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Topical treatment with mastic (resin from *Pistacia lentiscus*) elicits anti-inflammatory and anti-pruritic responses by modulating keratinocyte activation in a mouse model of allergic dermatitis

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ABSTRACT

Background: As the number of patients with skin allergies, including atopic dermatitis, has increased rapidly, therapeutic options such as anti-IL-31 antibody and Janus kinase inhibitor have been developed recently. However, many concerns remain regarding the adverse effects and cost of these drugs; therefore, development of supplements that could support the effect of therapeutic agents is always required.

Purpose: The aim of this study was to develop preventive and supportive options for skin allergies by focusing on a natural product called "Mastic". **Methods:** Initially, the anti-inflammatory and anti-pruritic responses of 3% and 30% Mastic topical treatment were investigated in a mouse model of allergic contact dermatitis, generated by topical application of toluene-2,4-diisocyanate (TDI), a hapten that induces type 2 helper T cells. After itch behaviour and ear-swelling response were monitored, serum, auricular lymph nodes, and skin tissues were collected to analyse immunocyte differentiation, cytokine determination, and histological changes.

Results: Our findings indicated that topical treatment with mastic significantly ameliorated ear swelling, itch behaviour, immunocyte infiltration, and cytokine production. Histological evaluation confirmed the occurrence of anti-inflammatory responses. The anti-inflammatory and anti-pruritic effects of topical treatment with mastic (3% and 5%) were further confirmed in a mouse model of atopic dermatitis which was generated by topical application of TDI in NC/Nga mice. Thickness of the back skin, AD score, transepidermal water loss (TEWL), and itch behaviour were measured weekly, and immunocyte differentiation, cytokine determination, and histological changes were also analysed. Mastic treatment significantly attenuated the skin thickness, AD score, TEWL, and itch behaviour. Corroborated reduction was observed in the numbers of T cells and IgE-B cells, as well as in pro-inflammatory cytokine production. The reproducibility of the effects of mastic was confirmed with 1% mastic ointment in a setting similar to the AD mouse model. *In vitro* evaluation of keratinocytes indicated that mastic pre-exposure induced a significant dose-dependent decrease in cytokine production.

Conclusion: Our findings thus demonstrate that topical treatment with mastic significantly ameliorate inflammatory and pruritic responses in a mouse model of allergic dermatitis.

Introduction

Mastic is a natural resin obtained from the stem and leaves of the

Pistacia lentiscus tree, which exists only on the Greek island of Chios (Amerikanou et al., 2021; Papada and Kaliora, 2019). In the 10th century, tourists visiting Chios discovered that Chios produced mastic.

Abbreviations: ACD, allergic contact dermatitis; AD, atopic dermatitis; ELISA, enzyme linked immunosorbent assay; gk2, glycyrrhizic acid dipotassium salt; IL, interleukin; IgE, immunoglobulin E; LN, lymph node; TARC, thymus and activation-regulated chemokine; TDI, toluene-2,4-diisocyanate; Th, T helper; TEWL, transepidermal water loss; TSLP, thymic stromal lymphopoietin.

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Although mastic can be produced outside Chios, the process involving the sap dripping and falling is only conducted in Chios. Over time, the sap turns into a yellow mass, sometimes called “tears of Christ” because its shape resembles tears (Amerikanou et al., 2021; Papada and Kaliora, 2019). In Greece, chewing mastic has long been considered to prevent periodontal disease. Previous studies have shown that mastic extract significantly inhibits the growth of periodontal pathogens, and has beneficial effects on cell viability; thus, it could be considered an alternative antibacterial agent for preventing periodontal disease (Carrol et al., 2020; Koychev et al., 2017). Besides its anti-bacterial effects, the anti-inflammatory effects of mastic have also been demonstrated in several reports. Papalois et al. (2012) demonstrated that mastic treatment downregulated the levels of IL-8 and NF- κ B p65 *in vitro*. Triantafyllou et al. (2011) also indicated that the anti-inflammatory activity of Chios mastic gum was associated with the inhibition of TNF- α -induced oxidative stress. In addition, anti-inflammatory effects of plant-derived components such as green tea extract and glycyrrhizic acid have been used for centuries all over the world (Chu et al., 2017; Hasan et al., 2021). However, the anti-inflammatory effects of Chios mastic *in vivo*, using actual models of inflammatory diseases are not fully understood. Therefore, the initial aim of this study was to investigate the anti-inflammatory effects of Chios mastic *in vivo* using a mouse model of allergic dermatitis.

Dermatitis is a skin inflammatory condition that occurs in many forms including itching, eczema, dry red skin, rash, and swelling (Williams, 2005). Among these types of cutaneous allergy, atopic dermatitis (AD) is one of the most frequent forms of dermatitis in infants (Rizk et al., 2019). AD is a common skin disease in dogs and cats. Its clinical, immunological, histological, and pathological features are highly similar between dogs and humans; thus, canine AD has been suggested as an animal model for human AD (Mineshige et al., 2018). There are several therapeutic options for AD including oral supplements such as omega-3 fatty acids and lactobacilli; however topical treatment options are still limited, even though topical treatment is a more popular method to improve inflammatory symptoms. Therefore, this study aimed to introduce Chios mastic as a novel supplemental option for skin allergy. We investigated the anti-inflammatory and anti-itch responses of topical treatment with mastic ointment in a mouse model of allergic contact dermatitis (ACD) and AD.

Materials and Methods

Experimental animals

Six-week-old female BALB/c and NC/Nga mice were provided by Japan SLC, Inc. (Shizuoka, Japan) for developing the ACD and AD models, respectively. All mice were housed under a 12 h/12 h light/dark cycle at $22 \pm 3^\circ\text{C}$ and $50\% \pm 20\%$ humidity. The mice were provided with food and water *ad libitum*. All the aspects of this study were conducted in accordance with the Animal Care and Use Program of Azabu University (Approval No. 1910097).

Preparation of test items

Chios mastic resin was provided by Sosin Co., Ltd. (Tokyo, Japan). Highly purified mastic was dissolved in caprylic/capric triglycerides to prepare a final concentration of 1%, 3%, 5%, or 30%. Glycyrrhizic acid dipotassium salt (gk2, 0.1%) in caprylic/capric triglycerides was used as the positive control substance for the second experiment with the AD model.

Murine model of ACD

A murine model of ACD was generated by repetitive topical sensitisation and challenge with a Th (T helper) 2 type hapten, toluene-2,4-diisocyanate (TDI, Sigma-Aldrich, Tokyo, Japan) diluted in acetone

(5% for sensitisation, 0.5% for challenge) in BALB/c mice, based on a previously described method (Aihara et al., 2020; Makino et al., 2019; Watanabe et al., 2018) (Supplemental Fig. 1A). Mastic cream at 0% (vehicle cream only, $n = 6$), 3%, and 30% ($n = 6/\text{group}$) was topically administered to the mice once daily during the experimental period. The aim of this experiment was screening the anti-allergic potential of mastic with wide variety concentrations. Dose selection of mastic was based on a preliminary study examining the irritation of mouse skin. No irritation response was observed after treatment with 30% mastic. A murine model of ACD has been widely used to evaluate the drugs for the treatment of allergic skin diseases such as tacrolimus (calcineurin inhibitor), cilomilast (PDE4 inhibitor) and oclacitinib (Janus kinase inhibitor), and anti-allergic responses were well detected (Baumer et al., 2005; Fukuyama et al., 2015). As the adequacy of this model has been well validated, no positive control group was included in this study.

To evaluate the anti-inflammatory effect of mastic, the ear swelling response was calculated by subtracting the ear thickness before TDI challenge from the ear thickness at 24 h after TDI challenge. Immediately after TDI challenge to the auricle and the dorsal cervical, a video of scratching bouts was monitored for 60 min as an itch behaviour associated with TDI challenge. Immediately after measuring ear thickness, blood was collected from the posterior vena cava under isoflurane anaesthesia, and the animals were euthanized. Serum samples obtained from blood were used to measure the total immunoglobulin E (IgE) levels. A portion of the auricle was collected and cryopreserved to measure skin tissue cytokine levels. The remaining ear parts were fixed in formalin for histopathological examination. Isolated single-cell suspensions were prepared from auricular lymph nodes (LNs), and the immunocyte numbers and cytokine levels were measured.

Murine model of AD

A murine model of AD was generated using NC/Nga mice based on a previously described method with minor modifications (Fukuyama et al., 2018a; Fukuyama et al., 2018b) (Supplemental Fig. 1B). Briefly, depilated NC/Nga mice (on their backs) were topically sensitised with 100 μl of 5% TDI on their back. For the challenge, 0.5% TDI was topically applied to the back once weekly. In the initial setting, 0% (vehicle cream only), 3%, and 5% of the mastic ointment ($n = 6/\text{group}$) was applied to the AD model to determine the dose-dependent effects, and in the second setting, 0% (vehicle cream only) and 1% mastic treatment ($n = 6/\text{group}$) was used to confirm the repeatability and effects at lower concentrations. The second experiment also included a group treated with 0.1% gk2 for side-by-side comparison with another type of anti-inflammatory supplement. Experiments with murine AD model were performed to demonstrate more practical concentrations of mastic, therefore, mastic 30% that had greater anti-allergic response in first experiment in murine ACD model was not included in this experiment.

Scratching behaviour (30 min), transepidermal water loss (TEWL), ear and back skin thickness, and clinical scores were monitored once weekly during the experimental period. TEWL was measured using VAPO SCAN (AS-VT100RS, ASCH JAPAN Co., LTD, Tokyo, Japan), and a clinical score from 0–4 was assigned as follows: no symptoms, 0; mild, 1; moderate, 2; severe, 3; and extreme, 4 as described by Fukuyama et al. (2018b). The back skin, auricular LN, and serum samples were collected from each mouse 24 h after the last TDI challenge and test application. The collected samples were processed for histology, cytokine analysis, IgE detection, and cell differentiation.

Histopathology

A portion of skin samples was fixed in 10% formalin solution, embedded in paraffin wax, sectioned to 5 μm thickness, and stained with haematoxylin and eosin, or toluidine blue. A semi-quantitative histopathological evaluation of dermal oedema, cellular infiltration, folliculitis, and micro-abscesses was performed in a blinded fashion using the

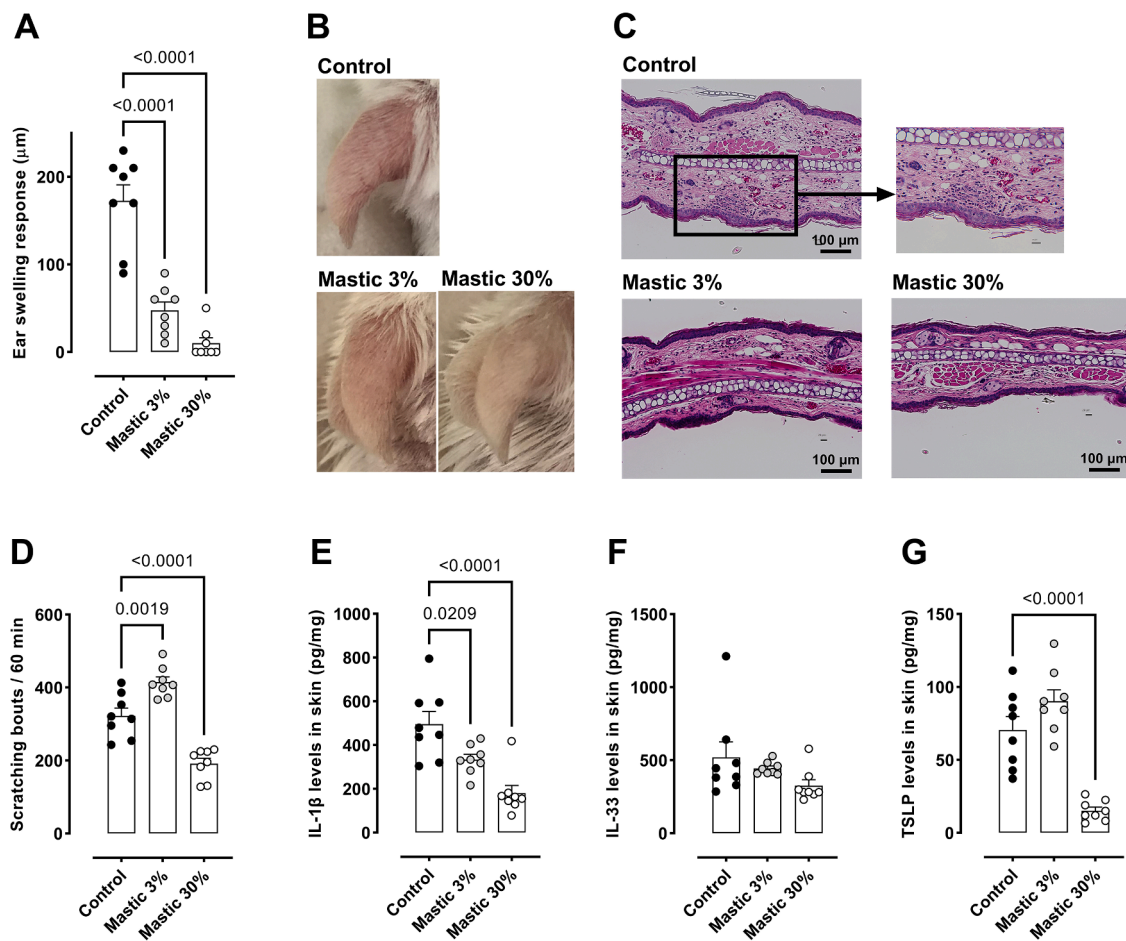


Fig. 1. Topical treatment with mastic ointment ameliorated the inflammatory and pruritic response in a mouse model of allergic contact dermatitis. (A) The ear swelling response was significantly suppressed by the topical treatment with mastic ointment. (B) Typical pictures of ear auricles from each group. (C) Representative histological pictures of ear skin in each group. (D) Scratching bouts were significantly reduced by 30% mastic application, whereas 3% mastic significantly enhanced the scratching behaviour. Inflammatory cytokines, IL-1β (E), IL-33 (F), and TSLP (G), in ear skin tissue were downregulated by topical mastic application. Each result is presented as the mean $\pm$ 1 SEM. n = 8 per group.

following grading system: 0, within normal limits; 1, mild; 2, moderate; 3, severe. The total score of the lesions was used for statistical evaluation. In the murine model of AD, microscopic measurement of dermal and epidermal thickness, and mast cell number were evaluated by 10 sections per mouse.

Cytokine determination in skin tissues

A portion of frozen skin was homogenised, and the supernatant was used to determine the cytokine levels as described previously (Stover et al., 2016; Tajima et al., 2019). The protein content in the supernatant was measured using a dendritic cell protein assay kit (Bio-Rad Laboratories, Tokyo, Japan). Interleukin (IL)-1β, -33, thymus and activation-regulated chemokine (TARC), and thymic stromal lymphopoietin (TSLP) were measured using respective enzyme-linked immunosorbent assays (ELISAs) (R&D Systems, Minneapolis, MN, USA), and the amount of each cytokine per milligram of protein was calculated.

Analysis of lymph nodes (LNs)

Single-cell suspensions isolated from the auricular LN were prepared as described in previous studies (Aihara et al., 2020; Ookawara et al., 2020), and the total number of cells was measured using a Tali® Image-Based Cytometer (Thermo Fisher Scientific, Inc., Kanagawa, Japan). To avoid nonspecific binding in flow cytometric analysis, 1×10^6 cells were first incubated with 1 μg of mouse Fc Block (Miltenyi

Biotec K.K., Tokyo, Japan) prior to incubation with monoclonal antibodies (FITC-conjugated anti-mouse CD3, PE-conjugated anti-mouse CD4, FITC-conjugated anti-mouse IgE, PE-conjugated anti-mouse CD19, FITC-conjugated anti-mouse CD11c, PE-conjugated anti-mouse CD40, Miltenyi Biotec K.K.). Regulatory T cells were also identified using a Treg detection kit according to the manufacturer's protocol (Miltenyi Biotec K.K.). Strategy of FACS analysis for gating cells is presented in the supplemental Fig. 2.

In addition to FACS analysis, cytokine production from T cells was measured by co-culturing isolated T cells using Dynabeads Mouse T-Activator CD3/CD28 for 24 or 96 h (Thermo Fisher Scientific, Inc., Kanagawa, Japan). Cytokine levels (IL-4, IL-5, IL-9, and IL-13) in the culture supernatant were measured using ELISA.

Measurements of total IgE in serum

The total IgE levels in serum samples were determined using ELISA according to the manufacturer's protocol (BD Biosciences, Tokyo, Japan). Optical density was measured using a microplate reader (iMark microplate reader, Bio-Rad Laboratories, Inc., Tokyo, Japan).

Cytokine release assay in HaKaT cell line (human epidermal keratinocyte cell line)

HaKaT cells were obtained from CLS (Cell Lines Service) GmbH (Eppelheim, Germany) and cultured in Dulbecco's modified Eagle's

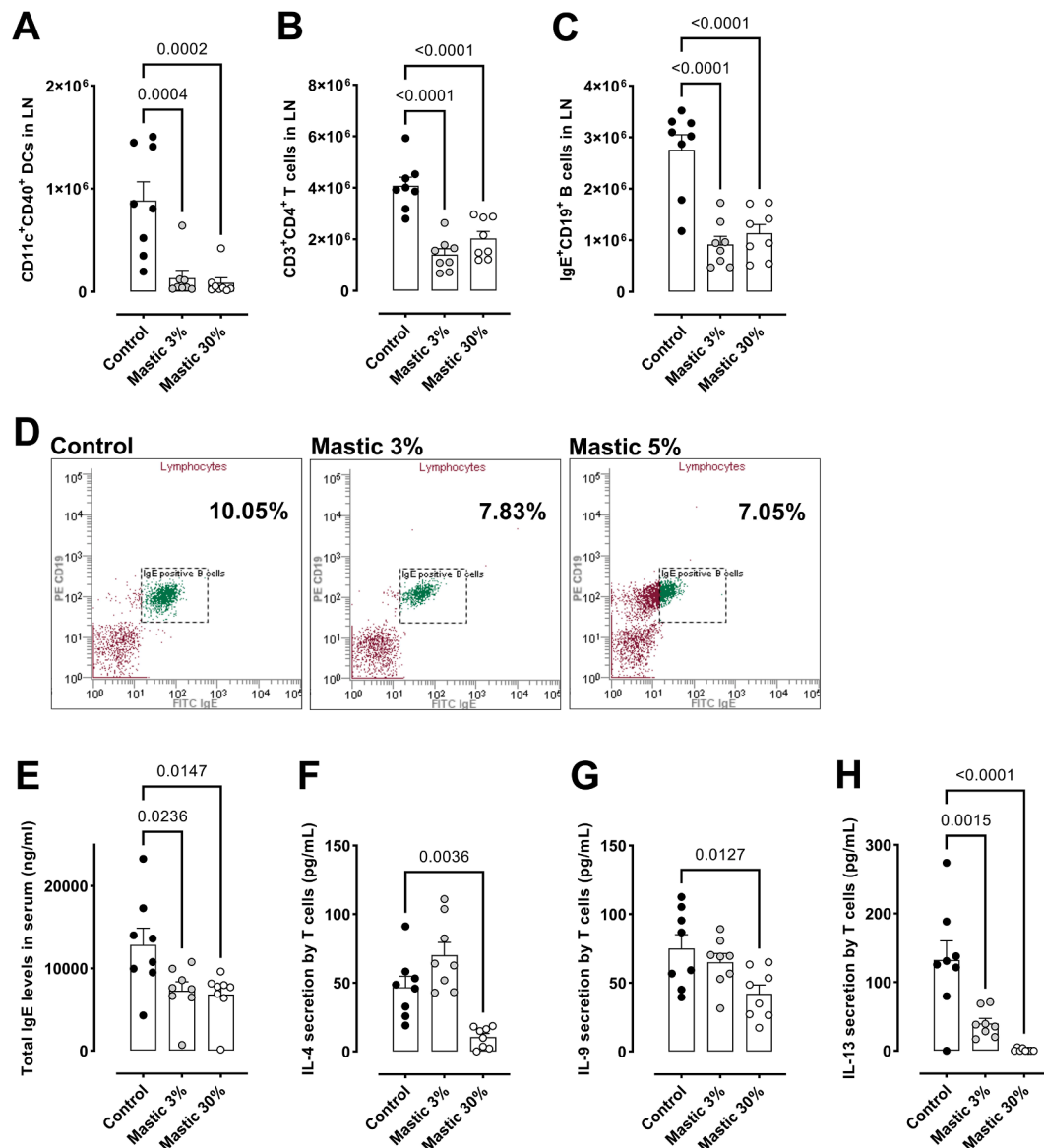


Fig. 2. The number of dendritic cells (A), helper T cells (B), and IgE positive B cells (C) in the auricular lymph node were significantly reduced by mastic treatment. (D) Total IgE levels in serum were also downregulated by mastic application. Production of IL-4 (E), IL-9 (F) and IL-13 (G) from CD3/CD28-stimulated T cells in the auricular lymph node was significantly reduced by mastic administration. Each result is presented as the mean $\pm$ 1 SEM. $n = 8$ per group.

medium (FUJIFILM Wako Pure Chemical Corporation) supplemented with 10% FCS (Sigma-Aldrich Co. LLC., Tokyo, Japan) and penicillin-streptomycin (FUJIFILM Wako Pure Chemical Corporation). HaKaT cells (1×10^4 cells) were seeded in 100 μ l of medium at 70% confluency in 96-well culture plates and pretreated for 24 h with 0.04, 0.2, or 1% of mastic before stimulation with recombinant human TNF α and IFN γ (PeproTech, Inc., Cranbury, NJ) for another 24 h. The concentrations of IL-1 β and IL-6 in the supernatants were quantified by ELISA. Cytotoxicity was not observed even with the highest mastic concentrations. A minimum of three independent experiments were performed to verify the reproducibility of the experiments.

Statistical analyses

Data are expressed as the mean $\pm$ 1 SEM. Analysis of variance (ANOVA) was used to evaluate the results. For significant results, differences between the control and treatment groups were assessed using Dunnett's multiple comparison test. Data analysis was performed using Prism 9 (GraphPad Software, San Diego, CA, USA).

Results

Topical treatment with mastic ointment significantly ameliorates both the ear swelling response and itch behaviour in a mouse model of ACD

To elucidate the possible anti-inflammatory and anti-pruritic effects of mastic on cutaneous allergy, topical treatment with 3% or 30% mastic ointment was initially applied to a mouse model of ACD. The ear swelling response was significantly suppressed by mastic administration in a dose-dependent manner (Fig. 1 A and B). Suppression of the ear swelling response by mastic application was corroborated by the histological evaluation. Oedema and cellular infiltration were dose-dependently ameliorated by mastic application, and significant changes were observed in the 30% mastic treatment group (Fig. 1C, Supplemental Table 1). Surprisingly, although treatment with 30% mastic significantly decreased the itch behaviour, 3% mastic significantly increased the scratching bouts induced by TDI challenge (Fig. 1D). A similar tendency was observed in the skin tissue cytokine levels. There was a significant decrease in the IL-1 β levels with both the

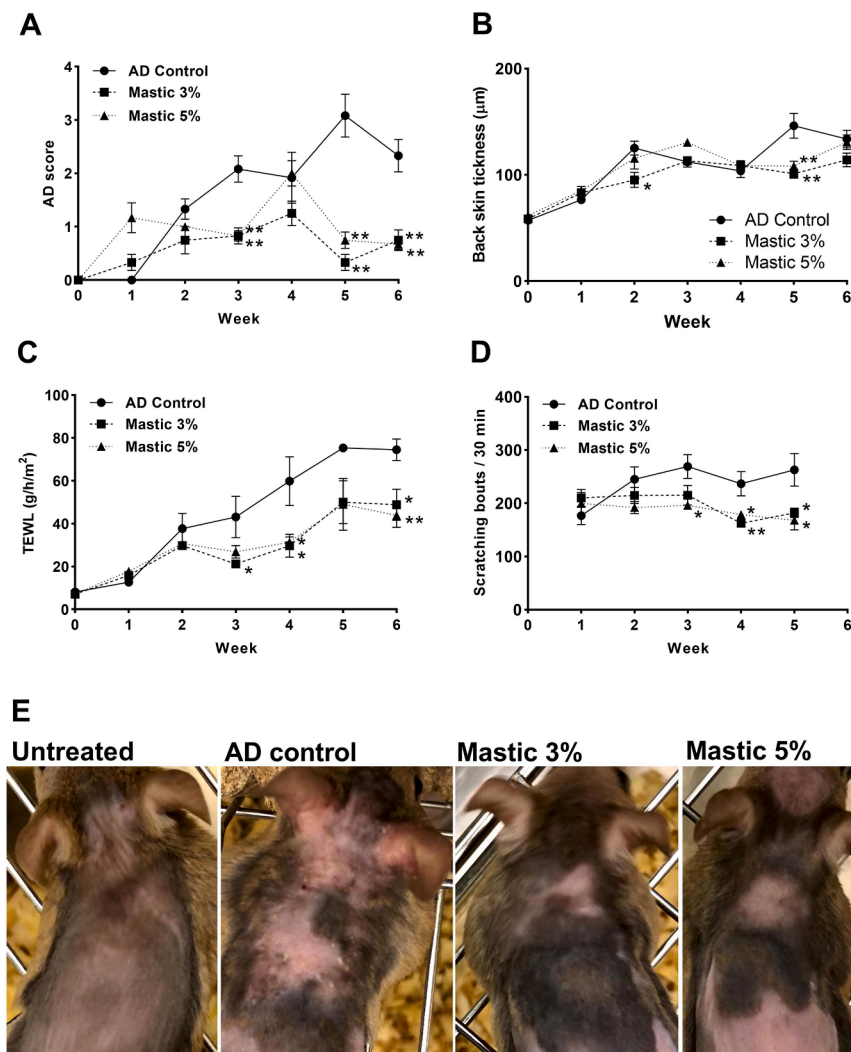


Fig. 3. Topical treatment with mastic ointment inhibits lesion formation and itch behaviour in a mouse model of atopic dermatitis. Vehicle-treated and hapten-challenged mice developed AD and were evaluated by their AD score (A), skin thickness (B), TEWL (C), and scratching bouts (D). Mastic-treated mice developed only mild lesions throughout the challenge, and there was a significant difference between the vehicle- and mastic-treated groups. (E) Typical pictures of back skin in each group. Each result is presented as the mean $\pm$ 1 SEM. $n = 6$ per group. * $p < 0.05$, ** $p < 0.01$ (Dunnett's multiple comparison test) vs. AD control group. TEWL = transepidermal water loss.

3% and 30% mastic treatment groups; however, a significant decrease in TSLP levels was found only in the 30% treatment group, whereas 3% mastic treatment enhanced the TSLP levels (Fig. E-G).

Effect of mastic ointment on immunocyte infiltration, inflammatory cells, inflammatory cytokines, and IgE levels in a mouse model of ACD

To analyse the efficacy of mastic ointment on the local immune reaction, the number of dendritic cells, helper T cells, and IgE-positive B cells in the auricular lymph nodes were measured by flow cytometric analysis. Corresponding to the ear swelling response, treatment with 3% and 30% mastic ointment significantly reduced the number of inflammatory cells compared to the vehicle control group (Fig. 2 A-D). Total IgE levels, produced by IgE-positive B cells, in the serum were also significantly reduced by mastic application (Fig. 2 E). Significant inhibition of Th2 and Th9 cytokine production (IL-4, IL-9, and IL-13) by T cells in mastic treatment groups indicates the direct influence of mastic on helper T cell activation (Fig. 2 F-H).

Topical treatment with mastic significantly exhibited the anti-inflammatory and anti-pruritic responses in a mouse model of AD

In the next step, the efficacy of 3% and 5% mastic was evaluated in a mouse model of AD. Daily application of mastic significantly reduced the AD score, skin thickness, TEWL, and scratching bouts compared to

the vehicle control group (Fig. 3). No dose-dependent changes were observed between the 3% and 5% mastic treatment groups in terms of the above endpoints. All findings of in-life measurements were confirmed by histological evaluation and cytokine levels in skin tissues. Both 3% and 5% mastic treatments significantly reduced folliculitis, cellular infiltration, and thickness of dermis and epidermis (Fig. 4 A, Table 1). IL-1 β , IL-33, TARC, and TSLP levels in skin tissue were also significantly downregulated by mastic treatment compared to the AD control group (Fig. 4 B-E).

Treatment with mastic ointment significantly suppressed the local immune function in a TDI-induced mouse model of AD

Corresponding to the outcomes from the ACD mouse model, 3% and 5% mastic treatment significantly reduced the local immune function including the migration of dendritic cells, helper T cells, and IgE-positive B cells into local lymph nodes (Fig. 5 A-C). Total IgE secreted by IgE-positive B cells was also downregulated by mastic treatment in a dose-dependent manner compared to the AD control group (Fig. 5 D, Supplemental Fig. 3A). Inflammatory cytokine production by stimulated T cells such as IL-4, 5, 9, and 13, was significantly reduced by mastic treatment compared to the AD control group in a dose-dependent manner (Fig. E-H). Mastic treatment slightly enhanced the number of regulatory T cells, which plays a pivotal role in regulating the immune function and allergic reaction, but significant difference was not found

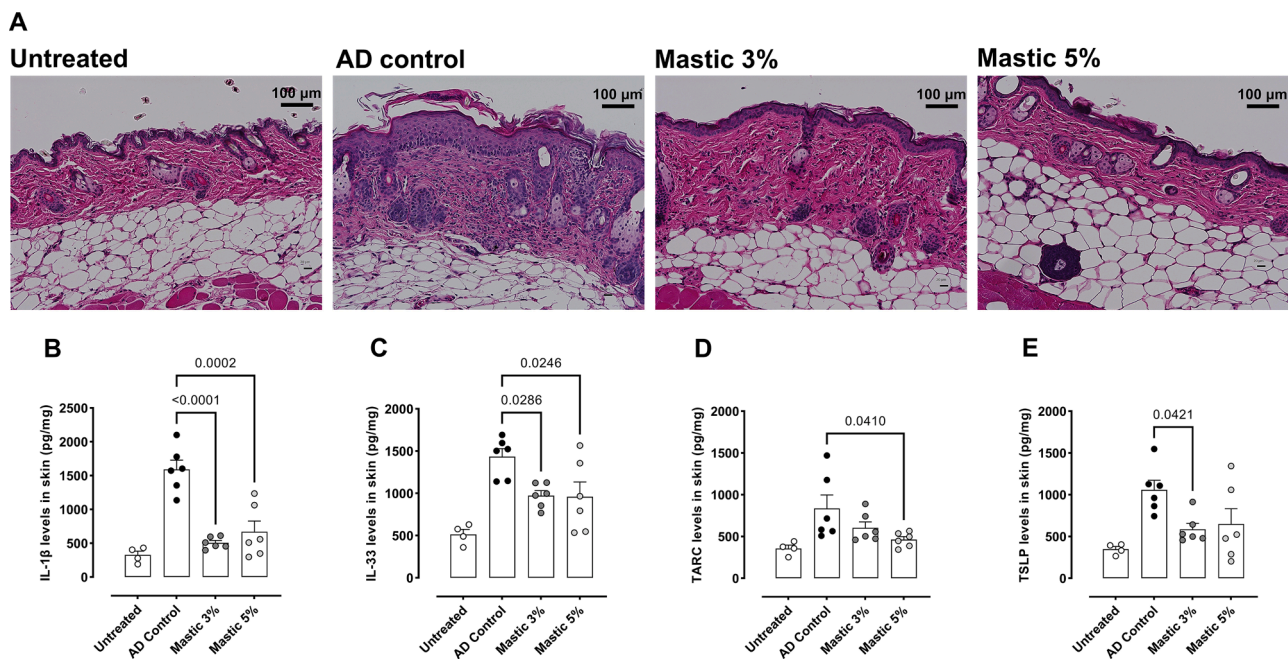


Fig. 4. Representative histological images of back skin at 6 weeks after treatment initiation (A). Mastic ointment-treated mice show significantly reduced inflammatory cytokines, IL-1β (B), IL-33 (C), TARC (D), and TSLP (E), in back skin tissue. Each result is presented as the mean $\pm$ 1 SEM. $n = 6$ per group.

TABLE 1

Histological evaluation of the back skin following topical treatment with mastic in a TDI-induced mouse model of atopic dermatitis.

Group	Untreated	AD control	Mastic 3%	Mastic 5%
Folliculitis	0.00 $\pm$ 0.00	0.67 $\pm$ 0.21	0.17 $\pm$ 0.17	0.00 $\pm$ 0.00
Cellular infiltration (epidermis)	0.00 $\pm$ 0.00	2.50 $\pm$ 0.22	1.17 $\pm$ 0.17	1.17 $\pm$ 0.17
Cellular infiltration (dermis)	0.25 $\pm$ 0.25	2.00 $\pm$ 0.00	1.17 $\pm$ 0.17	1.00 $\pm$ 0.00
Ulcer	0.00 $\pm$ 0.00	0.67 $\pm$ 0.42	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00
Total score	0.25 $\pm$ 0.25	5.83 $\pm$ 0.60	2.50 $\pm$ 0.34	2.17 $\pm$ 0.17
Epidermal thickness (um)	12.0 $\pm$ 0.98	65.6 $\pm$ 2.85	24.1 $\pm$ 0.81	16.3 $\pm$ 1.16
Dermal thickness (um)	147 $\pm$ 4.36	411 $\pm$ 8.91	267 $\pm$ 6.44	132 $\pm$ 4.32

A histological score (0, within normal limits; 1, mild; 2, moderate; and 3, severe) was given for each observation. Results are expressed as the mean $\pm$ SEM. $n = 6$ per group.

* $p < 0.05$,

** $p < 0.01$ (Dunnett's multiple comparison test) vs. the AD control group.

TDI = toluene-2,4-diisocyanate.

(Supplemental Fig. 3B).

Efficacy of mastic ointment was confirmed in a second setting of the AD model treated with 1% mastic ointment

The efficacy of mastic ointment was confirmed in a second setting of an AD mouse model treated with 1% mastic ointment or 0.1% gk2. Although there were no changes in back skin thickness and itch behaviour in both the mastic and gk2 treatment groups, the AD score and TEWL were significantly ameliorated by these treatments (Supplemental Fig. 4 A-E). Histological evaluation showed that 1% mastic treatment significantly decreased cellular infiltration into the epidermis and dermis, whereas there was no change in the 0.1% gk2 treatment group (Supplemental Table 2). Reproducibility experiments were

performed at a lower concentration of 1% mastic. The parameters measured in the reproducibility experiment included back skin symptoms, TEWL, and IgE production measurements. All parameters in the administration group was significantly reduced compared to the control group. The total serum IgE levels were downregulated by 1% mastic and 0.1% gk2 treatment; however, there were no significant changes in the immunocyte proliferation in LN, cytokine production by T cells, and cytokine levels in skin tissue (Supplemental Fig. 5).

Mastic pre-exposure significantly suppressed IL-1β and IL-6 secretion by stimulated keratinocytes

To demonstrate the possible interaction between topical mastic treatment and keratinocyte activation, IL-1β and IL-6 secretion by stimulated human keratinocytes (HaKaT cells) pre-treated with 0, 0.04, 0.2, and 1% of mastic was measured. Both IL-1β and IL-6 secretion was suppressed by mastic treatment in a dose-dependent manner. The mastic-treated groups showed a significant decrease compared to the vehicle control group (Supplemental Fig. 6).

Discussion

The prevalence of cutaneous allergies, including AD, has increased dramatically over the last century among humans and companion animals (David Boothe et al., 2017; Nassau and Fonacier, 2020). Glucocorticoids and calcineurin inhibitors have long been used to treat cutaneous allergies because of their immediate efficacy and competitive prices. However, they demonstrate various side effects depending on their dose and dosing period; prolonged dosing of these immunosuppressants requires extreme caution from doctors as well as patients. Similar concerns have been expressed in the veterinary field, and many case reports have provided warnings regarding the inappropriate usage of steroids, leading to an increased risk of Addison's disease (Stalder et al., 1994). Against this backdrop, novel therapeutics such as Janus kinase inhibitors (e.g., oclacitinib) and anti-IL-31 monoclonal antibody (e.g., Lokivetmab) have been recently launched for pruritus associated with AD (Cinats et al., 2018; He and Guttman-Yassky, 2019; Kabashima and Irie, 2021; Nakashima et al., 2018). However, these drugs are still of

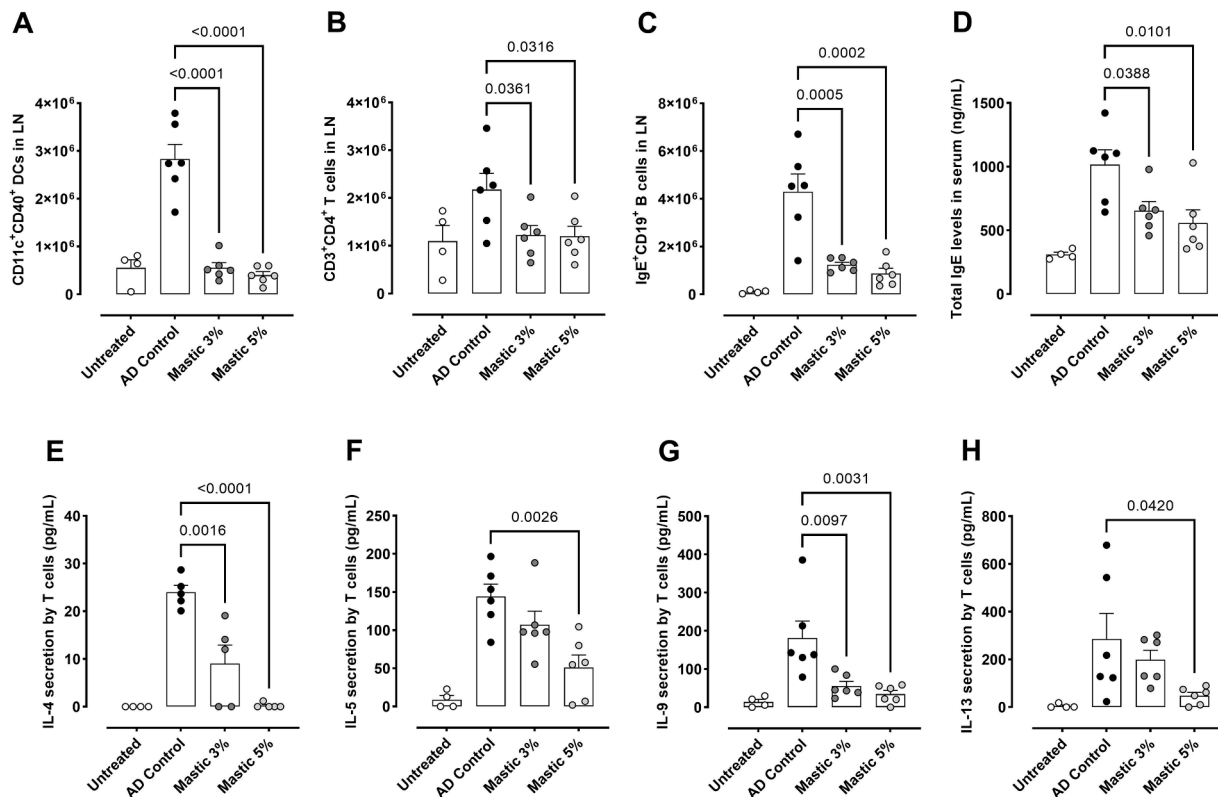


Fig. 5. The number of dendritic cells (A), helper T cells (B), and IgE positive B cells (C) in the auricular lymph node were significantly reduced by mastic treatment in the AD mouse model. Total IgE levels in serum (D), and production of IL-4 (E), IL-5 (F), IL-9 (G) and IL-13 (H) from CD3/CD28-stimulated T cells in the auricular lymph node were significantly reduced by mastic administration. Each result is presented as the mean $\pm$ 1 SEM. n = 6 per group.

concern because of cost and limited efficacy, particularly for inflammatory symptoms; therefore, development of supplements that can support the effect of these new therapeutics is strongly required. This study aimed to introduce a new supplemental option for skin allergy by focusing on the natural product, mastic, and evaluated the anti-allergic effects of topical treatment with mastic using *in vivo* mouse models and an *in vitro* experiment.

Initially, we examined whether topical mastic treatment directly affects inflammatory and itch responses in a mouse model of ACD. The anti-inflammatory property of mastic ointment was corroborated by significant downregulation of the ear swelling response, histological findings of inflammation, total IgE levels, lymphocyte proliferation in LNs, and cytokine production. Anti-itch property was also demonstrated in the 30% mastic treatment group, whereas the 3% mastic treatment enhanced scratching behaviour. This phenomenon was confirmed by the TSLP levels in skin tissues, which are reported to play a pivotal role in promoting itching by direct communication with cutaneous sensory neurons (Wilson et al., 2013). Based on the results of the initial experiment, an anti-inflammatory effect of 3% and 30% mastic and an anti-itch effect of 30% mastic were identified. However, using 30% mastic is not realistic for the commercialisation of products, and the efficacy of mastic on actual dermatitis symptoms such as AD is still unknown. Therefore, experiments with lower concentrations of mastic (3% and 5%) in a mouse model of AD were conducted in the next step.

In the AD mouse model, treatment with both 3% and 5% mastic treatment significantly improved the AD symptoms, itch behaviour, and cutaneous barrier function, which was corroborated by a reduction in TEWL. Skin dryness leading to the breakdown of the cutaneous barrier is recognised as a key trigger for the negative spiral of itch behaviour and inflammatory responses in patients with AD (Harrison and Spada, 2019). Therefore, significant improvement of cutaneous barrier function should be a strong point for use as a skin care supplement focusing on

cutaneous allergy. In contrast to the initial experiment in the ACD mouse model, lower concentrations of mastic also improved the histological findings and immune responses significantly, corresponding to the results with the AD symptoms. In particular, IL-33 and TSLP are emerging as crucial immune modulators with pleiotropic activities in type-2, -1, and regulatory immune responses, and they directly expand and/or activate Th2 cells, ILC2s, eosinophils, and other immune cells (Arae et al., 2021; Bell et al., 2013). Additionally, TARC is produced by dendritic cells, T cells, endothelial cells, and fibroblasts; it is considered a key chemokine in AD development, of AD and serves as one of the most reliable biomarkers that can reflect the AD activity (Morimura et al., 2021). Our findings indicate that the significant reduction of IL-33, TSLP, and TARC levels might directly inactivate eosinophils, neutrophils, macrophages, and other immune cells, resulting in the exacerbation of proinflammatory responses in AD. In contrast, there is only a slight upward trend in the number of regulatory T cells, which play a pivotal role in regulating immune responses during allergy. The reproducibility of mastic efficacy was confirmed by the second setting of an AD mouse model treated with 1% mastic ointment. The AD score and cutaneous barrier function were significantly reduced by 1% mastic treatment, whereas itch behaviour and immune reactions showed almost no influence. The limited impact of 1% mastic on immune responses suggests a dose-dependent relationship compared with the treatment at higher concentrations (3% and 5%) in the first setting. Side-by-side comparison of the existing topical treatment, gk2, with 1% mastic treatment showed similar efficacy.

Finally, we determined the direct effects of mastic on keratinocytes that play a pivotal role in the initiation and maintenance of AD (Steinhoff et al., 2001). Unexpectedly, IL-1 β and IL-6 production upon TNF α and IFN γ stimulation was reduced by mastic pretreatment. The concentrations used in this experiment did not induce cell toxicity, as determined in the pilot experiments.

Taken together, our findings showed that topical application of mastic ointment ameliorated either inflammatory or itch responses; these findings were corroborated by the significant reduction of AD score, TEWL, and immune reactions through the activation of keratinocytes. These data provide new mechanistic insights into the anti-allergic properties of mastic. However, its precise cellular targets in neurons, keratinocytes, and dendritic cells remain to be discovered. Clinical studies in dogs are also needed to verify the effect of mastic on higher mammals.

Conclusions

We herein evaluated the effect of mastic, which is a natural resin obtained from the stem and leaves of the *Pistacia lentiscus tree*, on the anti-inflammatory and anti-pruritic responses in a mouse model of ACD and AD. Topical treatment of mastic significantly ameliorated either inflammatory or itch responses; these findings were corroborated by the significant reduction of AD score, TEWL, and immune reactions through the activation of keratinocytes. Our findings provide new mechanistic insights into the anti-allergic properties of mastic.

Credit Author Statement

Risako Kishimoto and Tomoki Fukuyama wrote the paper draft. Narumi Kato, Mayuka Koike, Naoki Iwashita, and Yoshiichi Takagi corrected the draft. Tomoki Fukuyama supervised the experimenters. Risako Kishimoto, Narumi Kato, Mayuka Koike, Naoki Iwashita, and Yoshiichi Takagi performed the experiments. All data were generated in-house, and no paper mill was used. All authors agree to be accountable for all aspects of work ensuring integrity and accuracy.

Declaration of Competing Interest

The authors state no conflicts of interest.

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Supplementary materials

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1 **Original Research Article (Phytomedicine)**

2 **Topical treatment with mastic (resin from *Pistacia lentiscus*) elicits anti-**
3 **inflammatory and anti-pruritic responses by modulating keratinocyte activation in**
4 **a mouse model of allergic dermatitis**

5

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20

21Abstract

22Background: As the number of patients with skin allergies, including atopic dermatitis,
23has increased rapidly, therapeutic options such as anti-IL-31 antibody and Janus kinase
24inhibitor have been developed recently. However, many concerns remain regarding the
25adverse effects and cost of these drugs; therefore, development of supplements that
26could support the effect of therapeutic agents is always required.

27Purpose: The aim of this study was to develop preventive and supportive options for
28skin allergies by focusing on a natural product called “Mastic”. Methods: Initially, the
29anti-inflammatory and anti-pruritic responses of 3% and 30% Mastic topical treatment
30were investigated in a mouse model of allergic contact dermatitis, generated by topical
31application of toluene-2,4-diisocyanate (TDI), a hapten that induces type 2 helper T
32cells. After itch behaviour and ear-swelling response were monitored, serum, auricular
33lymph nodes, and skin tissues were collected to analyse immunocyte differentiation,
34cytokine determination, and histological changes.

35Results: Our findings indicated that topical treatment with mastic significantly
36ameliorated ear swelling, itch behaviour, immunocyte infiltration, and cytokine
37production. Histological evaluation confirmed the occurrence of anti-inflammatory
38responses. The anti-inflammatory and anti-pruritic effects of topical treatment with
39mastic (3% and 5%) were further confirmed in a mouse model of atopic dermatitis which
40was generated by topical application of TDI in NC/Nga mice. Thickness of the back skin,
41AD score, transepidermal water loss (TEWL), and itch behaviour were measured
42weekly, and immunocyte differentiation, cytokine determination, and histological
43changes were also analysed. Mastic treatment significantly attenuated the skin

44thickness, AD score, TEWL, and itch behaviour. Corroborated reduction was observed
45in the numbers of T cells and IgE-B cells, as well as in pro-inflammatory cytokine
46production. The reproducibility of the effects of mastic was confirmed with 1% mastic
47ointment in a setting similar to the AD mouse model. *In vitro* evaluation of keratinocytes
48indicated that mastic pre-exposure induced a significant dose-dependent decrease in
49cytokine production.

50Conclusion: Our findings thus demonstrate that topical treatment with mastic
51significantly ameliorate inflammatory and pruritic responses in a mouse model of allergic
52dermatitis.

53

54**Keywords:** Mastic; topical treatment; allergic contact dermatitis; atopic dermatitis;
55transepidermal water loss; keratinocyte

56

57**Abbreviations:** ACD, allergic contact dermatitis; AD, atopic dermatitis; gk2, glycyrrhizic
58acid dipotassium salt; TARC, thymus and activation-regulated chemokine; TDI, toluene-
592,4-diisocyanate; Th, T helper; TEWL, transepidermal water loss; TSLP, thymic stromal
60lymphopoietin; LN, lymph node; IL, interleukin; ELISA, enzyme linked immunosorbent
61assay; IgE, immunoglobulin E

62

63

64Introduction

65 Mastic is a natural resin obtained from the stem and leaves of the *Pistacia*
66*lentiscus* tree, which exists only on the Greek island of Chios (Amerikanou et al., 2021;
67Papada and Kaliora, 2019). In the 10th century, tourists visiting Chios discovered that
68Chios produced mastic. Although mastic can be produced outside Chios, the process
69involving the sap dripping and falling is only conducted in Chios. Over time, the sap
70turns into a yellow mass, sometimes called “tears of Christ” because its shape
71resembles tears (Amerikanou et al., 2021; Papada and Kaliora, 2019). In Greece,
72chewing mastic has long been considered to prevent periodontal disease. Previous
73studies have shown that mastic extract significantly inhibits the growth of periodontal
74pathogens, and has beneficial effects on cell viability; thus, it could be considered an
75alternative antibacterial agent for preventing periodontal disease (Carrol et al., 2020;
76Koychev et al., 2017). Besides its anti-bacterial effects, the anti-inflammatory effects of
77mastic have also been demonstrated in several reports. Papalois et al. (2012)
78demonstrated that mastic treatment downregulated the levels of IL-8 and NF- κ B p65 *in*
79*vitro*. Triantafyllou et al. (2011) also indicated that the anti-inflammatory activity of Chios
80mastic gum was associated with the inhibition of TNF- α -induced oxidative stress. In
81addition, anti-inflammatory effects of plant-derived components such as green tea
82extract and glycyrrhizic acid have been used for centuries all over the world (Chu et al.,
832017; Hasan et al., 2021). However, the anti-inflammatory effects of Chios mastic *in*
84*vivo*, using actual models of inflammatory diseases are not fully understood. Therefore,
85the initial aim of this study was to investigate the anti-inflammatory effects of Chios
86mastic *in vivo* using a mouse model of allergic dermatitis.

87 Dermatitis is a skin inflammatory condition that occurs in many forms including
88itching, eczema, dry red skin, rash, and swelling (Williams, 2005). Among these types of
89cutaneous allergy, atopic dermatitis (AD) is one of the most frequent forms of dermatitis
90in infants (Rizk et al., 2019). AD is a common skin disease in dogs and cats. Its clinical,
91immunological, histological, and pathological features are highly similar between dogs
92and humans; thus, canine AD has been suggested as an animal model for human AD
93(Mineshige et al., 2018). There are several therapeutic options for AD including oral
94supplements such as omega-3 fatty acids and lactobacilli; however topical treatment
95options are still limited, even though topical treatment is a more popular method to
96improve inflammatory symptoms. Therefore, this study aimed to introduce Chios mastic
97as a novel supplemental option for skin allergy. We investigated the anti-inflammatory
98and anti-itch responses of topical treatment with mastic ointment in a mouse model of
99allergic contact dermatitis (ACD) and AD.

100

101**Materials and Methods**

102*Experimental animals*

103 Six-week-old female BALB/c and NC/Nga mice were provided by Japan SLC, Inc.
104(Shizuoka, Japan) for developing the ACD and AD models, respectively. All mice were
105housed under a 12 h/12 h light/dark cycle at $22 \pm 3^{\circ}\text{C}$ and $50\% \pm 20\%$ humidity. The
106mice were provided with food and water *ad libitum*. All the aspects of this study were
107conducted in accordance with the Animal Care and Use Program of Azabu University
108(Approval No. 1910097).

109

110

111

110 *Preparation of test items*

111 Chios mastic resin was provided by Sosin Co., Ltd. (Tokyo, Japan). Highly purified
112 mastic was dissolved in caprylic/capric triglycerides to prepare a final concentration of
113 1%, 3%, 5%, or 30%. Glycyrrhizic acid dipotassium salt (gk2, 0.1%) in caprylic/capric
114 triglycerides was used as the positive control substance for the second experiment with
115 the AD model.

116

117 *Murine model of ACD*

118 A murine model of ACD was generated by repetitive topical sensitisation and
119 challenge with a Th (T helper) 2 type hapten, toluene-2,4-diisocyanate (TDI, Sigma-
120 Aldrich, Tokyo, Japan) diluted in acetone (5% for sensitisation, 0.5% for challenge) in
121 BALB/c mice, based on a previously described method (Aihara et al., 2020; Makino et
122 al., 2019; Watanabe et al., 2018) (Supplemental Fig. 1A). Mastic cream at 0% (vehicle
123 cream only, n = 6), 3%, and 30% (n = 6/group) was topically administered to the mice
124 once daily during the experimental period. The aim of this experiment was screening the
125 anti-allergic potential of mastic with wide variety concentrations. Dose selection of
126 mastic was based on a preliminary study examining the irritation of mouse skin. No
127 irritation response was observed after treatment with 30% mastic. A murine model of
128 ACD has been widely used to evaluate the drugs for the treatment of allergic skin
129 diseases such as tacrolimus (calcineurin inhibitor), cilomilast (PDE4 inhibitor) and
130 oclacitinib (Janus kinase inhibitor), and anti-allergic responses were well detected
131 (Baumer et al., 2005; Fukuyama et al., 2015). As the adequacy of this model has been
132 well validated, no positive control group was included in this study.

133 To evaluate the anti-inflammatory effect of mastic, the ear swelling response was
134calculated by subtracting the ear thickness before TDI challenge from the ear thickness
135at 24 h after TDI challenge. Immediately after TDI challenge to the auricle and the
136dorsal cervical, a video of scratching bouts was monitored for 60 min as an itch
137behaviour associated with TDI challenge. Immediately after measuring ear thickness,
138blood was collected from the posterior vena cava under isoflurane anaesthesia, and the
139animals were euthanized. Serum samples obtained from blood were used to measure
140the total immunoglobulin E (IgE) levels. A portion of the auricle was collected and
141cryopreserved to measure skin tissue cytokine levels. The remaining ear parts were
142fixed in formalin for histopathological examination. Isolated single-cell suspensions were
143prepared from auricular lymph nodes (LNs), and the immunocyte numbers and cytokine
144levels were measured.

145

146*Murine model of AD*

147 A murine model of AD was generated using NC/Nga mice based on a previously
148described method with minor modifications (Fukuyama et al., 2018a; Fukuyama et al.,
1492018b) (Supplemental Fig. 1B). Briefly, depilated NC/Nga mice (on their backs) were
150topically sensitised with 100 μ L of 5% TDI on their back. For the challenge, 0.5% TDI
151was topically applied to the back once weekly. In the initial setting, 0% (vehicle cream
152only), 3%, and 5% of the mastic ointment (n = 6/group) was applied to the AD model to
153determine the dose-dependent effects, and in the second setting, 0% (vehicle cream
154only) and 1% mastic treatment (n = 6/group) was used to confirm the repeatability and
155effects at lower concentrations. The second experiment also included a group treated

156with 0.1% gk2 for side-by-side comparison with another type of anti-inflammatory
157supplement. Experiments with murine AD model were performed to demonstrate more
158practical concentrations of mastic, therefore, mastic 30% that had greater anti-allergic
159response in first experiment in murine ACD model was not included in this experiment.

160 Scratching behaviour (30 min), transepidermal water loss (TEWL), ear and back
161skin thickness, and clinical scores were monitored once weekly during the experimental
162period. TEWL was measured using VAPO SCAN (AS-VT100RS, ASCH JAPAN Co.,
163LTD, Tokyo, Japan), and a clinical score from 0–4 was assigned as follows: no
164symptoms, 0; mild, 1; moderate, 2; severe, 3; and extreme, 4 as described by
165Fukuyama et al. (2018b). The back skin, auricular LN, and serum samples were
166collected from each mouse 24 h after the last TDI challenge and test application. The
167collected samples were processed for histology, cytokine analysis, IgE detection, and
168cell differentiation.

169

170*Histopathology*

171 A portion of skin samples was fixed in 10% formalin solution, embedded in paraffin
172wax, sectioned to 5 µm thickness, and stained with haematoxylin and eosin, or toluidine
173blue. A semi-quantitative histopathological evaluation of dermal oedema, cellular
174infiltration, folliculitis, and micro-abscesses was performed in a blinded fashion using the
175following grading system: 0, within normal limits; 1, mild; 2, moderate; 3, severe. The
176total score of the lesions was used for statistical evaluation. In the murine model of AD,
177microscopic measurement of dermal and epidermal thickness, and mast cell number
178were evaluated by 10 sections per mouse.

179

180 *Cytokine determination in skin tissues*

181 A portion of frozen skin was homogenised, and the supernatant was used to
182 determine the cytokine levels as described previously (Stover et al., 2016; Tajima et al.,
183 2019). The protein content in the supernatant was measured using a dendritic cell
184 protein assay kit (Bio-Rad Laboratories, Tokyo, Japan). Interleukin (IL)-1 β , -33, thymus
185 and activation-regulated chemokine (TARC), and thymic stromal lymphopoietin (TSLP)
186 were measured using respective enzyme-linked immunosorbent assays (ELISAs) (R&D
187 Systems, Minneapolis, MN, USA), and the amount of each cytokine per milligram of
188 protein was calculated.

189

190 *Analysis of lymph nodes (LNs)*

191 Single-cell suspensions isolated from the auricular LN were prepared as described
192 in previous studies (Aihara et al., 2020; Ookawara et al., 2020), and the total number of
193 cells was measured using a Tali® Image-Based Cytometer (Thermo Fisher Scientific,
194 Inc., Kanagawa, Japan). To avoid nonspecific binding in flow cytometric analysis, 1 $\times$
195 10⁶ cells were first incubated with 1 μ g of mouse Fc Block (Miltenyi Biotec K.K., Tokyo,
196 Japan) prior to incubation with monoclonal antibodies (FITC-conjugated anti-mouse
197 CD3, PE-conjugated anti-mouse CD4, FITC-conjugated anti-mouse IgE, PE-conjugated
198 anti-mouse CD19, FITC-conjugated anti-mouse CD11c, PE-conjugated anti-mouse
199 CD40, Miltenyi Biotec K.K.). Regulatory T cells were also identified using a Treg
200 detection kit according to the manufacturer's protocol (Miltenyi Biotec K. K.). **Strategy of**
201 **FACS analysis for gating cells is presented in the supplemental Fig. 2.**

18

9

19

202 In addition to FACS analysis, cytokine production from T cells was measured by co-
203culturing isolated T cells using Dynabeads Mouse T-Activator CD3/CD28 for 24 or 96 h
204(Thermo Fisher Scientific, Inc., Kanagawa, Japan). Cytokine levels (IL-4, IL-5, IL-9, and
205IL-13) in the culture supernatant were measured using ELISA.

206

207*Measurements of total IgE in serum*

208 The total IgE levels in serum samples were determined using ELISA according to
209the manufacturer's protocol (BD Biosciences, Tokyo, Japan). Optical density was
210measured using a microplate reader (iMark microplate reader, Bio-Rad Laboratories,
211Inc., Tokyo, Japan).

212

213*Cytokine release assay in HaKaT cell line (human epidermal keratinocyte cell line)*

214 HaKaT cells were obtained from CLS (Cell Lines Service) GmbH (Eppelheim,
215Germany) and cultured in Dulbecco's modified Eagle's medium (FUJIFILM Wako Pure
216Chemical Corporation) supplemented with 10% FCS (Sigma-Aldrich Co. LLC., Tokyo,
217Japan) and penicillin-streptomycin (FUJIFILM Wako Pure Chemical Corporation).
218HaKaT cells (1×10^4 cells) were seeded in 100 μ L of medium at 70% confluency in 96-
219well culture plates and pretreated for 24 h with 0.04, 0.2, or 1% of mastic before
220stimulation with recombinant human TNF α and IFN γ (PeproTech, Inc., Cranbury, NJ) for
221another 24 h. The concentrations of IL-1 β and IL-6 in the supernatants were quantified
222by ELISA. Cytotoxicity was not observed even with the highest mastic concentrations. A
223minimum of three independent experiments were performed to verify the reproducibility
224of the experiments.

225

226 *Statistical analyses*

227 Data are expressed as the mean $\pm$ 1 SEM. Analysis of variance (ANOVA) was
228 used to evaluate the results. For significant results, differences between the control and
229 treatment groups were assessed using Dunnett's multiple comparison test. Data
230 analysis was performed using Prism 9 (GraphPad Software, San Diego, CA, USA).

231

232 **Results**

233 ***Topical treatment with mastic ointment significantly ameliorates both the ear*** 234 ***swelling response and itch behaviour in a mouse model of ACD***

235 To elucidate the possible anti-inflammatory and anti-pruritic effects of mastic on
236 cutaneous allergy, topical treatment with 3% or 30% mastic ointment was initially
237 applied to a mouse model of ACD. The ear swelling response was significantly
238 suppressed by mastic administration in a dose-dependent manner (Fig. 1 A and B).
239 Suppression of the ear swelling response by mastic application was corroborated by the
240 histological evaluation. Oedema and cellular infiltration were dose-dependently
241 ameliorated by mastic application, and significant changes were observed in the 30%
242 mastic treatment group (Fig. 1C, Supplemental Table 1). Surprisingly, although
243 treatment with 30% mastic significantly decreased the itch behaviour, 3% mastic
244 significantly increased the scratching bouts induced by TDI challenge (Fig. 1D). A
245 similar tendency was observed in the skin tissue cytokine levels. There was a significant
246 decrease in the IL-1 β levels with both the 3% and 30% mastic treatment groups;

247however, a significant decrease in TSLP levels was found only in the 30% treatment
248group, whereas 3% mastic treatment enhanced the TSLP levels (Fig. E-G).

249

250***Effect of mastic ointment on immunocyte infiltration, inflammatory cells,***
251***inflammatory cytokines, and IgE levels in a mouse model of ACD***

252 To analyse the efficacy of mastic ointment on the local immune reaction, the
253number of dendritic cells, helper T cells, and IgE-positive B cells in the auricular lymph
254nodes were measured by flow cytometric analysis. Corresponding to the ear swelling
255response, treatment with 3% and 30% mastic ointment significantly reduced the number
256of inflammatory cells compared to the vehicle control group (Fig. 2 A-D). Total IgE
257levels, produced by IgE-positive B cells, in the serum were also significantly reduced by
258mastic application (Fig. 2 E). Significant inhibition of Th2 and Th9 cytokine production
259(IL-4, IL-9, and IL-13) by T cells in mastic treatment groups indicates the direct influence
260of mastic on helper T cell activation (Fig. 2 F-H).

261

262***Topical treatment with mastic significantly exhibited the anti-inflammatory and***
263***anti-pruritic responses in a mouse model of AD***

264 In the next step, the efficacy of 3% and 5% mastic was evaluated in a mouse
265model of AD. Daily application of mastic significantly reduced the AD score, skin
266thickness, TEWL, and scratching bouts compared to the vehicle control group (Fig. 3).
267No dose-dependent changes were observed between the 3% and 5% mastic treatment
268groups in terms of the above endpoints. All findings of in-life measurements were
269confirmed by histological evaluation and cytokine levels in skin tissues. Both 3% and

270 5% mastic treatments significantly reduced folliculitis, cellular infiltration, and thickness
271 of dermis and epidermis (Fig. 4 A, Table 1). IL-1 β , IL-33, TARC, and TSLP levels in skin
272 tissue were also significantly downregulated by mastic treatment compared to the AD
273 control group (Fig. 4 B-E).

274

275 ***Treatment with mastic ointment significantly suppressed the local immune***
276 ***function in a TDI-induced mouse model of AD***

277 Corresponding to the outcomes from the ACD mouse model, 3% and 5% mastic
278 treatment significantly reduced the local immune function including the migration of
279 dendritic cells, helper T cells, and IgE-positive B cells into local lymph nodes (Fig. 5 A-
280 C). Total IgE secreted by IgE-positive B cells was also downregulated by mastic
281 treatment in a dose-dependent manner compared to the AD control group (Fig. 5 D,
282 Supplemental Fig. 3A). Inflammatory cytokine production by stimulated T cells such as
283 IL-4, 5, 9, and 13, was significantly reduced by mastic treatment compared to the AD
284 control group in a dose-dependent manner (Fig. E-H). Mastic treatment slightly
285 enhanced the number of regulatory T cells, which plays a pivotal role in regulating the
286 immune function and allergic reaction, but significant difference was not found
287 (Supplemental Fig. 3B).

288

289 ***Efficacy of mastic ointment was confirmed in a second setting of the AD model***
290 ***treated with 1% mastic ointment.***

291 The efficacy of mastic ointment was confirmed in a second setting of an AD
292 mouse model treated with 1% mastic ointment or 0.1% gk2. Although there were no

changes in back skin thickness and itch behaviour in both the mastic and gk2 treatment groups, the AD score and TEWL were significantly ameliorated by these treatments (Supplemental Fig. 4 A-E). Histological evaluation showed that 1% mastic treatment significantly decreased cellular infiltration into the epidermis and dermis, whereas there was no change in the 0.1% gk2 treatment group (Supplemental Table 2). Reproducibility experiments were performed at a lower concentration of 1% mastic. The parameters measured in the reproducibility experiment included back skin symptoms, TEWL, and IgE production measurements. All parameters in the administration group was significantly reduced compared to the control group. The total serum IgE levels were downregulated by 1% mastic and 0.1% gk2 treatment; however, there were no significant changes in the immunocyte proliferation in LN, cytokine production by T cells, and cytokine levels in skin tissue (Supplemental Fig. 5).

305

Mastic pre-exposure significantly suppressed IL-1 β and IL-6 secretion by stimulated keratinocytes

To demonstrate the possible interaction between topical mastic treatment and keratinocyte activation, IL-1 β and IL-6 secretion by stimulated human keratinocytes (HaKaT cells) pre-treated with 0, 0.04, 0.2, and 1% of mastic was measured. Both IL-1 β and IL-6 secretion was suppressed by mastic treatment in a dose-dependent manner. The mastic-treated groups showed a significant decrease compared to the vehicle control group (Supplemental Fig. 6).

314

Discussion

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14

29

316 The prevalence of cutaneous allergies, including AD, has increased dramatically
317over the last century among humans and companion animals (David Boothe et al.,
3182017; Nassau and Fonacier, 2020). Glucocorticoids and calcineurin inhibitors have long
319been used to treat cutaneous allergies because of their immediate efficacy and
320competitive prices. However, they demonstrate various side effects depending on their
321dose and dosing period; prolonged dosing of these immunosuppressants requires
322extreme caution from doctors as well as patients. Similar concerns have been
323expressed in the veterinary field, and many case reports have provided warnings
324regarding the inappropriate usage of steroids, leading to an increased risk of Addison's
325disease (Stalder et al., 1994). Against this backdrop, novel therapeutics such as Janus
326kinase inhibitors (e.g., oclacitinib) and anti-IL-31 monoclonal antibody (e.g.,
327Lokivetmab) have been recently launched for pruritus associated with AD (Cinats et al.,
3282018; He and Guttman-Yassky, 2019; Kabashima and Irie, 2021; Nakashima et al.,
3292018). However, these drugs are still of concern because of cost and limited efficacy,
330particularly for inflammatory symptoms; therefore, development of supplements that can
331support the effect of these new therapeutics is strongly required. This study aimed to
332introduce a new supplemental option for skin allergy by focusing on the natural product,
333mastic, and evaluated the anti-allergic effects of topical treatment with mastic using *in*
334*vivo* mouse models and an *in vitro* experiment.

335 Initially, we examined whether topical mastic treatment directly affects
336inflammatory and itch responses in a mouse model of ACD. The anti-inflammatory
337property of mastic ointment was corroborated by significant downregulation of the ear
338swelling response, histological findings of inflammation, total IgE levels, lymphocyte

339proliferation in LNs, and cytokine production. Anti-itch property was also demonstrated
340in the 30% mastic treatment group, whereas the 3% mastic treatment enhanced
341scratching behaviour. This phenomenon was confirmed by the TSLP levels in skin
342tissues, which are reported to play a pivotal role in promoting itching by direct
343communication with cutaneous sensory neurons (Wilson et al., 2013). Based on the
344results of the initial experiment, an anti-inflammatory effect of 3% and 30% mastic and
345an anti-itch effect of 30% mastic were identified. However, using 30% mastic is not
346realistic for the commercialisation of products, and the efficacy of mastic on actual
347dermatitis symptoms such as AD is still unknown. Therefore, experiments with lower
348concentrations of mastic (3% and 5%) in a mouse model of AD were conducted in the
349next step.

350 In the AD mouse model, treatment with both 3% and 5% mastic treatment
351significantly improved the AD symptoms, itch behaviour, and cutaneous barrier function,
352which was corroborated by a reduction in TEWL. Skin dryness leading to the breakdown
353of the cutaneous barrier is recognised as a key trigger for the negative spiral of itch
354behaviour and inflammatory responses in patients with AD (Harrison and Spada, 2019).
355Therefore, significant improvement of cutaneous barrier function should be a strong
356point for use as a skin care supplement focusing on cutaneous allergy. In contrast to the
357initial experiment in the ACD mouse model, lower concentrations of mastic also
358improved the histological findings and immune responses significantly, corresponding to
359the results with the AD symptoms. In particular, IL-33 and TSLP are emerging as crucial
360immune modulators with pleiotropic activities in type-2, -1, and regulatory immune
361responses, and they directly expand and/or activate Th2 cells, ILC2s, eosinophils, and

362 other immune cells (Arae et al., 2021; Bell et al., 2013). Additionally, TARC is produced
363 by dendritic cells, T cells, endothelial cells, and fibroblasts; it is considered a key
364 chemokine in AD development, of AD and serves as one of the most reliable
365 biomarkers that can reflect the AD activity (Morimura et al., 2021). Our findings indicate
366 that the significant reduction of IL-33, TSLP, and TARC levels might directly inactivate
367 eosinophils, neutrophils, macrophages, and other immune cells, resulting in the
368 exacerbation of proinflammatory responses in AD. In contrast, there is only a slight
369 upward trend in the number of regulatory T cells, which play a pivotal role in regulating
370 immune responses during allergy. The reproducibility of mastic efficacy was confirmed
371 by the second setting of an AD mouse model treated with 1% mastic ointment. The AD
372 score and cutaneous barrier function were significantly reduced by 1% mastic
373 treatment, whereas itch behaviour and immune reactions showed almost no influence.
374 The limited impact of 1% mastic on immune responses suggests a dose-dependent
375 relationship compared with the treatment at higher concentrations (3% and 5%) in the
376 first setting. Side-by-side comparison of the existing topical treatment, gk2, with 1%
377 mastic treatment showed similar efficacy.

378 Finally, we determined the direct effects of mastic on keratinocytes that play a
379 pivotal role in the initiation and maintenance of AD (Steinhoff et al., 2001).
380 Unexpectedly, IL-1 β and IL-6 production upon TNF α and IFN γ stimulation was reduced
381 by mastic pretreatment. The concentrations used in this experiment did not induce cell
382 toxicity, as determined in the pilot experiments.

383 Taken together, our findings showed that topical application of mastic ointment
384 ameliorated either inflammatory or itch responses; these findings were corroborated by

385the significant reduction of AD score, TEWL, and immune reactions through the
386activation of keratinocytes. These data provide new mechanistic insights into the anti-
387allergic properties of mastic. However, its precise cellular targets in neurons,
388keratinocytes, and dendritic cells remain to be discovered. Clinical studies in dogs are
389also needed to verify the effect of mastic on higher mammals.

390

391**Conclusions**

392We herein evaluated the effect of mastic, which is a natural resin obtained from the
393stem and leaves of the *Pistacia lentiscus* tree, on the anti-inflammatory and anti-pruritic
394responses in a mouse model of ACD and AD. Topical treatment of mastic significantly
395ameliorated either inflammatory or itch responses; these findings were corroborated by
396the significant reduction of AD score, TEWL, and immune reactions through the
397activation of keratinocytes. Our findings provide new mechanistic insights into the anti-
398allergic properties of mastic.

399

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402

403**Conflict of Interest**

404The authors state no conflicts of interest.

405

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409

410**CRedit author statement**

411 Risako Kishimoto and Tomoki Fukuyama wrote the paper draft. Narumi Kato,
412Mayuka Koike, Naoki Iwashita, and Yoshiichi Takagi corrected the draft. Tomoki
413Fukuyama supervised the experimentators. Risako Kishimoto, Narumi Kato, Mayuka
414Koike, Naoki Iwashita, and Yoshiichi Takagi performed the experiments. All data were
415generated in-house, and no paper mill was used. All authors agree to be accountable
416for all aspects of work ensuring integrity and accuracy.

417

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505 induce itch. *Cell* 155, 285-295.

506

507

508 **Figure Captions**

509 **Fig. 1.** Topical treatment with mastic ointment ameliorated the inflammatory and pruritic
510 response in a mouse model of allergic contact dermatitis. (A) The ear swelling response
511 was significantly suppressed by the topical treatment with mastic ointment. (B) Typical
512 pictures of ear auricles from each group. (C) Representative histological pictures of ear
513 skin in each group. (D) Scratching bouts were significantly reduced by 30% mastic
514 application, whereas 3% mastic significantly enhanced the scratching behaviour.
515 Inflammatory cytokines, IL-1 β (E), IL-33 (F), and TSLP (G), in ear skin tissue were
516 downregulated by topical mastic application. Each result is presented as the mean $\pm$ 1
517 SEM. n = 8 per group.

518

519 **Fig. 2.** The number of dendritic cells (A), helper T cells (B), and IgE positive B cells (C)
520 in the auricular lymph node were significantly reduced by mastic treatment. (D) Total
521 IgE levels in serum were also downregulated by mastic application. Production of IL-4
522 (E), IL-9 (F) and IL-13 (G) from CD3/CD28-stimulated T cells in the auricular lymph
523 node was significantly reduced by mastic administration. Each result is presented as the
524 mean $\pm$ 1 SEM. n = 8 per group.

525

526 **Fig. 3.** Topical treatment with mastic ointment inhibits lesion formation and itch
527 behaviour in a mouse model of atopic dermatitis. Vehicle-treated and hapten-challenged
528 mice developed AD and were evaluated by their AD score (A), skin thickness (B), TEWL
529 (C), and scratching bouts (D). Mastic-treated mice developed only mild lesions
530 throughout the challenge, and there was a significant difference between the vehicle-

531and mastic-treated groups. (E) Typical pictures of back skin in each group. Each result
532is presented as the mean $\pm$ 1 SEM. n = 6 per group. *p < 0.05, **p < 0.01 (Dunnett's
533multiple comparison test) vs. AD control group. TEWL = transepidermal water loss.

534

535**Fig. 4.** Representative histological images of back skin at 6 weeks after treatment
536initiation (A). Mastic ointment-treated mice show significantly reduced inflammatory
537cytokines, IL-1 β (B), IL-33 (C), TARC (D), and TSLP (E), in back skin tissue. Each result
538is presented as the mean $\pm$ 1 SEM. n = 6 per group.

539

540**Fig. 5.** The number of dendritic cells (A), helper T cells (B), and IgE positive B cells (C)
541in the auricular lymph node were significantly reduced by mastic treatment in the AD
542mouse model. Total IgE levels in serum (D), and production of IL-4 (E), IL-5 (F), IL-9 (G)
543and IL-13 (H) from CD3/CD28-stimulated T cells in the auricular lymph node were
544significantly reduced by mastic administration. Each result is presented as the mean $\pm$ 1
545SEM. n = 6 per group.

546

547**Supplemental Fig. 1** Experimental protocol used to sensitise and challenge BALB/c
548mice with TDI in acetone. ACD mouse model (A) and AD mouse model (B). ACD,
549allergic contact dermatitis; AD, atopic dermatitis; TDI, toluene-2,4-diisocyanate

550

551**Supplemental Fig. 2** Strategy of flowcytometric analysis for gating cells.

552

553**Supplemental Fig. 3** Representative IgE positive B cells staining of FACS analysis (A).

554A slight upward trend with mastic treatment was seen in the number of regulatory T
555cells in auricular lymph nodes in the AD mouse model (B). Each result is presented as
556the mean $\pm$ 1 SEM. n = 6 per group. Treg = regulatory T cells

557

558**Supplemental Fig. 4.** The reproducibility of mastic efficacy was confirmed by the
559second setting of an AD mouse model treated with 1% mastic or 0.1% gk2. The AD
560score (A) and TEWL (B) were significantly ameliorated by both mastic and gk2
561treatment, whereas skin thickness (B) and scratching bouts (D) were not influenced. (E)
562Typical pictures of the back skin in each group. (F) Representative histological pictures
563of the back skin in each group. Each result is presented as the mean $\pm$ 1 SEM. n = 6 per
564group. **p < 0.01 (Dunnett's multiple comparison test) vs. AD control group.

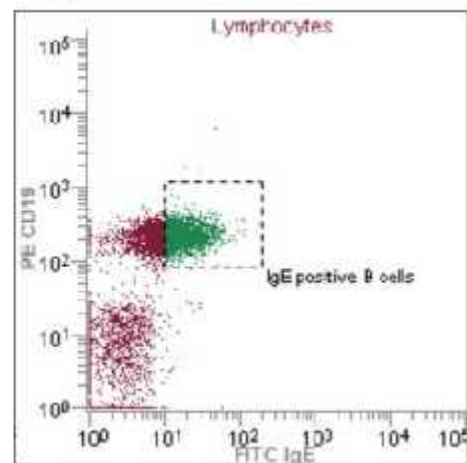
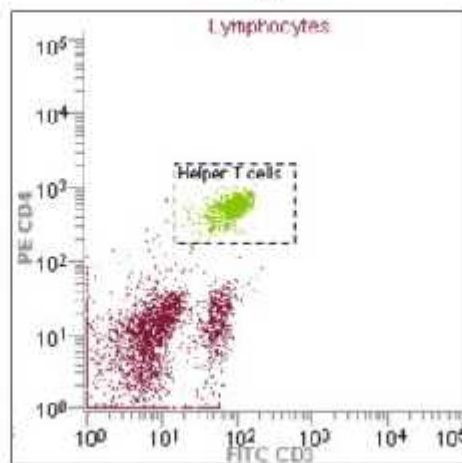
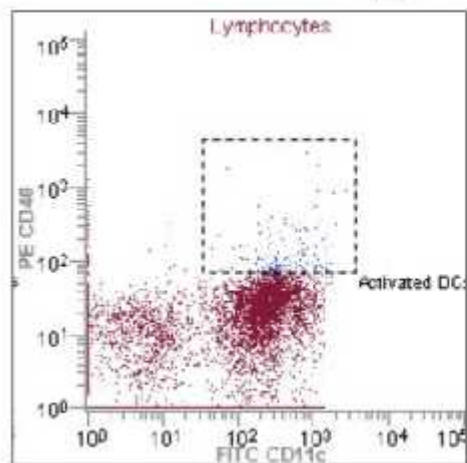
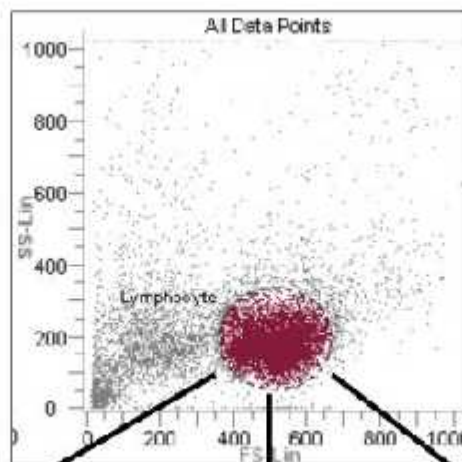
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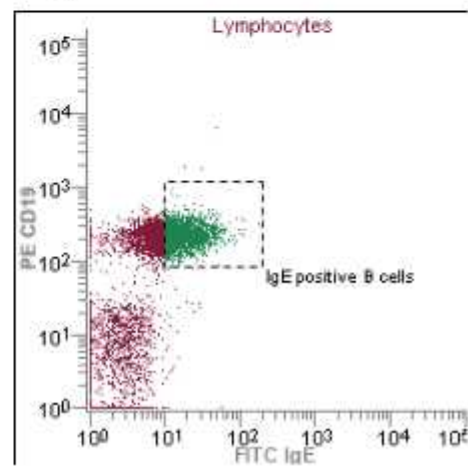
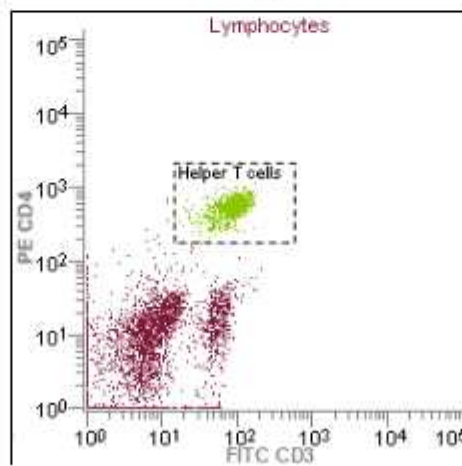
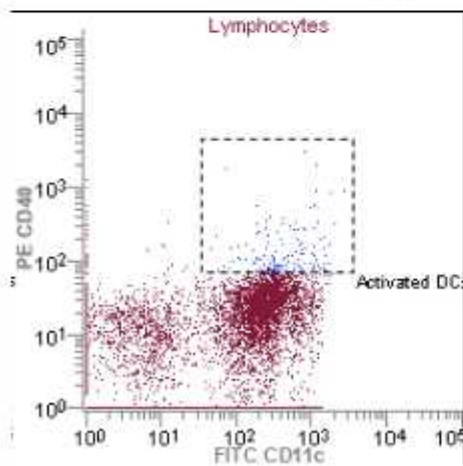
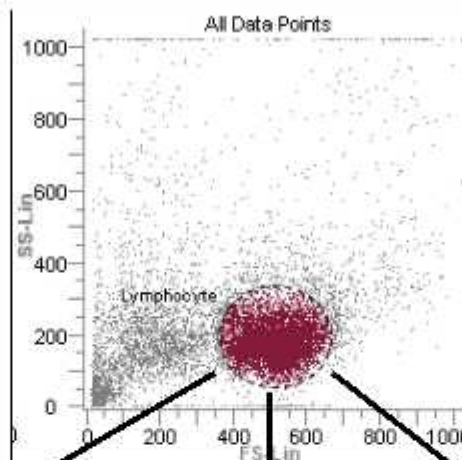
566**Supplemental Fig. 5.** The number of dendritic cells (A), helper T cells (B), IgE-positive
567B cells (C), and the production of IL-4 (D) and IL-5 (E) from CD3/CD28-stimulated T
568cells in the auricular lymph node were unchanged by 1% mastic treatment in the AD
569mouse model. Total IgE levels in serum (F) were significantly reduced by mastic
570administration; however, IL-1 β (G), IL-33 (H), TSLP (I), and TARC (J) levels in the back
571skin tissue were not affected. Each result is presented as the mean $\pm$ 1 SEM. n = 6 per
572group.

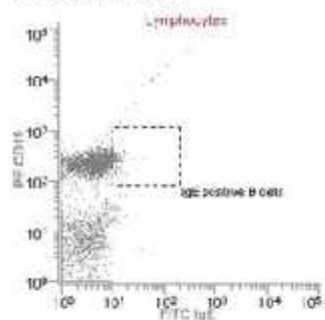
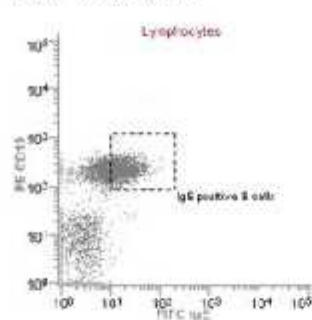
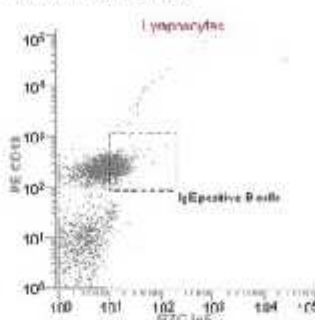
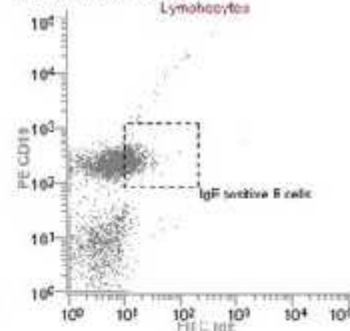
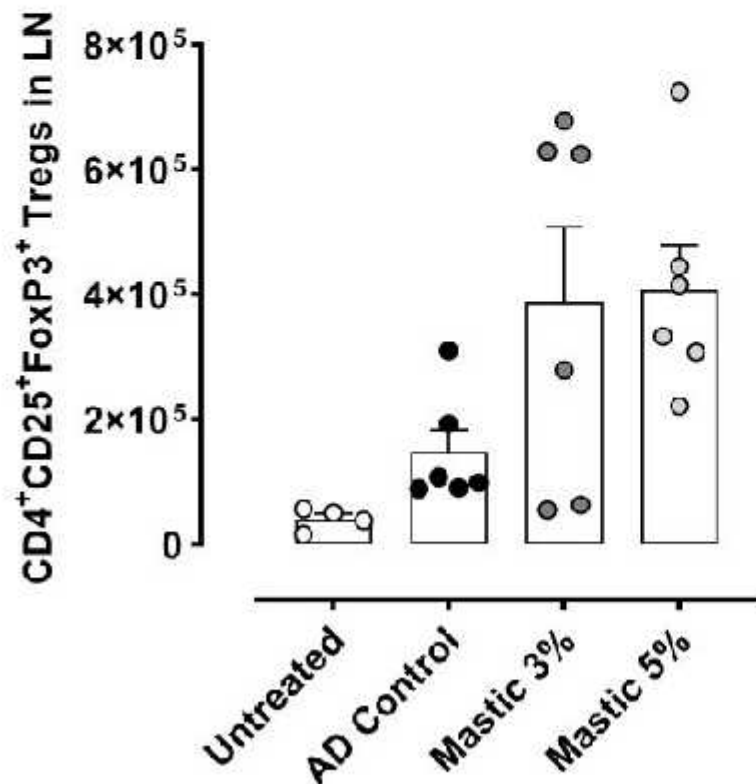
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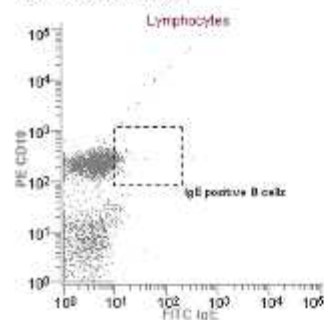
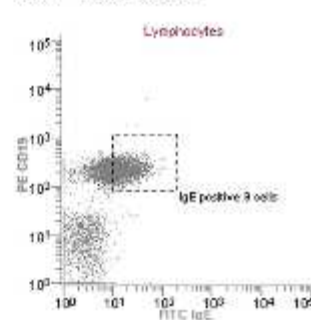
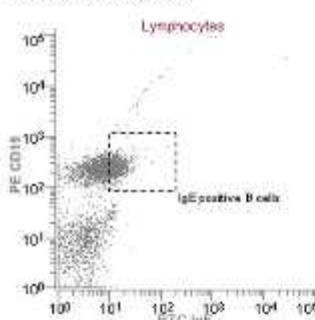
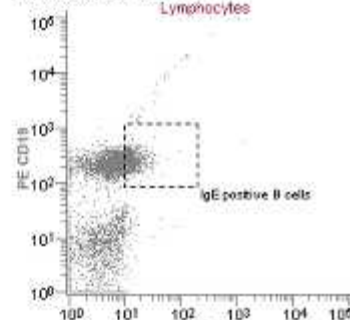
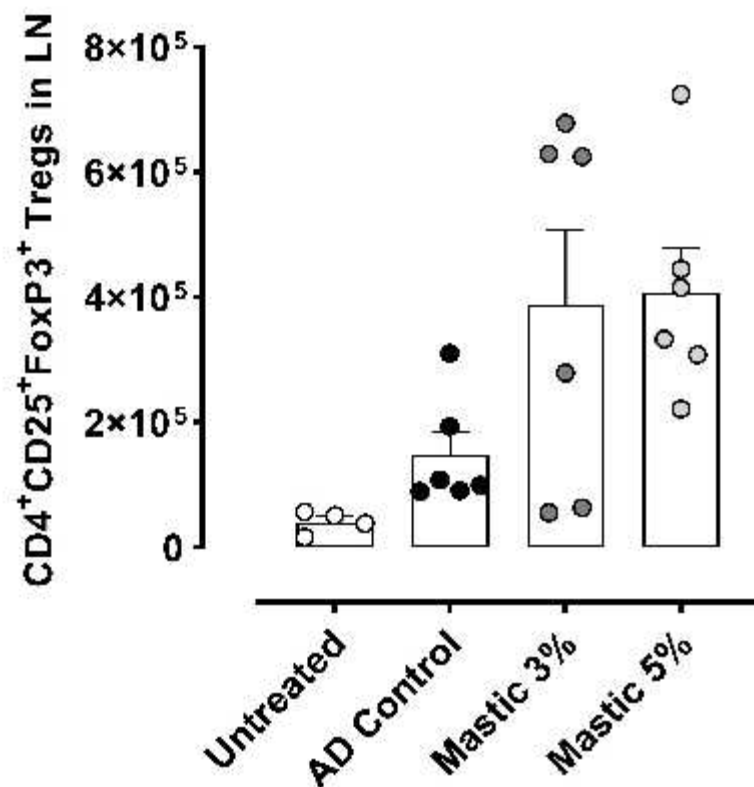
574**Supplemental Fig. 6.** *In vitro* IL-1 β (A) and IL-6 (B) secretion by human keratinocytes
575after TNF α and IFN γ stimulation was significantly reduced by pre-treatment with mastic

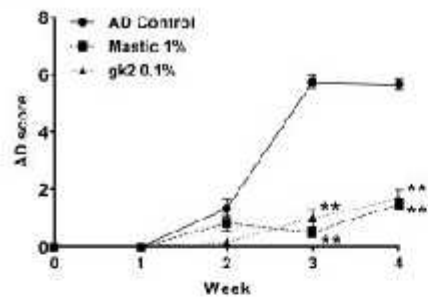
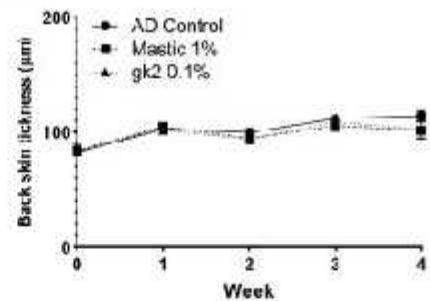
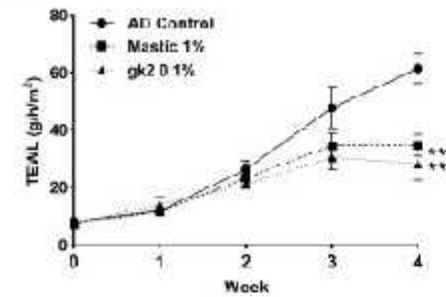
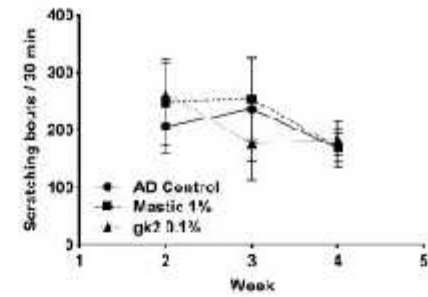
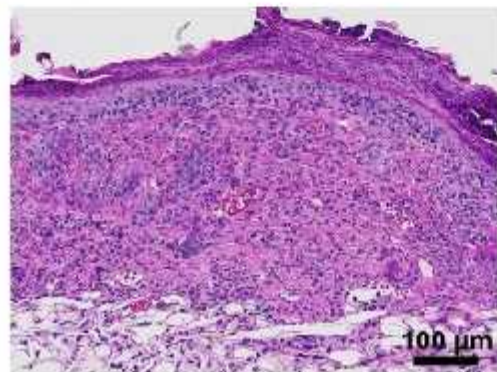
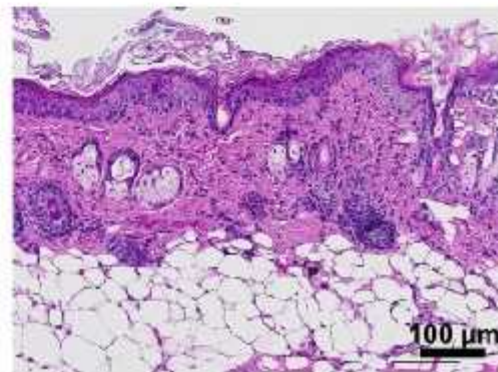
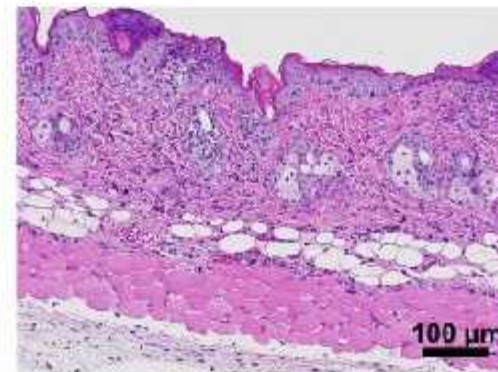
576for 24 h. Each result is presented as the mean $\pm$ 1 SEM. n = 7 per group.

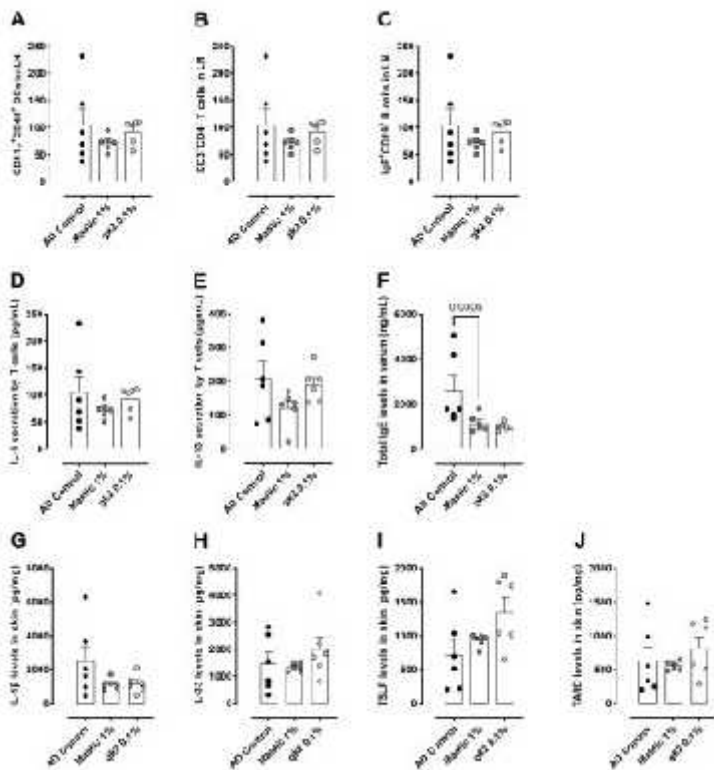


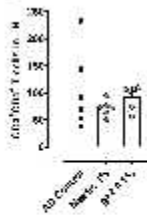
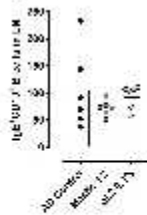
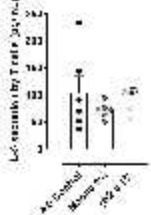
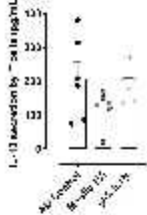
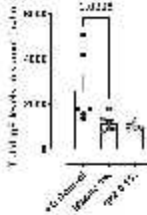
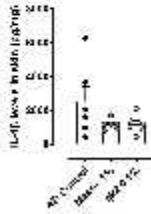
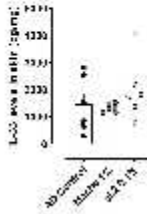
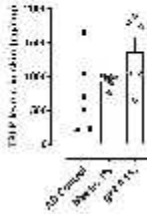
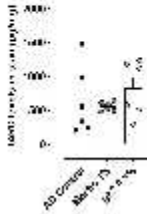


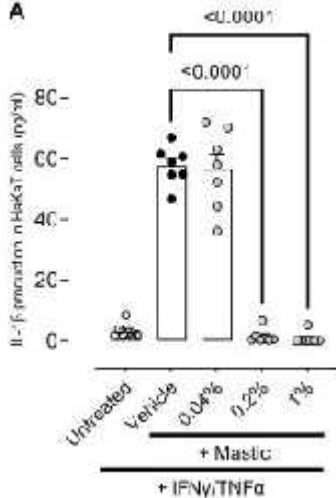
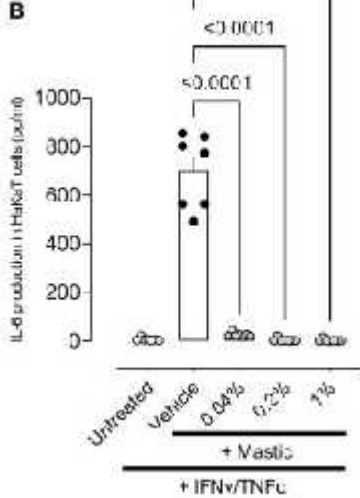
A**Untreated****AD control****Mastic 3%****Mastic 5%****B**

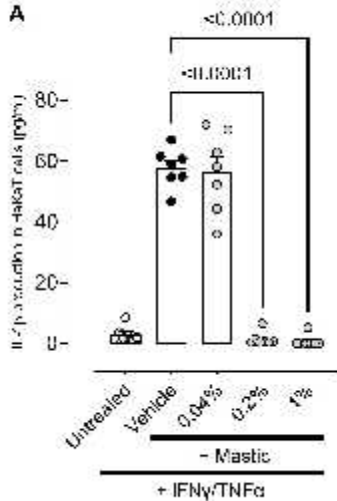
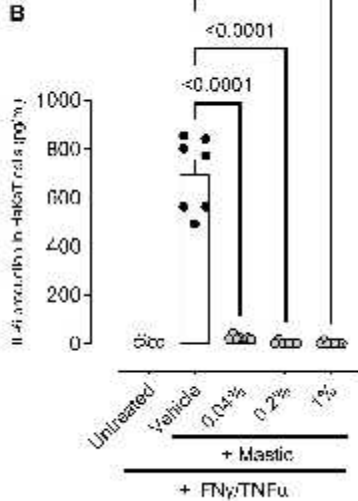
A**Untreated****AD control****Mastic 3%****Mastic 5%****B**

A**B****C****D****E****AD control****Mastitic 1%****gk2 0.1%****F****AD control****Mastitic 1%****gk2 0.1%**



A**B****C****D****E****F****G****H****I****J**

A**B**

A**B**

A. ACD mouse model



B. AD mouse model



● : 0.5% TDI

○ : 5% TDI



Mastic ointment

A. ACD mouse model



B. AD mouse model



● : 0.5% TDI

○ : 5% TDI



Mastic ointment