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Full Paper

Immunomodulatory effects of *Ulva*-derived polysaccharides, oligosaccharides, and residues in a murine model of delayed-type hypersensitivity

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***Ulva*, an edible green alga, contains sulfated polysaccharides and oligosaccharides that possess immunomodulatory and anti-inflammatory properties. The objective of this study was to investigate the anti-allergic effects of *Ulva*-derived samples of polysaccharides (UP), oligosaccharides (UO), and residues (UR) on delayed-type hypersensitivity (DTH) in mice. Oral treatment of mice with UP, UO, and UR (250 mg/kg body weight) daily noticeably improved the DTH reaction as evidenced by attenuation of footpad swelling and cell infiltration at the allergen-challenge site. Although the *Ulva* samples had limited impacts on the production of serum total IgG, decreased concentrations of allergen-specific IgG and IgG_{2a} and an increased concentration of IgG_1 were observed in the treated mice. Moreover, treatment with them suppressed allergen-induced IFN-γ and TNF-α secretion and elevated IL-4 secretion. However, none of the *Ulva* sample treatments could modulate the production of IL-10. Concordantly, the *in situ* data reveal that the *Ulva* sample treatments suppressed IFN-γ and TNF-α expression at the allergen-injection site. These findings collectively suggest the potential of UP, UO, and UR as functional food candidates for the management of delayed-type hypersensitivity.**

Key words: delayed-type hypersensitivity, oligosaccharides, polysaccharides, residues, *Ulva*

INTRODUCTION

Ulva has a high tolerance to environmental adversities and is rich in sulfated polysaccharides, which contributes to its various physiological activities, including antioxidant, hypolipidemic, anti-inflammatory, anti-cancer, and immunomodulatory activities [1–4]. The primary constituents of *Ulva* polysaccharides are rhamnose, xylose, and glucuronic acid, forming the backbone. The monosaccharides found in this context are organized into repeating disaccharide units, specifically α-$_L$-Rhaρ-(1→4)-$_D$-Xyl and (→4)-β-$_D$-GlcρA-(1→4)-α-$_L$-Rhaρ. Additionally, other monosaccharides, such as galactose, arabinose, mannose, and glucose, are also present, albeit in smaller proportions. The backbone of *Ulva* polysaccharides exhibits sulfate group substitutions at the O-2, O-3, or O-4 positions [5–7]. With respect to immunostimulatory activity, *Ulva* polysaccharides have been observed to stimulate Raw 264.7 cells, resulting in the induction of nitric oxide production and the synthesis of various cytokines, indicating that *Ulva* polysaccharides are immunostimulators [1]. In regard to anti-inflammatory activity, treatment with *Ulva* polysaccharides reduced the production of pro-inflammatory cytokines and suppressed the activities of nitric oxide synthase and cyclooxygenase in a murine model of colitis [8]. Nevertheless, there is no information available about the impact of *Ulva* or its derived components on allergy.

Delayed-type hypersensitivity (DTH) represents a form of immune response known as type IV hypersensitivity. This reaction involves the participation of various immune cells, especially T cells and macrophages [9, 10]. DTH is classified as the second most prevalent allergic disorder and is characterized by an inflammatory response that typically takes more than 24 to 48 hr to develop [10]. The primary histopathological characteristic of DTH is the infiltration of leukocytes into affected tissues. As a therapeutic approach, topical corticosteroids are commonly utilized for the management of DTH [11]. The immune response in DTH is primarily orchestrated by the induction of T helper (Th) 1 cells. Following the recognition of an allergen, presented by antigen-presenting cells through MHC-II molecules, by naïve T cells, the secretion of cytokines such as IL-2 induces the differentiation of naïve $CD4^+$ Th cells into Th1 cells.

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Consequently, various cytokines, especially IFN-γ, are released by Th1 cells, thereby activating cytotoxic activity. This cascade of events attracts macrophages to the site of the allergen through chemotaxis, leading to pro-inflammatory TNF-α production and subsequently localized and pronounced inflammation [9, 10, 12]. IL-4 actively supports the differentiation and stability of Th2 cells while impeding the differentiation of Th1 cells. Conversely, the inhibitory effect of IFN-γ on Th2 cell differentiation and its role in stabilizing Th1 cells are widely acknowledged. Thus, modulation of the Th1/Th2 balance is a potential strategy for treating DTH. Moreover, regulatory T (Treg) cells are known to suppress the allergic response via secretion of inhibitory cytokines, such as IL-10 [13].

In the current study, a sample of polysaccharides was extracted from *Ulva* (UP), and a sample of oligosaccharides was obtained by enzymatic hydrolysis of UP (UO). To achieve our sustainable development goals, the residues of *Ulva* (UR) were also harvested. To assess the potential use of UP, UO, and UR in managing type IV hypersensitivity, this study aimed to investigate their anti-allergic effects in a murine model of DTH. Furthermore, the profile of serum antibody production and splenic and local cytokine expression was clarified to unravel their influences on modulation of humoral and cellular immune responses.

MATERIALS AND METHODS

Chemicals, reagents, detection kits, and preparation of UP, UO, and UR

The chemicals and reagents were acquired from Sigma Chemical (St. Louis, MO, USA). Cell culture reagents were obtained from GE Healthcare Life Sciences (Marlborough, MA, USA). To measure cytokines and immunoglobulins (Ig), enzyme-linked immunosorbent assay (ELISA) kits were purchased from eBioscience, Inc. (San Diego, CA, USA). Reagents and antibodies for immunohistochemical (IHC) staining were procured from Vector Laboratories, Inc. (Newark, CA, USA) and Thermo Fisher Scientific Inc. (Waltham, MA, USA). The *Ulva* sp. used in this study was supplied by Professor Chorng-Liang Pan of the Department of Food Science at National Taiwan Ocean University (Keelung, Taiwan), transformed into a powder form using an electric grinder, and filtered through a 40-mesh sieve. The process for preparing UP and UO followed the method described in a previous study [14]. However, in this study, a commercial enzyme called Cellulase AP3 (2000 U/g; Amano Enzyme, Aichi, Japan) was used for the hydrolysis of UP. After extraction of UP, the residues of *Ulva* were collected and lyophilized as UR powder.

Animal experiment

All animal experiments strictly followed the guidelines of the National Research Council's Guide for the Care and Use of Laboratory Animals and received approval from the Institutional Animal Care and Use Committee of National Taiwan Ocean University (NTOU IACUC-108040). Female BALB/c mice were obtained from the National Laboratory Animal Center of Taiwan at 5 weeks of age. Prior to the experiment, the mice were housed in the laboratory animal facility at the NTOU and allowed unrestricted access to water and food for 1 week. As illustrated in Fig. 1A, the mice were categorized into distinct groups (consisting of 5 mice per group): a naïve group (NA), vehicle group (VH), and three treatment groups. In the VH group, the mice were subjected to daily oral administration of 0.1 mL of phosphate-buffered saline (PBS). Conversely, the mice in the treatment groups were administered 250 mg/kg body weight of UP, UO, or UR, which was dissolved in 0.1 mL of PBS, via oral gavage on a daily basis. The protocol and methods used for the induction of DTH were in accordance with those reported in a previous study, with slight modifications [15, 16]. Excluding the mice in the NA group, all remaining mice were sensitized with ovalbumin (OVA) by intraperitoneal injection of 0.1 mL of a sensitization solution comprising 100 μg OVA and 2 mg alum in PBS on the third day of the experiment. By the ninth day, the footpads of all mice underwent a subcutaneous challenge with OVA, which was administered at a dosage of 200 μg in 20 μL of saline. The degree of footpad swelling, representing the DTH reactions, was measured using an electronic caliper before and 24 hr after the OVA challenge. After the measurement of swelling, serum samples were collected, and the mice were euthanized to obtain footpad tissues for histopathological examination. IHC staining was performed to detect IFN-γ and TNF-α using the same protocol and methods as previously reported in other studies [16–18]. The stained sections of footpad tissues were observed with an upright microscope (Olympus BX53). IHC-stained sections were analyzed using the ImageJ software to determine the ratios of the areas of IFN-γ- and TNF-α-positive signals to the total tissue area, following previously described methods [16–18]. Spleen samples were individually isolated to prepare splenocyte suspensions [14].

Culture of murine splenocytes and determination of splenic cytokine and serum antibodies

Splenocytes prepared from the spleen samples isolated from each mouse were seeded into 24-well culture plates at a concentration of 5×10^6 cells/mL. These cells were then treated with OVA for a period of 24 to 72 hr. After the designated treatment time, the supernatants from the culture plates were collected. The levels of IFN-γ, IL-4, IL-10, and TNF-α were measured using ELISA. Furthermore, the concentrations of antibodies in the serum samples were quantified using ELISA. To detect OVA-specific antibodies, the coating of capture antibodies for IgG or IgE was substituted with the coating of OVA. The ELISA protocol was conducted according to the instructions provided by the ELISA kit supplier.

Statistical analysis

Comparison between groups was conducted by use of Dunnett's test (SigmaPlot V14, Systat Software Inc.). Differences were considered significant at $p<0.05$.

RESULTS

Mitigation of DTH reactions by treatment with UP, UO, or UR

It has been reported that 24 hr after injecting OVA into the footpads of OVA-sensitized mice, DTH reactions were elicited and characterized by swollen footpads with inflammatory cell infiltration [15]. In this study, we utilized a murine model of DTH and quantified the alterations in footpad thickness between before and after the challenge with OVA to evaluate the effects of UP, UO, and UR (250 mg/kg body weight daily by gavage) on mitigation of DTH reactions (Fig. 1A). As depicted in Fig. 1B,

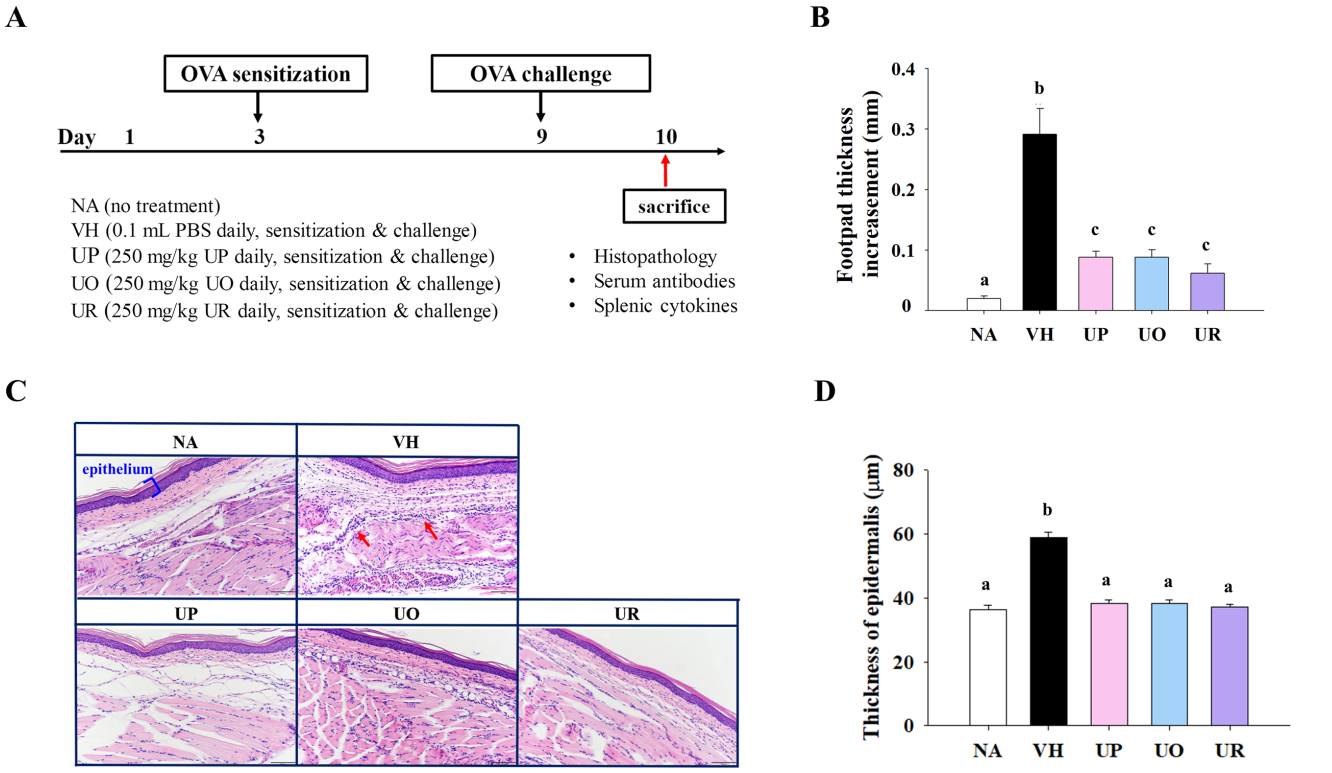

Fig. 1. Protocol of the animal experiment and effects of the *Ulva* samples on mitigation of delayed-type hypersensitivity (DTH) reactions. A) BALB/c mice were divided into groups (each consisting of 5 mice): a naïve group (NA), vehicle group (VH), and three treatment groups. In the VH group, the mice received daily oral administration of 0.1 mL of phosphate-buffered saline (PBS) by gavage. Mice in the treatment groups were given daily oral doses of 250 mg/kg body weight of polysaccharides (UP), oligosaccharides (UO), or residues (UR) dissolved in 0.1 mL of PBS by gavage. Except for the mice in the NA group, all other mice were sensitized with ovalbumin (OVA) by intraperitoneal injection on day 3. On day 9, the footpads of all mice were subcutaneously challenged with OVA to induce DTH. B) The degree of footpad swelling in response to DTH was determined by measurements of footpad thickness before challenge and at 24 hr post-challenge. To gain further insights, histopathological examination of the footpad tissues was conducted after sacrificing the mice. C) Representative images of footpad tissues subjected to hematoxylin and eosin staining are shown, and red arrows indicate cell infiltration. D) The epithelial thickness of footpad was measured with the ImageJ software. The data presented here are expressed as mean ± standard error of the mean (SEM) (n=5) values derived from three separate and independent experiments. Values with different letters are significantly different ($p<0.05$).

there was a notable increase in footpad thickness among mice in the VH group, in contrast to the NA group. Nevertheless, the mice in the UP, UO, and UR groups exhibited significantly reduced changes in footpad thickness compared with the VH group. Upon examination of sections subjected to hematoxylin and eosin staining, the mice in both the NA group and treatment groups displayed minimal lymphocyte infiltration. On the other hand, there was obvious cell infiltration at the challenge sites in the mice of the VH group (Fig. 1C). Simultaneously, the VH group displayed a noticeable increase in epithelial thickness at the challenge sites compared with the NA group. Conversely, the mice in the UP, UO, and UR groups exhibited a reduction in epithelial thickness (Fig. 1D). The histopathology of the footpad tissues demonstrated that administration with UP, UO, or UR to OVA-sensitized mice could alleviate the local inflammatory response induced by the OVA challenge, providing solid evidence demonstrating the protective effects of UP, UO, and UR against DTH reactions.

Influence of UP, UO, and UR on serum antibody production in mice with DTH

In the DTH mice, the serum concentrations of total IgG, IgG_1, IgG_{2a}, and OVA-specific IgG were measured to understand the impacts of the *Ulva* samples on humoral immunity. Remarkably, the VH group mice exhibited significantly higher levels of antibodies compared with the NA group (Fig. 2), indicating successful OVA sensitization and challenge. Interestingly, administration of the *Ulva* samples resulted in a noteworthy suppression of the production of OVA-specific IgG (Fig. 2A and 2B). Additionally, treatment with the *Ulva* samples led to the up-regulation of IgG_1 production and down-regulation of IgG_{2a} production (Fig. 2C and 2D). Nonetheless, treatment with the *Ulva* samples did not result in significant changes in total IgE or OVA-specific IgE production (Fig. 2E and 2F).

Modulatory effects of UP, UO, and UR on splenic and local cytokine production

The levels of cytokines in the supernatants of OVA-stimulated splenocytes were quantified to investigate whether treatment with the *Ulva* samples modulated allergen-specific T cell immune responses and pro-inflammatory cytokine production.

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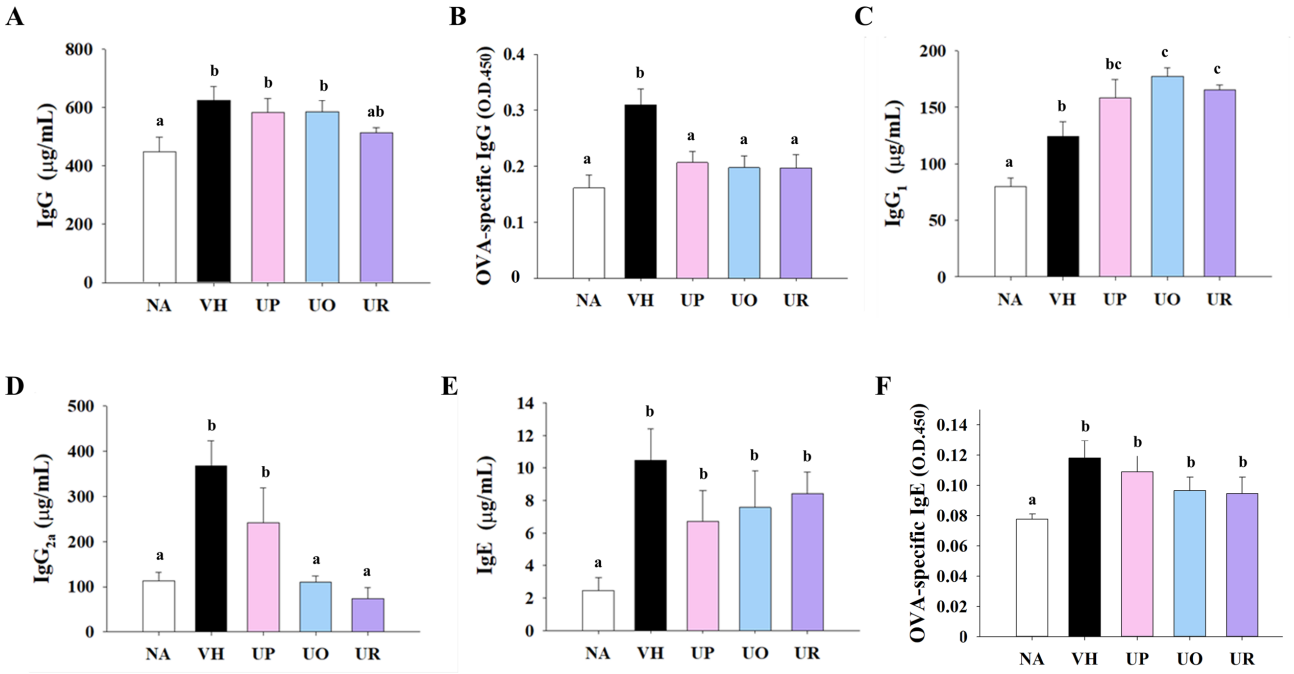

Fig. 2. Impact of the *Ulva* samples on serum antibody production in mice with delayed-type hypersensitivity (DTH). Individual serum samples were collected from each mouse before sacrifice, and the concentrations of A) total IgG, B) ovalbumin (OVA)-specific IgG, C) IgG_1, D) IgG_{2a}, E) total IgE, and (F) OVA-specific IgE were determined by enzyme-linked immunosorbent assay (ELISA). The data presented here are expressed as mean ± standard error of the mean (SEM) (n=5) values derived from three separate and independent experiments. Values with different letters are significantly different ($p<0.05$). NA: naïve group; VH: vehicle group; UP: polysaccharides; UO: oligosaccharides; UR: residues.

As shown in Fig. 3, a substantial increase in IFN-γ, IL-4, IL-10, and TNF-α production was observed in the VH group, signifying the successful induction of allergen-specific immune responses. In line with the profile of serum antibody production, treatment with the *Ulva* samples down-regulated IFN-γ secretion and up-regulated IL-4 secretion, further supporting their activities related to modulation of the Th1/Th2 immune balance (Fig. 3A and 3B). Furthermore, administration of the *Ulva* samples did not result in any significant alteration in the production of IL-10 but did lessen the level of TNF-α (Fig. 3C and 3D), suggesting that the anti-inflammatory effects of the *Ulva* samples may not result from enhancement of Treg cell activity.

Since Th1 cells and macrophages are essential for triggering the DTH reaction at the allergen exposure site, the levels of IFN-γ and TNF-α, which are major cytokines secreted by activated Th1 cells and macrophages, in the footpad tissue were determined by IHC staining [9, 10, 12]. OVA sensitization and challenge resulted in a significant up-regulation of IFN-γ and TNF-α expression in the footpad tissues (Fig. 4). However, the levels of IFN-γ and TNF-α were significantly diminished in mice treated with the *Ulva* samples (Fig. 4), strengthening the linkage between the improvement of footpad inflammation and suppression of Th1 cell and macrophage activation.

DISCUSSION

Currently, there is limited information available regarding the impact of *Ulva* on allergic disorders. However, a few studies have revealed immunostimulatory activities of *Ulva* extracts. For example, treatment with the water-soluble fraction of a methanol extract from *Ulva* (10 μg/mL) induced nitric oxide production from Raw 264.7 macrophages and increased alkaline phosphatase activity in murine splenocytes [19]. Oral treatment of *Ulva* polysaccharides (200 or 400 mg/kg) restored the proliferation of splenocytes and production of nitric oxide and cytokines in cyclophosphamide-induced immunodeficient mice [20]. In contrast, immunomodulatory and anti-inflammatory activities of *Ulva* extracts have been reported in previous studies. COX-2 activity and human red blood cell degradation were inhibited by an *Ulva* extract tested at a concentration of 500 μg/mL [21]. Oral treatment with an *Ulva* polysaccharide extract (300 mg/kg) reduced the TNF-α production in a murine model of kidney injury [22]. In a rat model of carrageenan-induced acute inflammation, *Ulva* polysaccharide extracts (100 mg/kg) attenuated paw edema and suppressed the levels of myeloperoxidase and malondialdehyde [23]. Moreover, in a murine model of dextran sulfate sodium-induced inflammatory bowel disease, oral treatment with selenized *Ulva* polysaccharides (100 mg/kg) inhibited the infiltration of white blood cells into the intestine and the production of pro-inflammatory cytokines [8]. The outcomes of our study are in line with these findings, providing additional evidence for the anti-inflammatory potential of the *Ulva* samples. It is worth noting that in our pilot study, a dosage of 250 mg/kg body weight was found to significantly alleviate footpad swelling, and the dosages of UP, UO, and UR used in this study fell within the range reported in previous studies, as mentioned earlier.

The composition of immunoglobulin isotypes undergoes significant modulation depending on the balance of Th1 and Th2 responses within the immune system. This area has been extensively explored, particularly in murine studies, where the

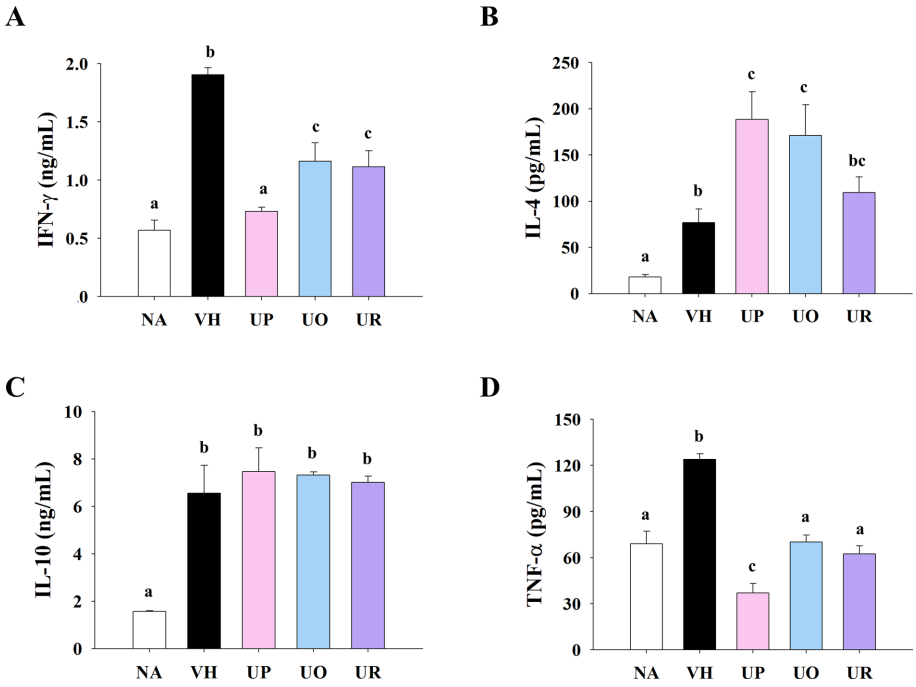

Fig. 3. Influence of the *Ulva* samples on modulation of splenic cytokine production. Splenocytes were isolated from the spleen of each mouse and subsequently prepared for experimentation. These prepared splenocytes were incubated in the presence of ovalbumin (OVA) for a period of 24 to 72 hr. After the incubation period, the supernatants from the cultured splenocytes were collected for further analysis. The concentrations of various cytokines, namely A) IFN-γ, B) IL-4, C) IL-10, and D) TNF-α, were measured by enzyme-linked immunosorbent assay (ELISA). The data presented here are expressed as mean ± standard error of the mean (SEM) (n=5) values derived from three separate and independent experiments. Values with different letters are significantly different ($p<0.05$). NA: naïve group; VH: vehicle group; UP: polysaccharides; UO: oligosaccharides; UR: residues.

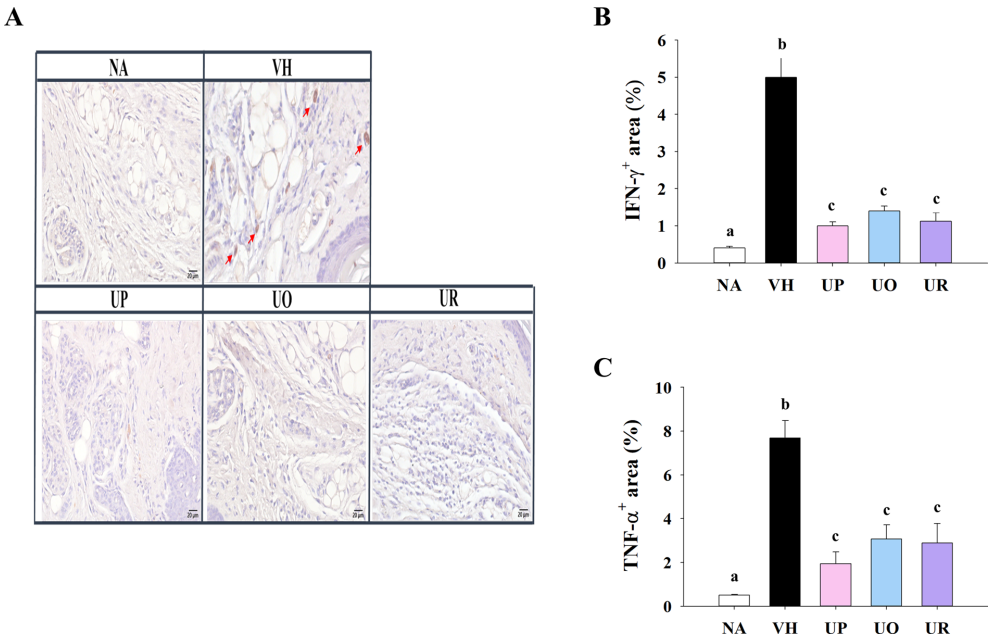

Fig. 4. Immunohistochemical (IHC) staining of duodenal sections was conducted to investigate the levels of IFN-γ and TNF-α expression. A) Representative images of IHC stained sections for IFN-γ are shown. Red arrows (brown signals) indicate the IFN-γ positive area. The ratios of the areas of B) IFN-γ- and C) TNF-α-positive signals to the total tissue area were determined with the ImageJ software. The data presented here are expressed as mean ± standard error of the mean (SEM) (n=5) values derived from three separate and independent experiments. Values with different letters are significantly different ($p<0.05$). NA: naïve group; VH: vehicle group; UP: polysaccharides; UO: oligosaccharides; UR: residues.

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levels of IgG_1 (with a limited Fcγ receptor affinity) and IgG_{2a} (possessing strong complement fixing ability and a high Fcγ receptor affinity) have been linked to the prevalence of Th1/Th2 immune responses. Th2-dominant responses are influenced by IL-4 and tend to foster higher IgG1/IgG2a ratios, whereas Th1 patterns are driven by IL-12 and IFN-γ and exhibit elevated IgG2a/IgG1 ratios [24–26]. Based on the results of the serological tests in this study, the *Ulva* samples may have the potential to shift the Th1/Th2 balance toward Th2 polarization. Therefore, their impact on the Th1/Th2 balance was further explored by measuring the levels of relevant cytokines of T cell subsets.

There are only a few studies available that have investigated the impacts of algae and algal derivatives on DTH responses, and their results are controversial. For instance, polysaccharides from *Petalonia binghamiae* and *Spirulina pacifica* complex were found to reduce the production of inflammatory cytokines through TLR4, thus mitigating the DTH response [27]. In a rat model of DTH, treatment with a *Eucheuma cottonii* extract (150 and 300 mg/kg) attenuated paw inflammation [28]. Previous studies have also shown the effect of *Porphyra* polysaccharides on suppression of serum IgE production in rodents with DTH [29, 30]. In contrast, protein hydrolysates from the alga *Chlorella vulgaris* (500 mg/kg) enhanced the reconstitution of the DTH response [31].

As shown above, it is clear that previous studies have yielded contradictory results regarding the effects of *Ulva* polysaccharides on the immune system. These inconsistencies may be attributed, in part, to the use of different cell and/or animal models in evaluating the impact of *Ulva* polysaccharides on the immune system. This variability in experimental approaches is a potential contributing factor to the contradictions observed in previous studies. Traditionally, polysaccharides and oligosaccharides have been viewed as T cell-independent antigens that do not directly stimulate T cells. Instead, their immunomodulatory effects typically rely on processes involving antigen processing and presentation by antigen-presenting cells, the activation of $CD4^+$ T cells, and the modulation of host cytokine responses [32]. Moreover, it is worth noting that the gut microbiota represents a potential target for non-digestible carbohydrates to exert their biological activities [33]. To gain a more comprehensive understanding of the mechanisms involved in enhancing Th2-type responses in this model, further in-depth investigations of antigen-presenting cells and gut microbiota are essential. In line with the findings of our current study, previous research showed that low-molecular-weight sulfated *Ulva* polysaccharides could inhibit the Th1 cell response and enhance the Th2 cell response, which was advantageous in mitigating the inflammatory damage induced by dextran sulfate sodium [34]. Currently, there is a lack of information regarding the influence of UP, UO, and UR on Th2-mediated type I hypersensitivity. Given that the pathogeneses of type I and type IV hypersensitivity differ, it is challenging to speculate about the effects of UP, UO, and UR on type I hypersensitivity based solely on our current results. Further investigations utilizing a type I hypersensitivity model are imperative to gain insights into the impacts of UP, UO, and UR on type I hypersensitivity.

Among the *Ulva* samples, UP had the least impact on modulating IgG_1 and IgG_{2a} production. In contrast, UP was the most potent in reducing IFN-γ and TNF-α production. As UP, UO, and UR exerted differences in potency in modulating antibody and cytokine production, it is suggested that specific subsets of immune cells targeted by UP, UO, and UR may be distinct due to their different contents of bioactive components. T cells and possibly macrophages, rather than B cells, are suggested to be the potential targets of UP. Previous studies have also revealed the critical role that molecular weight and functional groups play in the biological activities of polysaccharides and oligosaccharides [14, 34, 35]. In another unpublished study, we found differences in molecular weight and functional groups between UP and UO and significant differences in the composition of potential bioactive components, including total sugar, reducing sugar, peptides, total phenolics, and sulfate, within samples of UP, UO, and UR. Nonetheless, additional research into the structure-activity relationships will be necessary to confirm this hypothesis.

The results obtained from this study emphasize the ability of UP, UO, and UR to down-regulate allergen-specific Th1 and Th2 immune responses and suppress systemic and local pro-inflammatory cytokine TNF-α production, leading to the suppression of DTH reactions. Accordingly, UP, UO, and UR hold promise as potential therapeutic agents for managing Th1-dominant immune disorders. However, further investigations are necessary to fully understand the underlying mechanisms and long-term effects of UP, UO, and UR in the context of allergic responses and immune regulation.

CONFLICT OF INTEREST

The authors declare no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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