

Regular Article

Ephedrine Alkaloid-Independent High-Affinity Immunoglobulin-E Receptor (FcεRI) Internalization Results in CCL2 Production without Inducing Mast Cell Degranulation

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Mast cells (MCs) play an important role in allergies, leading to the development of MC-targeted therapies. Ephedra herb (Mao) has potent anti-allergic activity, but contains ephedrine alkaloids (EAs); therefore, its hazardous effects are taken into consideration during its clinical use. We previously reported that Mao attenuates robust MC degranulation by an allergen through high-affinity immunoglobulin E (IgE) receptor (FcεRI) internalization, in which an EA-independent mechanism was suggested to be at play. This study aimed to deepen our understanding of the potential of Mao against FcεRI internalization using two strains with different EA contents. Mao extracts were administered to bone marrow-derived MCs (BMMCs), and their cellular responses, including FcεRI internalization, were analyzed. In addition, physiological events were evaluated using a passive cutaneous anaphylactic (PCA) reaction mouse model. BMMCs mediate the production of diverse inflammatory mediators. Among these, the potent chemokine CCL2 is thought to be differentially regulated from other pro-inflammatory mediators. We found that Mao significantly induces CCL2 expression in BMMCs despite suppressing robust degranulation through FcεRI internalization. Importantly, this was a distinctly EAs-independent response. In the PCA reaction, local MC activation following allergen challenge was suppressed by Mao treatment, which strengthened the view that Mao sufficiently decreased the rapid activation of MCs and promoted CCL2 secretion. Collectively, these observations provide additional insights into the mechanism of Mao-induced silent FcεRI internalization in MCs and the complex and heterogeneous secretory responses operating in MCs.

Key words Mao, ephedra herb, ephedrine alkaloid, chemokine, mast cell

INTRODUCTION

Allergen-induced mast cell (MC) activation initiates and shapes allergic inflammation.^{1,2)} FcεRI, the high-affinity immunoglobulin E (IgE) receptor, is considered a key regulator of MC-mediated allergic inflammation; therefore, the therapeutic benefits of FcεRI-targeting strategies have been explored.³⁾ Allergens cross-linked with FcεRI-bound IgE trigger elaborate signaling cascades in MCs, leading to the heterogeneous secretion of several biologically active mediators that influence disease development.⁴⁾

CCL2 is a chemoattractant of monocytes and macrophages and is involved in tissue damage and repair.⁵⁾ MCs are capable of secreting CCL2 chemokines; however, the secretion mechanism is likely to be different from that of other pro-inflammatory mediators such as histamine, leukotriene, and lysosomal enzymes like β-hexosaminidase.^{6–8)} MCs can respond differentially to various degrees of allergen stimuli, where FcεRI plays an important role in translating the stimulus intensity into an appropriate response.⁹⁾ FcεRI displays diverse responses to allergens, both on the plasma membrane and in the intracellular compartment (*i.e.*, distribution, clustering, and internalization).^{10,11)} These intrinsic dynamics of FcεRI in MCs contribute to deciphering various stimuli, both qualitatively and quantitatively.¹²⁾ Even under less than optimal stimulation, FcεRI undergoes dynamic changes, likely

representing small and mobile clusters and a slow internalization,^{11,13,14)} which is also recognized as a mechanism for the allergen-specific desensitization that the most effective therapy for allergies.¹⁵⁾ CCL2 is considered to be dominantly regulated in such an insignificant situation (*i.e.*, low allergen concentrations). In fact, stimulation with low-concentration and low-affinity allergens is known to induce CCL2 production in MCs without robust activation.^{6,7)} Thus, although CCL2 is a critical chemokine for monocytes/macrophages and mediates allergic inflammation, MC-produced CCL2 does not appear to be a simple activation marker for MC allergic responses. Overall, these observations suggest that CCL2 production in MCs might be a potential feature of the silent dynamics of FcεRI, which is considered a fundamental and critical mechanism for allergen-induced desensitization and can ultimately induce tolerance or sustained unresponsiveness.

Ephedra herb, called “Mao” in Japanese, is defined as the terrestrial stem of *Ephedra sinica* Stapf (Ephedraceae), which is an important medicinal plant containing ephedrine alkaloids (EAs). Mao is listed in the Japanese Pharmacopeia as a medicinal herb used in traditional Japanese medicine called “Kampo” and is found in several Kampo formulas, especially those prescribed for allergies, including Kakkonto, Shoseiryuto, Daiseiryuto, and Maoto. Mao exerts potent anti-allergic effects^{16,17)}; however, the precise mechanism by which Mao attenuates allergic inflammation remains to be

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elucidated. We previously demonstrated that Mao attenuates allergen-induced MC degranulation through FcεRI internalization, in which ephedrine alkaloid-independent mechanisms have been suggested but not fully validated.¹⁸⁾ These observations were made using murine bone marrow-derived mast cells (BMMCs). We applied Mao extract to BMMCs and analyzed the effects of Mao on the allergic responses of BMMCs by examining several cellular reactions, including the secretion of cytokines and chemokines.

Mao is known to induce FcεRI internalization in MCs without activation,¹²⁾ suggesting that Mao has the potential to induce CCL2 expression in MCs. The present study explored this possibility and further investigated the mechanisms underlying Mao-induced FcεRI silent internalization. We demonstrate ephedrine alkaloid-independent CCL2 induction by Mao, which may provide further supporting evidence for the potential of Mao to maintain allergen-induced desensitization.

MATERIALS AND METHODS

Mice and Cell Culture C57BL/6N mice were utilized for this study and housed in the animal facility of Kanazawa University. All animal studies were approved by the Animal Experiment Committee of Kanazawa University, Japan. BMMCs were generated from mice using an established method.⁷⁾ Differentiation was confirmed as previously described,¹⁸⁾ in which more than 90% of the cells were c-Kit⁺ FcεRI⁺.

Crude Extract Preparation A commercial Mao sample was obtained from Uchida Wakanyaku Ltd. (Tokyo, Japan, Lot. 452226). This drug has been approved by the Ministry of Health, Labour, and Welfare of Japan (MHLWJ). The original Mao strains were grown in a cultivation station in Ishikawa Prefecture, which is controlled by Kanazawa University. Preparation and ultra-performance LC-mass spectrometry (UPLC-MS) analysis of the crude extracts were performed as described in our previous publication.¹⁸⁾ All materials used in this study were stored in the Specimen Room, Kanazawa University, Japan.

Cell Stimulation and Analysis The BMMCs were sensitized with anti-2,4-dinitrophenol (DNP)-IgE antibody (1 μg/mL) and then challenged with DNP-human serum albumin (HSA) with or without Mao (100 μg/mL, 0.1% water). For stimulation with both Mao and DNP-HSA, the IgE-sensitized cells were pretreated with Mao for 30 min before DNP-HSA was added into the culture medium in the presence of Mao. While in the only Mao stimulation, the cells were incubated with Mao for 30 min. Degranulation was determined using a β-hexosaminidase assay, according to an established method.⁷⁾ The concentrations indicated in this study are the final concentrations after the experimental dilution. RNA isolation and relative quantification by quantitative reverse transcription PCR (qRT-PCR) were performed as previously described.¹⁸⁾ The primer sequences are available upon request. An enzyme-linked immunosorbent assay (ELISA) was performed on cell supernatants collected 60 min after DNP-HSA stimulation. The release of CCL2 was determined using an ELISA kit (R&D Systems, Minneapolis, MN, U.S.A.) according to the manufacturer's instructions. Fluorescence-activated cell sorting (FACS) and fluorescein-isothiocyanate (FITC)-conjugated antibodies were used to determine surface IgE levels as previously described.¹⁸⁾

Immunocytochemistry BMMCs were fixed with 4%

paraformaldehyde, permeabilized with 0.5% Triton X-100, and labeled with anti-IgE-fluorescein-isothiocyanate (FITC). For plasma membrane staining, wheat germ agglutinin (WGA)-Alexa Fluor 647 was added before fixation/permeabilization. Images were acquired using an LSM710 confocal laser scanning microscope (CLSM; Carl Zeiss, Oberkochen, Germany) with a ×63 magnification objective lens. Fluorescence intensity was analyzed using ImageJ software (NIH Image, Bethesda, MD, U.S.A.). None of the images were modified by nonlinear adjustments.

Passive Cutaneous Anaphylaxis IgE-mediated passive cutaneous anaphylaxis (PCA) in mice was performed as previously described.⁷⁾ Anti-DNP-IgE dissolved in normal saline were administered subcutaneously. Additionally, after 18 h, 30 μL of Mao extract dissolved in normal saline (100 μg/mL, 0.1% water) was administered subcutaneously. Mice were challenged with DNP-HSA 24 h after anti-DNP-IgE administration. The ears were whole-mount immunostained as previously described.⁷⁾ This study used anti-CD31-Alexa 647 (BioLegend, San Diego, CA, U.S.A.) and FITC-conjugated Avidin. Images were acquired using an LSM710 confocal laser scanning microscope (CLSM) (Carl Zeiss) with a ×20 magnification objective lens.

Statistical Analysis Statistical significance was assessed using GraphPad Prism version 7.0 (Graphpad Software, San Diego, CA, U.S.A.). Statistical differences were determined by one-way ANOVA followed by Tukey's or Dunnett's multiple-comparison test, as appropriate (for comparing multiple groups). Statistical significance was determined at *p* values less than 0.05.

RESULTS

Mao Induces Significant CCL2 Production in BMMCs, Which Is Independent of EAs Cultured BMMCs were treated with Mao extract—a commercial product containing a significant amount (>0.7%) of EAs—and subsequently stimulated with DNP-HSA allergen in the presence of Mao for 60 min. Mao treatment significantly induced CCL2 mRNA expression and protein production in BMMCs. Specifically, mRNA induction was a 20 to 30-fold higher in Mao treatment, even without allergen stimulation (Fig. 1). Considering the contradiction between CCL2 release and mRNA expression in DNP-HSA-stimulated cells, allergen-induced MC activation resulted in the secretion of pre-existing and newly synthesized inflammatory mediators.¹⁹⁾ CCL2 is stored within MC secretory granules and is promptly released following the allergen stimulation, whereas CCL2 mRNA synthesis occurs at a later time. Figure 1 shows that the stimulation of MCs with the optimal concentration of DNP-HSA for 60 min resulted in the release of pre-existing CCL2; however, no increase in CCL2 mRNA expression was observed at this point in time. The content of EAs in *Ephedra sinica* Stapf (Ephedraceae) vary depending on the strain. We previously evaluated the difference in the content of EAs in several strains of originally cultivated *Ephedra sinica* Stapf (Ephedraceae).¹⁸⁾ In this study, we used two strains: one containing sufficient (0.7%) EAs (denoted by Suf-EAs) and the other deficient (0.02%) in EAs (denoted by Def-EAs). Crude extracts were prepared from these strains. The procedures for the extraction and compositional analysis of EAs using UPLC-MS have been previously described.¹⁸⁾ RT-PCR analysis of BMMCs treated

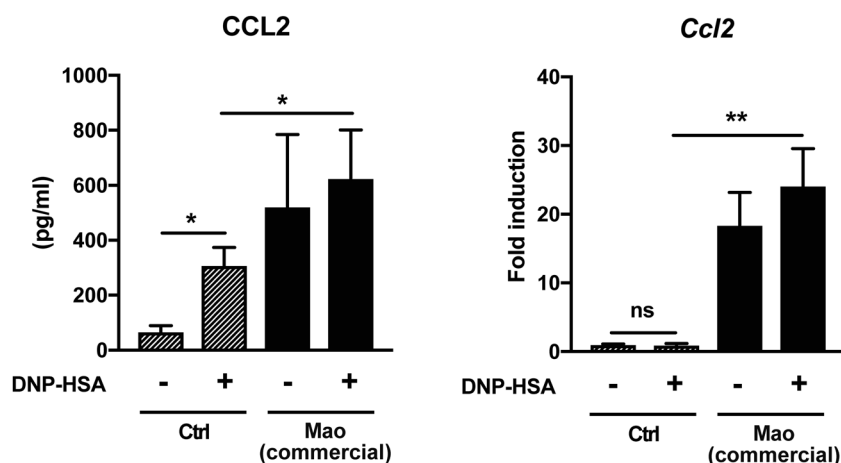


Fig. 1. Mao Induces CCL2 Production in BMMCs

IgE-sensitized BMMCs were treated with Mao (100 μ g/mL) for 30 min without washout and stimulated with DNP-HSA (50 ng/mL) or control medium for 60 min. Cell pellets and supernatants were analyzed for mRNA expression of CCL2 and extracellularly released CCL2, respectively. Data are presented as the mean $\pm$ standard error. $n = 6$ (left panel) and $n = 3$ (right panel) independent experiments. * $p < 0.05$, ** $p < 0.01$; ns, not significant.

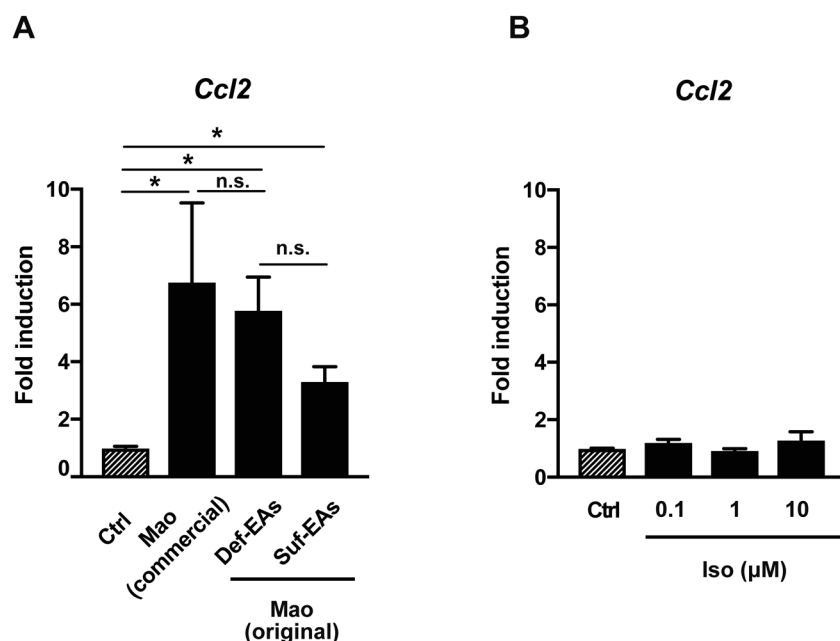


Fig. 2. Mao Induces CCL2 Independently of EAs in BMMCs

Naïve BMMCs were treated with Mao or the β -adrenergic receptor agonist isoprenaline and subjected to RT-PCR. (A) Cells were treated with three kinds of Mao extract (100 μ g/mL each), as described in Results, for 30 min. (B) Cells were treated with isoprenaline for 30 min. Data are presented as the mean $\pm$ standard error. $n = 4$ (A) and $n = 3$ (B) independent experiments. * $p < 0.05$; ns, not significant.

with each extract for 30 min revealed that Mao-derived CCL2 induction in BMMCs was not due to the EA content (Fig. 2A). Consistently, treatment with the β -adrenergic receptor agonist, isoprenaline, did not induce CCL2 production (Fig. 2B). These observations clearly demonstrate that Mao induces CCL2 expression in MCs, independent of its EA content.

The Mao-Induced Fc ϵ RI Internalization Is Independent of EAs The induction of CCL2 without allergen stimulation in Mao-treated MCs suggests internalization of Fc ϵ RI. We previously demonstrated that Mao has the potential to induce Fc ϵ RI internalization without considerable degranulation. However, whether this effect is due to EAs remains unknown. In this study, the effect of EAs on Mao-induced Fc ϵ RI internalization was analyzed using two different strains (Suf-EAs and Def-EAs) alongside isoprenaline. Intracellular (Fig. 3A) and cell surface

(Fig. 3B) clusters of IgE-Fc ϵ RI were analyzed in each treated BMMCs group. There were no significant differences in the rate of internalization of IgE-Fc ϵ RI between the Suf-EAs and Def-EAs groups, and isoprenaline treatment did not show the Fc ϵ RI internalization caused by Mao extracts (Figs. 3A, B). In addition, supernatants separated from the cell pellet, as shown in Fig. 3B, were subjected to a β -hexosaminidase assay (Fig. 3C), which confirmed that Mao induced significant internalization, but was not associated with robust activation of MCs.

Mao Significantly Suppresses the Local Activation of MCs in the PCA Reaction Mao induced both CCL2 induction and Fc ϵ RI internalization in an EA-independent manner. In the context of its pathophysiological role, we previously found that Mao suppressed a systemic anaphylactic reaction in mice. However, we did not explore whether the *in vivo* administration of Mao

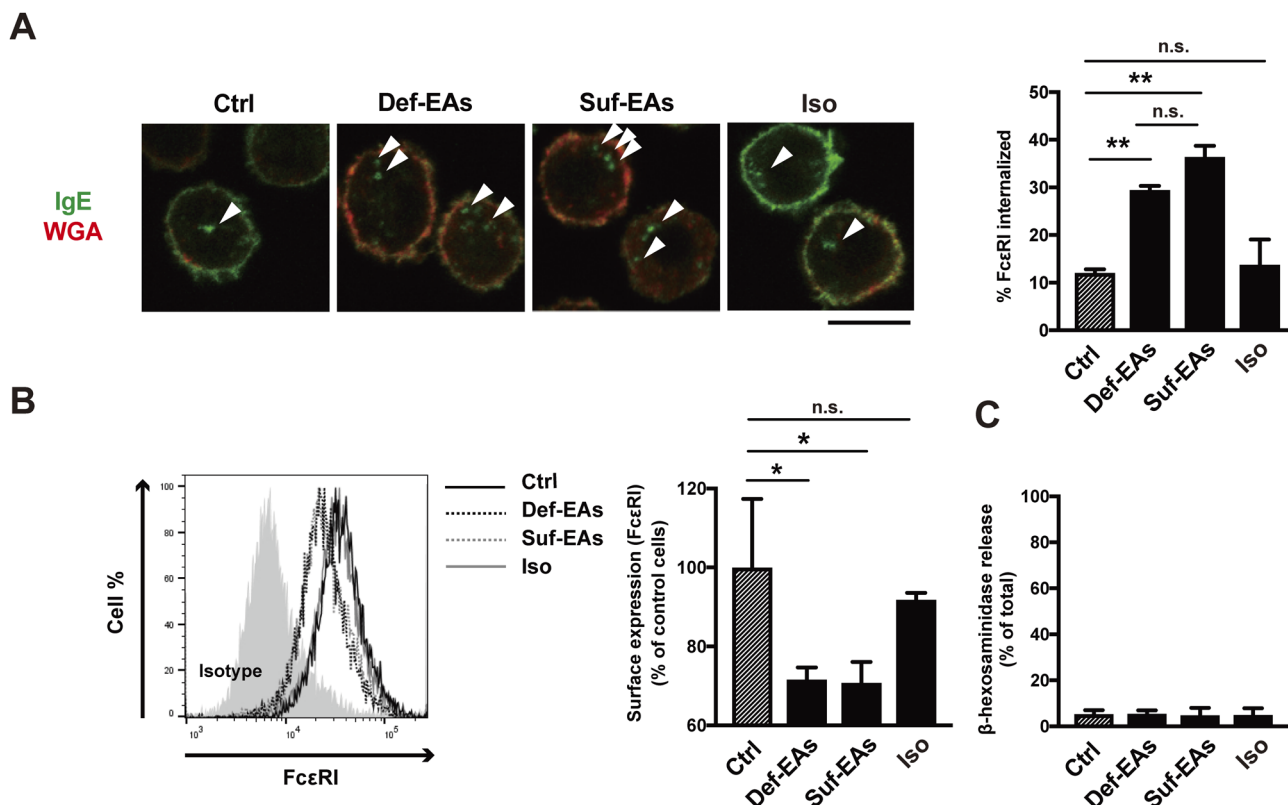


Fig. 3. Mao Induced FcεRI Internalization Independently of EAs in BMMCs

Naïve BMMCs were treated with Mao (100 μg/mL of both Def-EAs and Suf-EAs) or the β-adrenergic receptor agonist isoprenaline (10 μM) and subjected to CLSM, FACS, and degranulation assays. (A) Permeabilized cells were labeled with anti-IgE and wheat germ agglutinin (WGA) to identify the plasma membrane. Subcellular localization of FcεRI (arrowhead) was observed in Mao-treated and isoprenaline-treated cells (magnification × 63, scale bar = 20 μm, left). The rate of FcεRI (IgE-bound) internalization was expressed as the ratio of surface to intracellular FITC-IgE fluorescence intensity. (B) Non-permeabilized cells labeled with anti-IgE antibodies alone. Representative histograms of FACS (left panel) and fold changes in gMFI (right panel) are shown. Isotype control is shown in grey. (C) Degranulation responses were observed using the supernatant of the cells that were analyzed in B. Data represent the mean ± standard error from three independent experiments that were derived from 10 to 40 cells in each single experiment (A). Data are presented as the mean ± standard error. $n = 3$ independent experiments (B, C). * $p < 0.05$, ** $p < 0.01$; ns, not significant.

suppressed MC activation. This study evaluated the effects of Mao on local MC activation in passive cutaneous anaphylactic (PCA) reactions. When MCs were degranulated in tissue, morphological changes of MCs were observed after the PCA reaction using FITC-conjugated avidin, which specifically binds to the contents of MC granules. IgE-sensitized and Mao-administered mice were challenged with DNP-HSA. The local activation of MCs, followed by anaphylaxis, was analyzed using CLSM. We found that Mao pre-administration (Fig. 4A) significantly attenuated allergen-induced MC degranulation in the local dermal environment (Fig. 4B), providing additional information for the therapeutic potential of Mao through FcεRI internalization.

DISCUSSION

We demonstrated that Mao induced CCL2 production without robust activation of MCs, which was not mediated by EAs. The EA-independent functions of Mao have been discussed previously but have not yet been fully resolved. Our previous study demonstrated that Mao suppressed MC degranulation independent of EAs, and had significant potential for facilitating FcεRI internalization without degranulation in MCs. We further investigated this essential aspect of Mao in controlling allergic responses using two strains of Mao containing different amounts of EAs.

CCL2 is an essential chemokine involved in allergic inflam-

mation,²⁰⁾ and MC-produced CCL2 appears to be differentially characterized from other MC-produced allergic mediators.⁶⁻⁸⁾ CCL2 production in MCs is thought to be dominantly regulated at an insignificant level of stimulation, although the underlying mechanisms remain to be determined.⁶⁻⁸⁾ FcεRI can respond to insignificant stimulation, where FcεRI is competent to internalize despite the lack of considerable degranulation in MCs,^{21,22)} as described in Introduction (*i.e.*, silent internalization). Src-homology-2-containing inositol phosphatase (SHIP), a negative regulator of MCs,²³⁾ is supposed to be regulated in the context of small FcεRI cluster formation¹⁴⁾ during silent internalization. Thus, even under a simple and diminished (*i.e.*, weak) response caused by low allergen concentrations, valency, or affinity,^{7,13)} FcεRI displays dynamic changes corresponding to MC responses. In this process, a complex cascade of signaling events, including activation and recruitment of distinct signaling and scaffolding proteins, are involved,¹⁴⁾ and subsequently, heterogeneous secretion of inflammatory mediators occurs,^{6,7)} as we previously reported.²⁴⁾ Hence, various properties of the foreign stimulus and the corresponding FcεRI dynamics initiate diverse responses, where FcεRI is suggested to change the specific intracellular signaling pathway and drive different outcomes in MCs.¹²⁾ Given the prior studies on the behavior of the FcεRI and the MC secretion patterns following insignificant antigen stimulation,^{7,13,21)} it appears that CCL2 secretion in MCs may be a characteristic

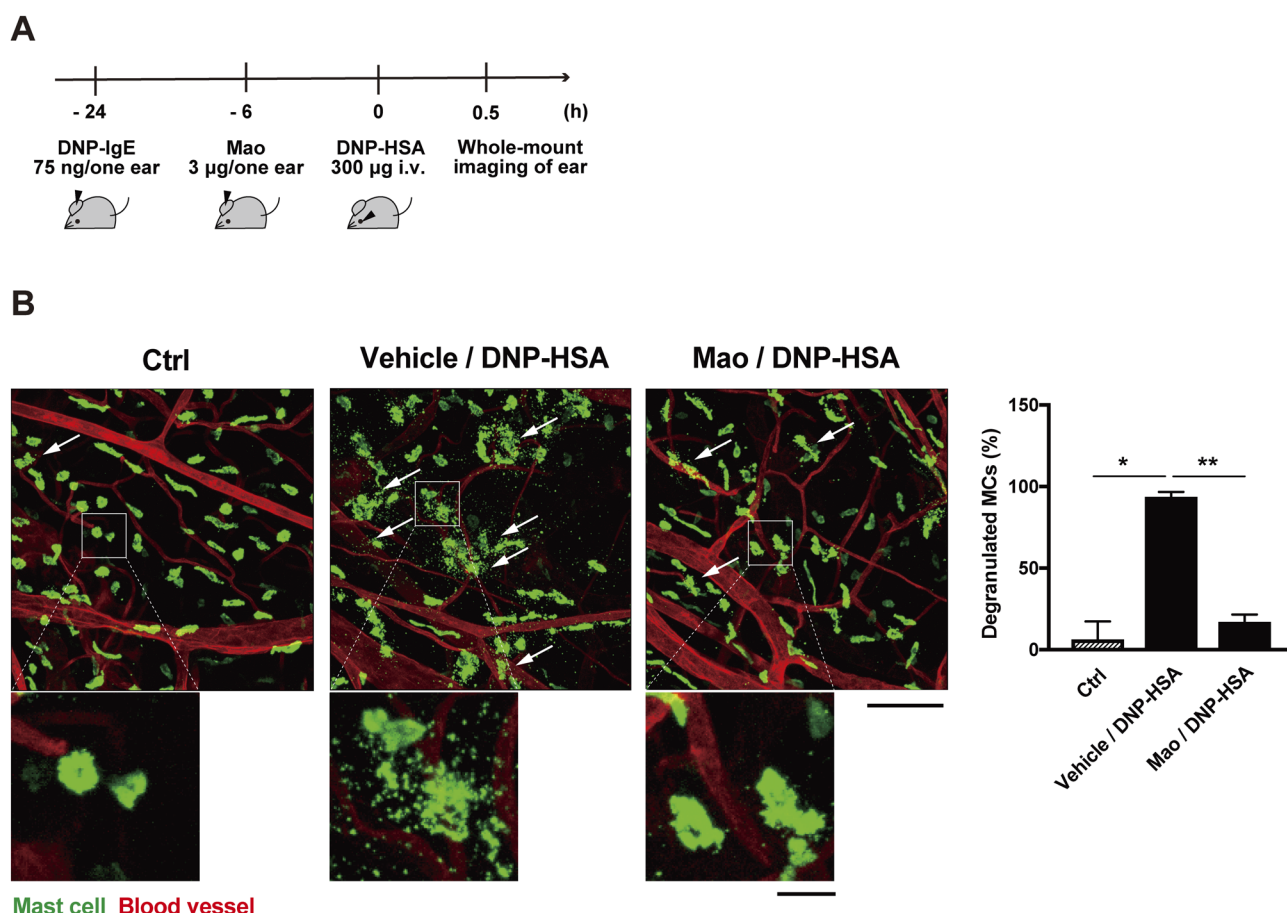


Fig. 4. Mao Prevents Allergen-Induced Cutaneous MC Activation

PCA was induced by sensitization with 75 ng/ear of anti-DNP IgE and by challenging 24 h later with DNP-HSA. Mao or vehicle was administered 6 h before the challenge. (A) Experimental protocol. (B) Whole-mount images of ear cells (magnification, $\times 20$; scale bar, upper panels: 100 μm ; lower panels: 20 μm). Fluorescent avidin-based MC staining (green) and CD31 staining of the blood vessels (red) were performed. Degranulated cells (white arrows) were visually counted. Lower panels show magnified images of individual cells designated in white squares. By employing avidin staining, avidin can specifically bind to individual MCs granules, and this staining method allows for the detection of degranulation events. In the mice treated with Vehicle/DNP-HSA, a clear mast cell degranulation event was observed. Data are presented as mean $\pm$ standard error. $n = 3$ mice per group. * $p < 0.05$, ** $p < 0.01$. i.v., intravenous.

feature that ensues following the silent internalization of Fc ϵ RI. Our finding that Mao is capable of inducing CCL2 production without MC activation seems to add further evidence to these interpretations. Additionally, we have previously reported that Mao can regulate Fc ϵ RI directly.¹⁸⁾ Treatment with Mao was found to significantly induce unsensitized Fc ϵ RI internalization, implying that one of the active components of Mao may have cross-linked Fc ϵ RI. While further investigation is required to determine the precise mechanisms that are responsible for the Mao-induced Fc ϵ RI internalization, we speculate that there exists a significant difference in cell surface Fc ϵ RI dynamics, particularly in the context of their mobility and clustering size, between Mao and allergen. These differences could have potentially initiated subsequent signaling and secretory responses of MCs, as we have reported.¹²⁾ However, in the case of both Mao and allergens, Fc ϵ RI cross-linking is essential for Fc ϵ RI internalization. Therefore, we speculated that the internalized Fc ϵ RI by Mao and allergen may be localized in the same intracellular compartment.

Although Mao induces a potent chemokine, CCL2, it suppresses immediate allergic responses in inflamed local tissues. PCA is an IgE-mediated type 1 hypersensitivity of the dermis. Subcutaneous administration of Mao, shown in Fig. 4, suppressed the rapid local activation of MCs. This is consistent

with our previous observation that Mao attenuates passive systemic anaphylaxis by regulating Fc ϵ RI internalization into peritoneal MCs. However, the previous results did not provide any evidence of the activation of peritoneal MCs. The present study observed significant suppression of the activation of MCs by Mao administration. In the PCA reaction, Mao was administered 6 h prior to DNP-HSA stimulation. The internalization of Fc ϵ RI by Mao is known to proceed in a gradual, time-dependent manner.¹⁸⁾ Time-course observations have revealed that the cell surface Fc ϵ RI levels were observed to decrease in a time-dependent manner, reaching a maximum internalization after 6 h of observation. Our data indicate that a treatment time of at least 30–60 min is sufficient to observe Mao-induced Fc ϵ RI internalization; however, a longer treatment duration may be desired for a more pronounced suppressive effect. Although further long-term observations may be needed to determine the consequence of CCL2 secretion by Mao, it appears to be clear that Mao has significant therapeutic effects on immediate allergic reactions in local inflamed tissues.

Mao contains EAs (l-ephedrine, pseudoephedrine, l-methylephedrine, and l-norephedrine), which are the most important ingredients. However, they can induce adverse effects, including hypertension, palpitations, insomnia, and dysuria, because they stimulate both the sympathetic and parasympathetic nerves. Al-

though Mao is one of the most important natural medicines in Kampo, these potentially hazardous effects are considered during its clinical use. EA-free ephedra herb extract has been developed to eliminate any adverse effects and is mainly used in Europe and the US. However, in addition to EAs, it is notable that recent studies have emphasized other active ingredients such as polyphenols (e.g., flavonoids and tannins) in Mao.^{25,26)} In addition, some studies have found that Mao extract exhibits alkaloid-independent pharmacological activity.^{27,28)} These observations collectively indicate that there are undefined aspects of Mao that cannot be explained by EAs and may be involved in its anti-allergic effects. This study also highlights the EA-independent property of Mao and may prove valuable for the use of EA-free ephedra herb. Further investigation of the active compounds in Mao and the precise mechanisms of FcεRI internalization by Mao may provide promising insights into the treatment and/or prevention of allergies using natural compounds.

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Conflict of Interest The authors declare no conflict of interest.

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