysis revealed shrinkage of villi in animals that were exposed to shrimp proteins along with oral challenge (Fig. 5D). Also, Allergic animals showed a significant reduction in crypt depth (Fig. 5E) and a slight increase in mucus production (Figure S1A-C).

4. Discussion

Although a plethora of experimental models of food allergy has been established, there is still a lack of data regarding shrimp allergy. This work aimed to establish a new mouse model to further investigate clinical and immunological aspects of shrimp allergy that could be a tool for the development of safer immunotherapies based on immunomodulation.

Several proteins from shrimp are reported to trigger an allergic response. Most of the allergens are also thermal stable. Taken specifically L. *vannamei*, according to Allergome (allergome.org) and Allergen (allergen.org) databases, approximately 8 proteins and their isoforms are identified as shrimp allergens, which are Lit v 1 (Tropomyosin, 36 kDa) [16], Lit v 2 (Arginine kinase, 40 kDa) [17], Lit v 4 (Sarcoplasmic calcium-binding protein, 20 kDa) [18], Lit v HC (Hemocyanin, 72-75 kDa) [19], Lit v 3 (Myosin, 20 kDa), Lit v 6 (Troponin, 20 kDa), Lit v PK (Pyruvate kinase, 63 kDa), and Lit v Trx (Thioredoxin, 12 kDa) [20].

The sensitization followed by oral challenge with total protein extract elicits the production of IgG antibodies against several well-described shrimp allergens. This analysis revealed reactivity to Lit v 1, Lit v 2, and Lit v HC. Similarly, there was another major band identified with ~20 kDa (Fig. 2A-B). As mentioned, Lit v 3, Lit v 4, and Lit v 6 are 20 kDa allergens found in shrimp meat.

The present analysis could not discriminate, which is(are) the allergen(s) seen at the 20 kDa band. However, based on these findings it is feasible to conclude that Allergic mice produced shrimp-specific IgG antibodies to at least 4 of the major allergens reported in L. *vannamei*. Therefore, as a non-single antigen-specific model, it shows a broader translational potential, and the model could be applied to different immunotherapies based on the induction of immunomodulation through regulatory response generation [21–23].

The microenvironment plays a crucial role in allergy reactions. The release of IL-4, IL-5, and IL-13 induces specific IgE production and eosinophil migration. Currently, IL-25 (also known as IL-17E) role has also been discussed. Susceptibility to anaphylaxis was associated with constitutive overexpression of IL-25, whereas IL-17rb$^{-/-}$ mice seemed less susceptible. Additionally, IL-5 and IL-13 were mainly released by type 2 lymphoid cells (ILC2s) in response to IL-25 [24]. Lymphoid tissues also participate in this process as a response to captured allergens. Fu and colleagues [25] observed a Th2 prominent pattern of cytokine release (IL-4, IL-5, IL-13, IL-10) in MLNs of animals allergic to tropomyosin from *Penaeus monodon*. Herein, we found a lymph node-coordinated Th2-driven response as a distinguished production of IL-5, and IL-10 in both CDLNs and MLNs. These data endorse the role of lymphoid tissues in the Th2 polarization in pairing an immune response against food allergens.

Hypersensitivity reactions are IgE-mediated. Specific IgE bound to the FCεRI receptor on eosinophils, mast cells, and basophils form a complex that activates tyrosine-based activation immunoreceptor domains (ITAMs) which leads to the release of intracellular calcium and, ultimately, the degranulation of mast cells and allergy reactions. After sensitization, serum anti-shrimp IgE was detected in high titers, and it progressively increased throughout 14 days of oral challenge (Fig. 3A-B). Saldanha and colleagues [10], using a similar protocol, detected an increase in allergen-specific IgE in animals allergic to OVA after 14 days of the oral challenge. Our data corroborate those observed in the study by Leung and colleagues [26], which reported a high IgE production towards tropomyosin Met-a 1 from *Metapenaeus ensis* in an anaphylaxis-induced model. Thus, these data demonstrate that the production of allergen-specific antibodies can be effectively induced through subcutaneous sensitization followed by continuous oral intake of small doses of the allergen.

To further explore the IgE essential role in mast cell degranulation and the triggering of an allergic reaction, a PCA assay was conducted. The data collected confirmed the IgE-mediated reaction as the injection of serum from Allergic mice induced the degranulation of mast cells resulting in a larger area of reaction on the back of the mice (Fig. 3E).

IgG1 contribution to allergic reactions remains debatable. However, some studies supported its role in allergic processes. During antibody class switching, endonucleases excise segments from the constant heavy chain gene locus to obtain the one that encodes a specific antibody class. In this perspective, the γ1 gene locus is closer to the donor region, which in turn may increase its recombination probability and antibody class switching in B cells [27]. Miyajima and colleagues [28] have shown IgG1-dependent anaphylactic reactions on γ chains (FcγRIII) knockout animals. IgG1 production against food allergens has been observed [29]. In our study, anti-shrimp IgG1 followed the same pattern as observed in anti-shrimp IgE. Interestingly, high titers of anti-shrimp IgG1 were sustained among sensitized animals that were not challenged (Fig. 3C). It suggests that IgG1 is likely to play a role in the immunopathogenesis of shrimp allergy as well.

Globet cells play an essential role in the intestinal mucosa as they secrete mucins contributing to the deposition of mucus onto epithelial cells' surface. The mucus serves as a first barrier preventing the entry of pathogens into the intestinal mucosa. Hyperplasia of globet cells has been observed in tropomyosin-sensitized mice [30]. In our study, we observed a slight increase in mucus detection in jejunum histological slides from Allergic mice (Figure S1A-C).

On the matter of intestinal humoral response, SIgA mediates the elimination of pathogenic microorganisms, consequently, preventing the intestinal epithelium from their access. In an anaphylaxis-induced model, low titers of SIgA were detected in the feces of animals sensitized to β-lactoglobulin [31]. On the other hand, Gomes-Santos and colleagues [32] observed augmented levels of anti-β-lactoglobulin SIgA in the intestinal lavage of allergic animals. Regarding shrimp allergens, significant production of SIgA was induced after successive intragastric sensitizations with tropomyosin from *Metapenaeus ensis* [30]. Similarly, we observed significant production of anti-shrimp SIgA after oral challenge (Fig. 3D). Thus, a protective role of SIgA in the elimination of shrimp allergens can be inferred. Moreover, the detection of high titers of specific immunoglobulins suggests that our experimental protocols were effective in inducing a local humoral response.

The immunological parameters involved with the allergic response may lead to histopathological alterations as a result of the tissue inflammation in the intestinal mucosa. Eosinophils can normally be found in mucosal membranes, however, it is known that in IgE-mediated food allergies, these cells can intensely infiltrate the intestinal lamina propria as a consequence of the release of chemotactic factors, such as CCL11. For instance, the eosinophilic infiltrate was observed in the duodenum and jejunum of animals allergic to *Met-a 1* shrimp after 24

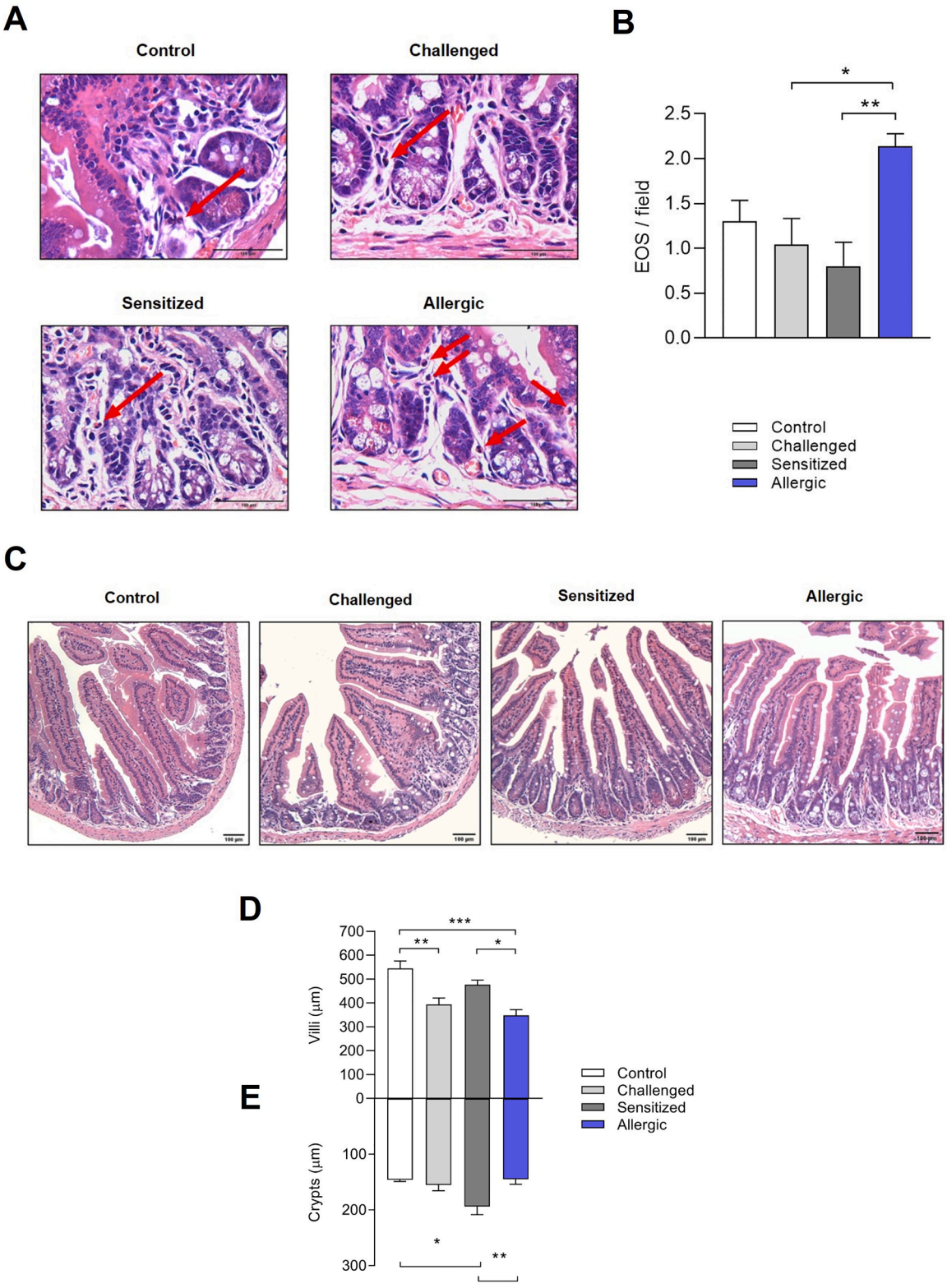

Fig. 5. Intestinal mucosa of allergic animals shows higher counting of eosinophils at the lamina propria and morphometric changes. After the oral challenge, fragments of jejunum were collected, processed into sections, and stained by HE for eosinophil counting, and morphometric analysis of villi height and crypt depth using 5 randomly captured fields. (A) Representative histological sections from each group at 40X magnification. Eosinophils are indicated by red arrows. (B) Representative histological section of villi and crypts at 10X magnification. (C) Quantification of eosinophil at lamina propria. (D/E) Measurement of villi and crypts extension. Values are expressed as in Mean ± SEM (n=4-6) and analyzed by One-Way ANOVA and Tukey's post-test. Scale bars = 100 µm.

and 72 h of intragastric challenge [30]. In our model, sensitized animals that continuously ingested shrimp proteins for 14 days during oral challenge showed higher eosinophil counting in their intestinal lamina propria (Fig. 5A-B). Our data corroborate the findings observed by Saldanha and colleagues [10] in a model of allergy to OVA. This study also demonstrated that an eosinophilic infiltrate in animals allergic to OVA was more prominent at the early stages of the oral challenge. Perhaps, markable differences could be observed in our study if earlier time points were evaluated.

Main Basic Protein 1 (MBP-1) and 2 (MBP-2), the Eosinophilic Cationic Protein (ECP), Eosinophil-Derived Neurotoxin (EDN), and the Eosinophilic Peroxide (EPO), which play a crucial role in defending against helminths, are the main secretory granules released by eosinophils. However, cytotoxicity to the epithelium cells has been associated with MCP, ECP, and EPO. An in vitro study showed histopathological alterations in the upper respiratory epithelium and ciliary stasis caused by MCP [33]. Cytotoxicity to the intestinal epithelium has also been suggested [reviewed in 34]. After continuous whey ingestion for 7 days, animals allergic to β-lactoglobulin showed not only eosinophilic infiltrate in the intestinal mucosa but also presented a reduction in villi extension [32]. In our model, continuous oral exposure to shrimp proteins induced the shortening of villi and crypts in the intestinal mucosa of Allergic animals (Fig. 5C-E). Thus, these data suggest that such morphometric changes in the intestinal mucosa could be associated with the recruitment of eosinophils due to their degranulation and prolonged exposure to allergenic proteins.

Bodyweight loss, and humoral and cellular response, besides intestinal morphological alterations, are the mainly observed clinical features in allergic individuals. Herein, we reproduced the physiological and immunological effects of shrimp hypersensitivity in an experimental model that is observed in patients reporting mild allergic reactions. Although we did not find any symptoms related to anaphylactic reactions, which could be considered a limitation in our experimental approach, the established model can be a helpful tool to further investigate not only the mechanisms associated with the immunopathogenesis of shrimp allergy but also the development of therapies based on oral immunomodulation.

Funding

IVN received a fellowship from CNPq (Centro Nacional de Pesquisa), PVG and CMA received fellowships from CAPES (Centro de Aperfeiçoamento do Pessoal do Ensino superior). ELFM is a Research Productivity fellow at CNPq. AMCF and CIB are senior investigators at CNPq. This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) - Finance Code 001

CRediT authorship contribution statement

Ivanéia Valeriano Nunes: Conceptualization, Methodology, Validation, Data curation, Visualization, Writing – review & editing. **Camila Mattos Andrade:** Validation, Visualization, Data curation. **Priscila Valera Guerra:** Validation, Visualization, Data curation. **Mariana Ivo Khouri:** Validation, Visualization, Data curation. **Maria Poliana Leite Galantini:** Validation, Visualization, Data curation. **Robson Amaro Augusto da Silva:** Conceptualization, Methodology, Data curation, Formal analysis, Writing – review & editing, Funding acquisition, Project administration, Supervision, Resources. **Eliana L. Faquim-Mauro:** Validation, Visualization, Data curation. **Leonardo Paiva Farias:** Conceptualization, Methodology, Data curation, Formal analysis, Writing – review & editing, Funding acquisition, Project administration, Supervision, Resources. **Juliana de Souza Rebouças:** Conceptualization, Methodology, Data curation, Formal analysis, Writing – review & editing, Funding acquisition, Project administration, Supervision, Resources. **Ana Maria Caetano Faria:** Conceptualization, Methodology, Data curation, Formal analysis, Writing – review & editing, Funding acquisition, Project administration, Supervision, Resources. **Cláudia Ida Brodskyn:** Conceptualization, Methodology, Data curation, Formal analysis, Writing – review & editing, Funding acquisition, Project administration, Supervision, Resources.

Declaration of Competing Interest

This study was not involved in any commercial or financial associations which could raise a potential conflict of interest.

Acknowledgements

We are thankful to Dr. Washington Luís Conrado dos Santos, Jonathan Luís Magalhães Fontes, and the technicians from the Histology Facility at Goncalo Moniz Institute for guidance with histological slides confection, and analysis. We are also thankful for the agencies that funded IVN, CMA, PVG, and ELFM fellowships.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.imlet.2023.06.007.

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