

FcεRI Cluster Size Determines Effective Mast Cell Desensitization without Effector Responses in vitro

Yuka Nagata Ryo Suzuki

Laboratory of Hygienic Chemistry, Institute of Medical, Pharmaceutical and Health Sciences, Faculty of Pharmaceutical Sciences, Kanazawa University, Kanazawa, Japan

Keywords

Ovalbumin · Mast cell · Desensitization · Immunoglobulin E · High-affinity receptor for immunoglobulin E

Abstract

Background: Allergen-specific desensitization of mast cell (MC) IgE receptors (FcεRI) is an important mechanism of allergen-specific immunotherapy that enables tolerance induction via systemic desensitization. Experimental in vitro IgE-mediated MC desensitization is a potential tool to understand the molecular mechanisms underlying this therapy. Desensitized MCs exhibit internalized IgE and its FcεRI receptors in response to suboptimal doses of allergen without provoking activation. The ovalbumin (OVA) allergen exhibits altered allergenicity upon heat treatment. MC reactions are fundamentally regulated by allergen features (i.e., allergenicity); however, the effects of allergenicity on desensitization remain unclear. **Objectives:** This study aimed to examine the impact of allergenicity on the establishment of in vitro MC desensitization using naive OVA (nOVA) and heated OVA (hOVA), which could induce varying MC effector responses. **Method:** Bone marrow-derived MCs (BMMCs) were sensitized with OVA-specific IgE, desensitized with sequentially increasing doses of nOVA or hOVA at 10-min intervals, and challenged with nOVA. To evaluate desensitization, the cell surface expression level and subcellular localization of FcεRI-bound IgE were analyzed before and after the final

nOVA challenge. MC activation was determined by measuring the release of β-hexosaminidase into supernatants. **Results:** Desensitized cells exhibited impaired activation following OVA challenge. Both nOVA and hOVA induced BMMC desensitization under different conditions. Formation of small IgE-FcεRI cluster BMMCs, which adequately represent the desensitized state, was significant. The size of the internalized IgE-FcεRI clusters might be correlated with the desensitized state of MCs. **Conclusions:** We demonstrate that the optimal size of IgE-FcεRI clusters for in vitro BMMC desensitization differed significantly depending on allergenicity, and the efficacy of desensitization was reflected by IgE-FcεRI cluster formation. Our study provides information on the characteristics of IgE-FcεRI internalization for successful desensitization in vitro.

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Introduction

Allergen-specific desensitization is the most effective therapy for allergies. Anaphylaxis is initiated by robust degranulation of mast cells (MCs) or basophils. Successful desensitization, which is induced by repeated treatments with a specific allergen, renders these cells less responsive or nonresponsive [1, 2]. The altered magnitude of mediators

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released from cells represents the first step in modulating the subsequent development of Th2-biased responses; this can lead to immunological tolerance to a specific allergen [3, 4]. Allergen-induced rapid desensitization of in vitro cultured MCs has been used to investigate molecular events and cell-specific activities during systematic desensitization [5–7]. In vitro MC desensitization is achieved by exposure to sequentially increasing suboptimal allergen doses, which results in a decrease in their cell surface IgE receptor (FcεRI)-bound IgE; this is explained by the internalization of IgE-FcεRI [5–8]. However, the mechanisms underlying successful desensitization are not fully understood.

The allergenic properties of food proteins often change through heating or digestion [9, 10]. For instance, ovalbumin (OVA), an abundant protein in egg whites and an ideal allergen to assess immune reactions, is easily denatured during thermal processing [11–13]. MC responses to heated OVA (hOVA) challenge have been reported to differ from those of naive OVA (nOVA) [14]. OVA undergoes denaturation and aggregation upon heating and exhibits altered allergenicity under in vitro experimental conditions [14]. OVA is also known to induce IgE-FcεRI internalization in a desensitization protocol [6]. MC desensitization requires sequential exposure to appropriate doses of allergen. Furthermore, an appropriate intensity of allergen against IgE may be an important factor for successful desensitization (i.e., without MC degranulation). However, the underlying molecular mechanisms, especially in the context of desensitized IgE-FcεRI dynamics and functions during desensitization, remain unclear. In this study, we utilized both nOVA and hOVA for allergen-specific desensitization and investigated the effects of allergenicity on successful desensitization in MCs through IgE-FcεRI internalization.

Materials and Methods

Mice

Female C57BL/6N mice aged 6–8 weeks were used in this study and housed in the animal facility of Kanazawa University under

controlled temperature ($23 \pm 2^\circ\text{C}$), humidity ($50 \pm 10\%$), and light (12/12-h light/dark cycle). All animal studies were approved by the Animal Experiment Committee of Kanazawa University and were conducted according to the “Guide for the Care and Use of Laboratory Animals” (NIH Publication No. 85-23, revised 1996).

Cells and Stimulation

Bone marrow-derived MCs (BMMCs) were generated from bone marrow cells harvested from C57BL/6N mice using a previously established method [15]. Differentiation was confirmed by flow cytometry after staining with anti-c-Kit-Alexa Fluor 647 (BioLegend, San Diego, CA, USA) and anti-FcεRIα-Alexa Fluor 488 (BioLegend) antibodies. More than 90% of the cells were c-Kit⁺ FcεRI⁺. BMMCs were then sensitized with 1.5 μg/mL OVA-specific IgE antibody (Chondrex, Redmond, WA, USA) and challenged with different doses of OVA (OVA grade V; Sigma, St. Louis, MO, USA). Degranulation was determined using the β-hexosaminidase release assay [15]. The concentrations indicated in this study were the final concentrations obtained after experimental dilution.

In vitro Desensitization

IgE-sensitized BMMCs were subjected to allergen-specific desensitization with increasing doses of OVA. The sensitized cells were washed and seeded at 5×10^4 cells/well in a V-bottom 96-well plate at a volume of 18 μL. Allergen-induced desensitization was performed by exposing the cells to increasing doses of nOVA/hOVA (low-dose protocol: 0.002, 0.005, 0.01, 0.05, 0.25, 0.5, 1, 2, 4, and 8 ng/mL; high-dose protocol: 0.2, 0.5, 1, 5, 25, 50, 100, 200, 400, and 800 ng/mL; all expressed as final concentrations) at 10-min intervals as described in the main figure (Fig. 1b, 2c). After the last 10 min of treatment, the cells were challenged with 2 μg/mL OVA without any washing step. All steps were performed with Tyrode's buffer. Desensitization of BMMCs was confirmed by the surface expression of FcεRI and degranulation responses.

Flow Cytometry

Challenged or unchallenged BMMCs were fixed with 4% paraformaldehyde. Anti-IgE-fluorescein isothiocyanate (BD Biosciences, San Jose, CA, USA) was used to label the fixed and nonpermeabilized cells. Flow cytometry was then performed using a FACSVerser flow cytometer (BD Biosciences).

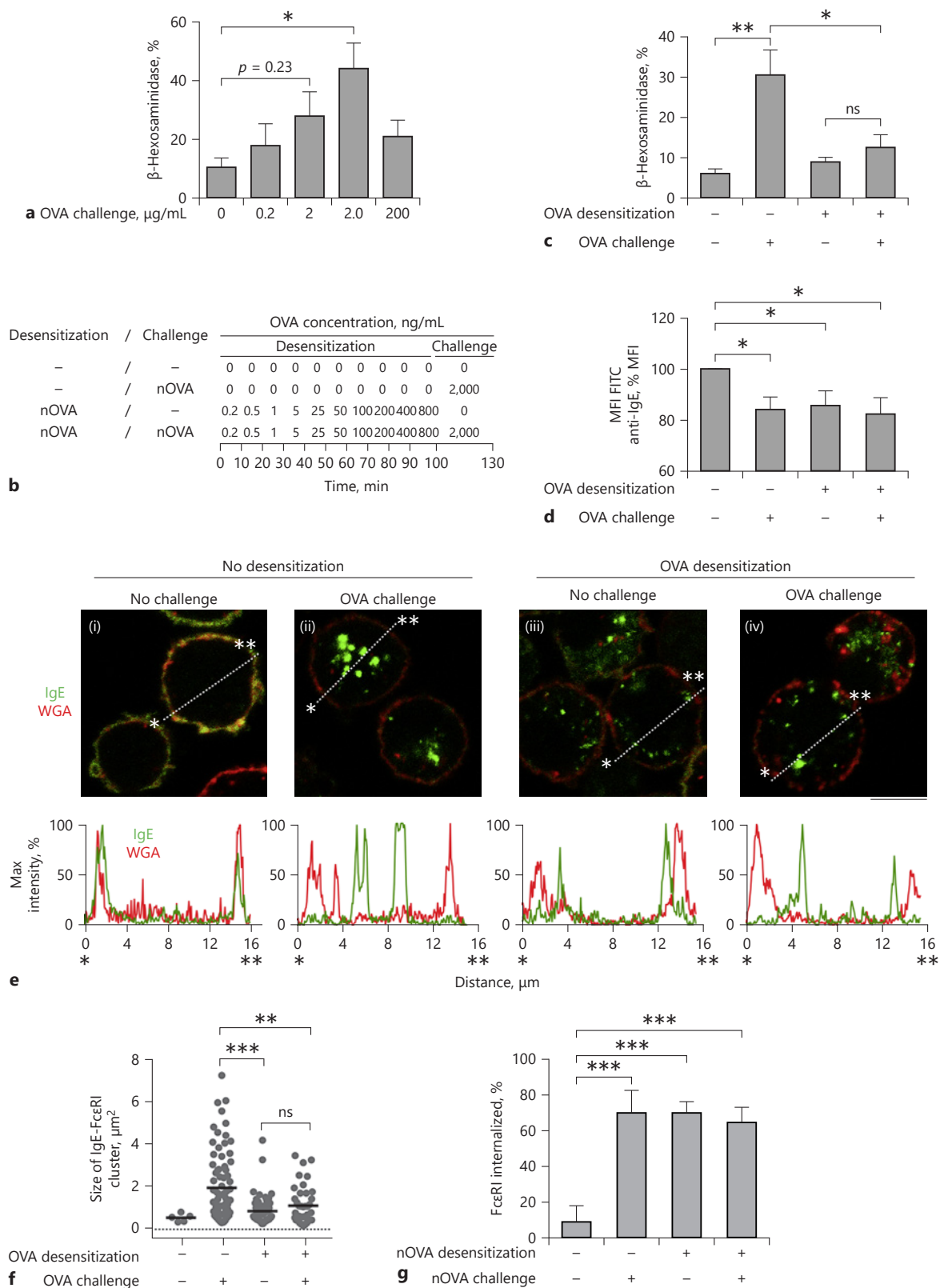
Immunocytochemistry

Challenged BMMCs were fixed with 4% paraformaldehyde, permeabilized with 0.5% Triton X-100, and labeled with anti-IgE-fluorescein isothiocyanate. For plasma membrane staining, wheat germ agglutinin (WGA)-Alexa Fluor 647 was added before fixation/permeabilization. Images were acquired using an LSM710

Fig. 1. OVA induced BMMC degranulation and desensitization. **a** BMMC degranulation rate following OVA challenge. **b** Desensitization protocol. OVA was dissolved in Tyrode's buffer and applied to sensitized cells at the indicated concentrations and time intervals. **c, d** BMMC degranulation rate (**c**) and surface IgE expression level (**d**) following OVA desensitization and OVA challenge. **e** Confocal microscopy images (scale bar, 10 μm) and line profiles of fluorescence intensity (green: IgE, red: WGA as a plasma membrane marker). **f** Average size of IgE-FcεRI clusters. **g** The

rate of FcεRI (IgE-bound) internalization is expressed as the ratio of surface to intracellular FITC-IgE fluorescence intensity. **a, c, d** Data are presented as mean $\pm$ SE of 3–5 independent experiments. Data are representative images (**e**) and 10 cells/sample (**f, g**) of 3 or 4 independent experiments. None of the images shown was modified by nonlinear adjustments. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; ns, nonsignificance; OVA, ovalbumin; BMMC, bone marrow-derived mast cell; WGA, Wheat Germ Agglutinin.

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confocal laser scanning microscope (CLSM; Carl Zeiss, Oberkochen, Germany) with a $\times 63$ magnification objective. Fluorescence intensity was analyzed using ImageJ software (NIH Image, Bethesda, MD, USA) and MIPAV software (NIH Image). None of the images were modified by nonlinear adjustments.

Immunoblotting

Native-PAGE was performed according to previously described methods with some modifications [16]. In brief, hOVA was prepared by boiling OVA for 1 min (OVA: 20 mg/mL, 100 μ L) at 100°C. The heat-treated and untreated OVAs were subjected to native-PAGE (Wako Chemicals, Osaka, Japan) followed by immunoblot analysis using anti-OVA antibody (Morinaga Institute of Biological Science, Kanagawa, Japan).

Statistical Analysis

Statistical significance was assessed using GraphPad Prism (version 7.0d; GraphPad Software, San Diego, CA, USA). Statistical differences were determined using the 1-way analysis of variance (ANOVA) followed by Tukey's or Dunnett's multiple-comparison test (for comparing multiple groups) and 2-way ANOVA followed by Fisher's least significant difference test (for pairwise comparisons). Statistical significance was set at $p < 0.05$.

Results

Cultured BMMCs Desensitized to OVA Showed Fc ϵ RI-IgE Internalization

We first evaluated degranulation responses under different concentrations of OVA (0–200 μ g/mL). OVA-challenged cells were degranulated with a maximum of 20 μ g/mL OVA (Fig. 1a). Based on these results, we established a desensitization protocol for OVA-specific IgE-sensitized BMMCs (Fig. 1b). After desensitization, BMMCs exhibited significantly decreased degranulation compared to untreated cells after the final OVA challenge (Fig. 1c). Furthermore, we observed a significant reduction in the cell surface Fc ϵ RI-bound IgE on the BMMCs (Fig. 1d). These data indicate that IgE-Fc ϵ RI internalization occurs during desensitization. To evaluate the subcellular localization of IgE-Fc ϵ RI after desensitization, we conducted confocal laser scanning microscopy experi-

ments. As shown in Figure 1e, surface IgE-Fc ϵ RI (Fig. 1e [i]) was internalized, and intracellular compartments were observed in both OVA-challenged (Fig. 1e [ii, iv]) and desensitized cells (Fig. 1e [iii]). The extent of internalization of IgE-Fc ϵ RI was analyzed by determining the ratio of fluorescence intensity for plasma membrane IgE and intracellular IgE; however, no significant differences were observed among the treatments (Fig. 1g). These data are consistent with the results of flow cytometric analysis shown in Figure 1d. However, the intracellular IgE-Fc ϵ RI clusters, especially in the context of cluster size, appeared to be different, as shown in Figure 1f; this suggests IgE-Fc ϵ RI cluster formation as a relevant characteristic of desensitization.

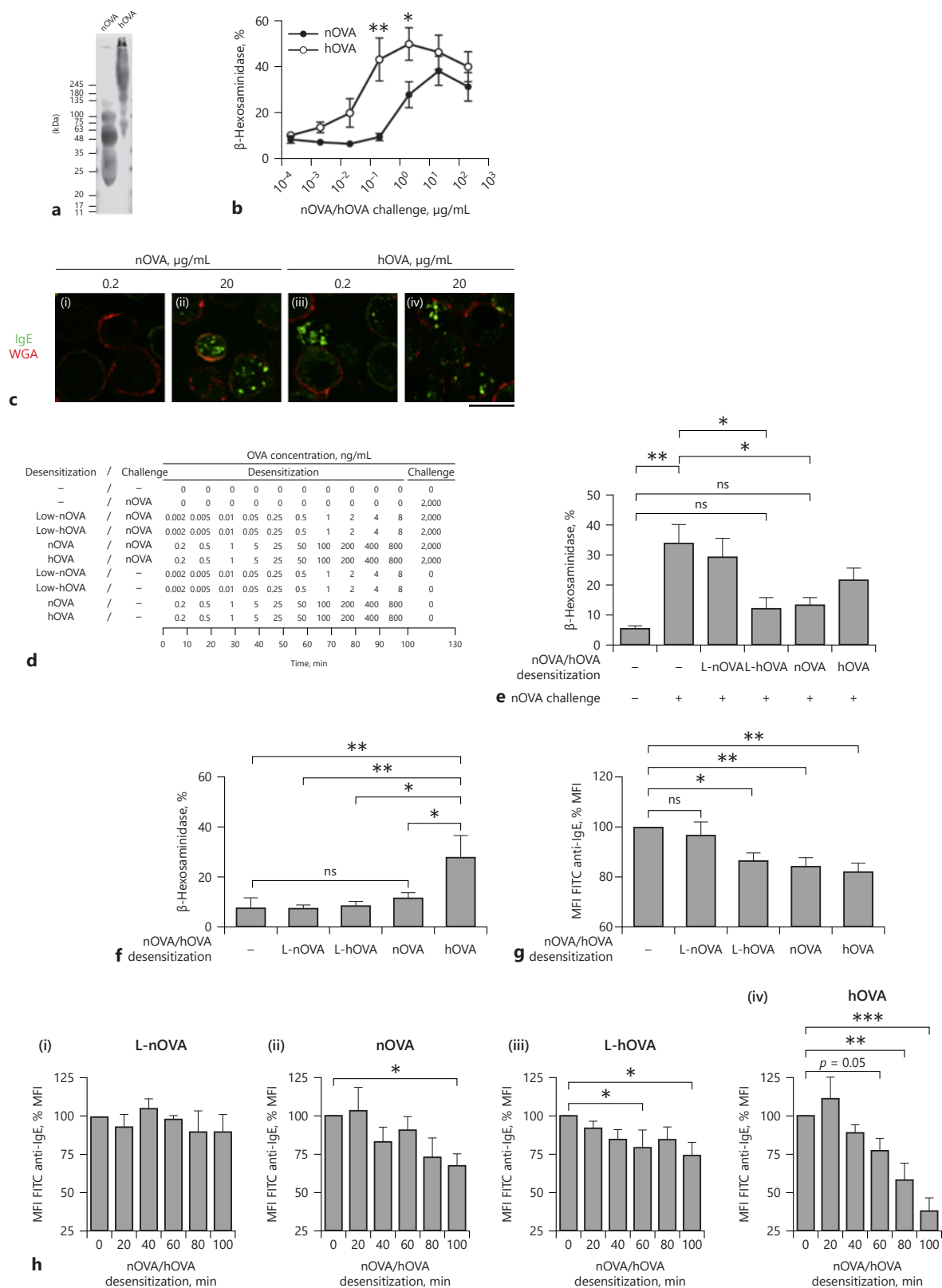
Desensitization Efficiency Differs between Naive and Heated OVA

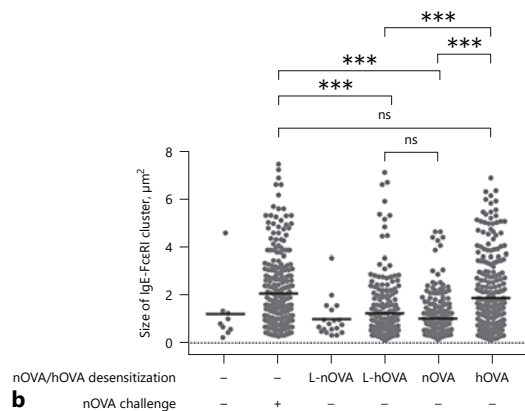
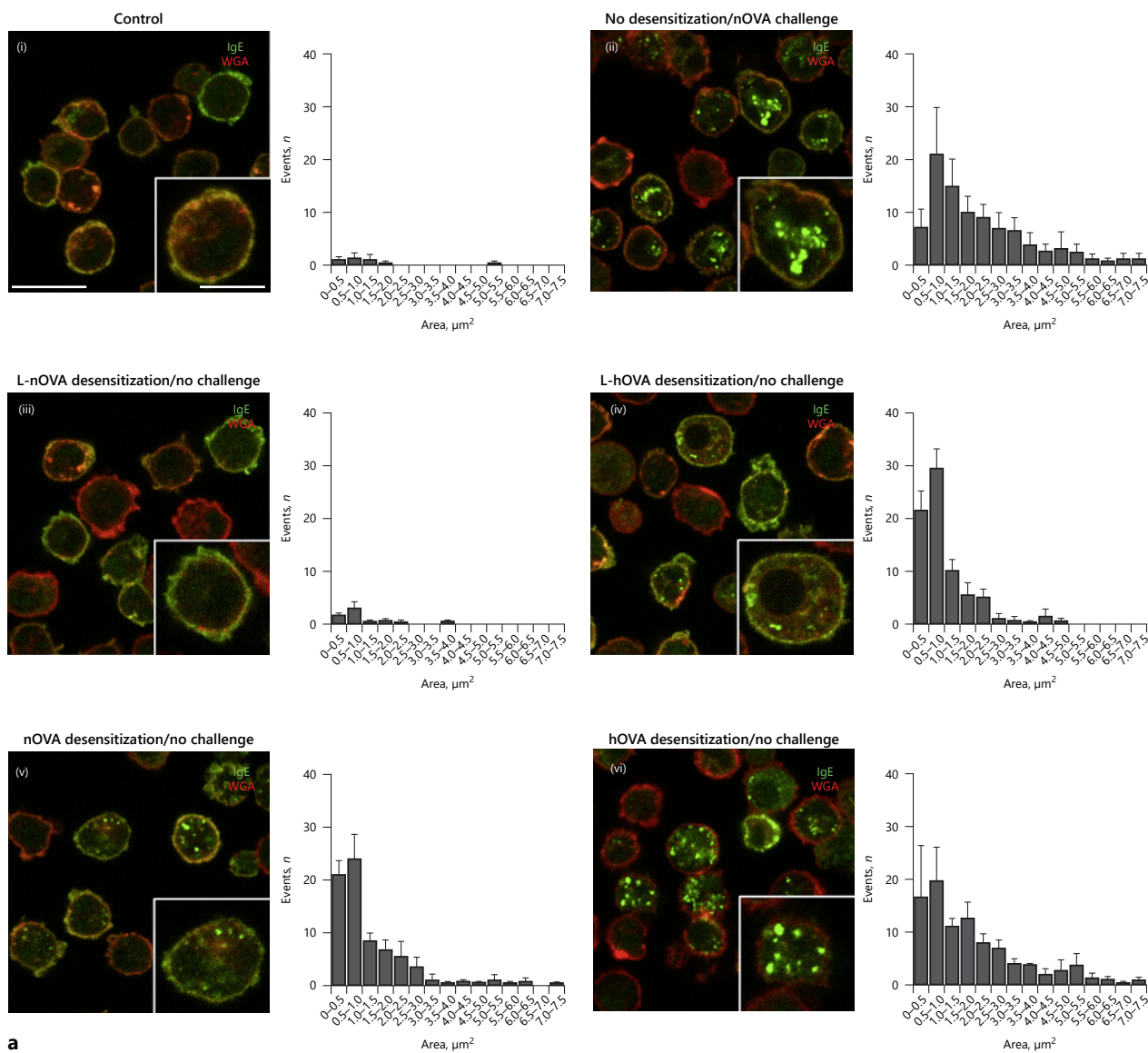
Thermal processing of OVA is known to enhance its allergenicity [17, 18], suggesting different potencies of naive OVA (nOVA) and heated OVA (hOVA) for MC desensitization in vitro. Therefore, we compared the consequences of nOVA and hOVA desensitization. Furthermore, we investigated the characteristics of BMMCs desensitized using allergens with different allergenicities. hOVA was prepared by boiling OVA for 1 min (OVA: 20 mg/mL, 100 μ L) at 100°C. The heat-treated and untreated OVAs were subjected to native-PAGE followed by immunoblot analysis using anti-OVA antibody. hOVA was much larger than the predicted molecular size of OVA (45 kDa), which indicated the heat-induced aggregation of OVA (Fig. 2a). As shown in Figure 2b, hOVA challenge clearly induced greater degranulation compared with nOVA challenge at all doses. The confocal microscopy images of IgE-Fc ϵ RI clusters in nOVA- and hOVA-challenged BMMCs appeared to be consistent with respect to the degree of degranulation shown in Figure 2b (Fig. 2c). nOVA challenge at 0.2 μ g/mL, which does not induce degranulation, did not induce significant internalization of IgE-Fc ϵ RI clusters, although the cell-surface IgE-Fc ϵ RI level seemed to be diminished (Fig. 2c [i]). However,

Fig. 2. Optimal desensitization settings differed with the thermal treatment of OVA. **a** Native-PAGE immunoblot analysis of nOVA and hOVA. **b** BMMC degranulation rate following nOVA and hOVA challenge. OVA was dissolved in water at the concentration of 20 mg/mL. Thereafter, hOVA and nOVA were diluted in Tyrode's buffer and added to sensitized cells at the indicated concentrations. **c** Confocal microscopic images (scale bar, 10 μ m). Data are presented as mean $\pm$ SE of 7–10 independent experiments (**b**) and a representative image from 3 independent experiments (**c**). **d** De-

sensitization protocol. OVA was dissolved in water at the concentration of 20 mg/mL. Thereafter, hOVA and nOVA were diluted in Tyrode's buffer and added to sensitized cells at the indicated concentrations and time intervals. **e–h** BMMC degranulation rate and cell surface IgE expression level following OVA desensitization with (**e**) or without (**f–h**) the final nOVA challenge. Data are presented as mean $\pm$ SE of 4–7 (**e–h**) independent experiments. * $p < 0.05$; ** $p < 0.01$. OVA, ovalbumin; hOVA, heated OVA; nOVA, naive OVA; BMMC, bone marrow-derived mast cell.

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BMMCs stimulated with nOVA (20 µg/mL; Fig. 2c [ii]) or hOVA (0.2 and 20 µg/mL; Fig. 2c [iii, iv]) showed significant numbers of IgE-FcεRI clusters and induction of degranulation responses to the same extent (Fig. 2b).

Allergenicity Determines the Optimal Conditions for FcεRI-IgE Desensitization

We sought to examine whether the differences between nOVA and hOVA could be reflected in their ability to induce BMMC desensitization. To verify this hypothesis, we designed 2 desensitization protocols with different OVA dose ranges, as shown in Figure 2d. We then examined the degranulation and surface IgE-FcεRI expression in BMMCs (Fig. 2e–h). For both nOVA and hOVA, one protocol included 1/100 times lower concentrations than those used in the other protocol (referred to as low-nOVA and low-hOVA) (Fig. 2d). Low-nOVA failed to induce desensitization (Fig. 2e, L-nOVA) and IgE-FcεRI internalization (Fig. 2g, L-nOVA). In contrast, low-hOVA successfully induced BMMC desensitization (Fig. 2e, L-hOVA), whereas hOVA failed to induce desensitization (Fig. 2e, hOVA). The high concentration range of the hOVA protocol tended to induce undesirable activation (i.e., degranulation) during desensitization (Fig. 2f, hOVA). The cell pellets separated from the supernatant used in the experiment presented in Figure 2e were also analyzed for their surface IgE-FcεRI expression, and the results showed that the desensitized and inactivated cells decreased the expression of IgE-FcεRI to the same extent as those of the challenged and activated cells (data not shown). Moreover, we performed flow cytometric analysis, which revealed varying, time-dependent reduction of surface IgE levels in the desensitized cells (Fig. 2h). Low-nOVA failed to induce IgE-FcεRI internalization (Fig. 2h [i]). Both nOVA and low-hOVA induced IgE-FcεRI internalization in lower rate compared with hOVA (Fig. 2h [ii–iv]). hOVA induced IgE-FcεRI internalization in higher rate accompanied with undesirable activation (i.e., degranulation) during desensitization

(Fig. 2h [iv]). Consistent with other data, varying allergenicity may affect IgE-FcεRI clustering and subsequent desensitization processes.

Clarification of FcεRI-IgE Cluster Size in OVA Desensitized in BMMCs

As shown above, the high- or low-concentration protocols of nOVA and hOVA differ in desensitization and effector MC response efficacies. Given the different conditions of nOVA/hOVA desensitization (successful or failed), we analyzed the distribution of IgE-FcεRI cluster sizes and evaluated the cluster sizes required for successful desensitization. To our knowledge, the relationship between the IgE-FcεRI cluster size and MC desensitization has not been fully investigated, although the IgE-FcεRI cluster is a critical factor for signal transduction and MC effector responses [15, 19–22]. As shown in Figure 3a, cells desensitized with nOVA (Fig. 3a [v]) and low-hOVA (Fig. 3a [iv]) exhibited small clusters, whereas cells activated with nOVA (Fig. 3a [ii]) and hOVA (Fig. 3a [vi]) exhibited large clusters of sizes >3.0 µm². Control cells (Fig. 3a [i]) and those exposed to low-nOVA treatment (Fig. 3a [iii]) did not exhibit obvious IgE-FcεRI clusters. The average size of the clusters was significantly different between the treatments (Fig. 3b), and this was consistent with their activation pattern after the final challenge, as shown in Figure 2e. These results indicated that small size and low content of IgE-FcεRI clusters are likely required to induce desensitization in BMMCs.

Discussion/Conclusion

We demonstrated that the optimal dose of OVA for in vitro MC desensitization differs significantly depending on the thermal treatment of OVA, wherein the efficacy of desensitization is reflected in intracellular IgE-FcεRI cluster formation. Food proteins are usually subjected to various heat treatments such as boiling and baking. Therefore, ap-

Fig. 3. Small but certain IgE-FcεRI clusters were formed in desensitized BMMCs. **a** nOVA/hOVA-desensitized cells used in the experiments were subjected to microscopic examination, and their cluster size distributions were analyzed using MIPAV software. IgE-sensitized control (**i**), nOVA-challenged control (no desensitization/nOVA challenge) (**ii**), and desensitized (low [L]-nOVA, L-hOVA, nOVA, and hOVA, respectively) and unchallenged cells (**iii–vi**). **b** Average size of the IgE-FcεRI clusters. Data are presented as mean ± SE of *n* = 30 cells from 3 independent experiments (5 cells per area, 2 areas per experiment), and

the representative images are shown (scale bar, 20 µm in large panels and 10 µm in inset panels). Average numbers of clusters per 10 cells are shown in (**a**). Average size of clusters (**b**) in all analyzed events (clusters) counted from 30 cells per sample from 3 independent experiments. None of the images shown was modified by nonlinear adjustments. Significance was measured using the 1-way ANOVA with Tukey's multiple-comparison test. ****p* < 0.001; ns, nonsignificance; OVA, ovalbumin; hOVA, heated OVA; nOVA, naive OVA; BMMC, bone marrow-derived mast cell.

appropriate strategies are necessary to achieve effective treatment [14]. Heating is known to induce protein denaturation, leading to reduced allergenicity of food proteins under normal physiological conditions [10]. However, in vitro studies have shown that allergenicity of food proteins is enhanced by heating [17, 18]. These contradictory observations could be explained by their differential susceptibility to digestion and/or absorption in the gastrointestinal tract [14, 23]. In addition, allergenicity appears to be either enhanced or reduced depending on the particular allergen [14]. OVA is widely used as an allergen to study FcεRI-mediated allergic responses. In this study, we evaluated the effects of allergenicity on the allergen-dependent desensitization of FcεRI. hOVA (1 min at 100°C) highly enhanced the degranulation responses of BMMCs (Fig. 2). As shown in Figure 2a, the OVA bands were observed at a higher molecular weight after heat treatment. These data indicated that heat treatment caused aggregation of OVA and may increase its valency, which resulted in highly enhanced degranulation responses of BMMCs (Fig. 2). The differential allergenicity of OVAs was reflected in their ability to induce IgE-FcεRI desensitization in BMMCs (Fig. 2e–h). hOVA required low-dose treatment compared with nOVA for successful desensitization in BMMCs. As shown in Figure 2e–h, an inappropriate dose of hOVA/nOVA failed to induce desensitization and caused degranulation responses during treatment or failed to induce IgE-FcεRI internalization (manifested as desensitization) throughout treatment.

We analyzed the localization of the IgE-FcεRI complex by confocal laser scanning microscopy and found that IgE-FcεRI cluster formation might be a relevant characteristic for successful desensitization. Indeed, the size of the IgE-FcεRI cluster is one of the critical factors for activating signal transduction and MC effector responses [19–22]. However, in the context of desensitization, it is uncertain whether IgE-FcεRI cluster formation is linked to the efficacy and/or mechanisms of allergen-induced desensitization. Small clusters of IgE-FcεRI induced by allergens have been observed in MCs with suboptimal and supraoptimal dose stimulations [19, 21]. Andrews et al. [19] revealed that small FcεRI clusters are mobile and signaling competent compared with large clusters stimulated with an optimal dose of allergen, suggesting that small IgE-FcεRI clusters may not be a simple diminished response. In the present study, we observed that cluster size may also be involved in successful desensitization (Fig. 3a [iv, v]). Mahajan et al. [21] reported that suboptimal/supraoptimal dose stimulations could induce tyrosine phosphorylation of Src homology 2-containing inositol phosphatase (SHIP) colocalized with FcεRI. The phosphorylation of SHIP after FcεRI aggregation is known

to negatively regulate MC degranulation [24–26] and is suggested to be crucial in the desensitization process [22, 27]. By comparing nOVA and hOVA, we found that allergenicity varies in its capacity to induce FcεRI desensitization in vitro. Our observations, combined with those of earlier studies, suggest that the small size of the IgE-FcεRI cluster may be a critical feature for successful desensitization.

Overall, this study using allergens with different allergenicities provides additional fundamental information regarding successful desensitization without effector responses in MCs. It also provides information regarding the characteristic features associated with tolerance to allergens. This is essential for establishing desensitization in MCs. However, a limitation of the current study is it does not reveal the molecular mechanisms following or accompanying small IgE-FcεRI cluster formation, including whether the formation is associated with immunoregulatory and tolerogenic properties. Furthermore, it does not identify a certain threshold in the context of cluster formation at which MC degranulation was induced and failed to establish desensitization state. Further studies are required to expand this study, overcome the limitations, and offer insights into the undetermined risks associated with failure in allergen-specific immunotherapy.

Statement of Ethics

All animal studies were approved by the Animal Experiment Committee of Kanazawa University and were conducted according to the “Guide for the Care and Use of Laboratory Animals” (NIH Publication No. 85-23, revised 1996).

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

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Author Contributions

Y.N. and R.S. designed and performed the research, analyzed the data, and wrote the manuscript.

Data Availability Statement

All data generated or analyzed during this study are included in this article. Further enquiries can be directed to the corresponding author.

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