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ORIGINAL PAPER

Orengedokuto exerts anti-allergic effects via inhibition of effector T cell activation in a murine model of contact hypersensitivity

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Abstract

Orengedokuto (OGT) is a Kampo prescription that has been used for the treatment of inflammation, hypertension, gastrointestinal disorders, and liver and cerebrovascular diseases. It is also used for the treatment of skin diseases such as urticaria and atopic dermatitis. We previously studied its anti-allergic effects of OGT on the murine model of 2,4,6-trinitrochlorobenzene (TNCB)-induced contact hypersensitivity (CHS) and demonstrated that it significantly suppresses ear swelling in a dose-dependent manner. However, the mechanism underlying this activity remained unknown. Here, we sought to identify the mechanism involved. Using a murine model of TNCB-induced CHS, together with adoptive cell transfer experiments, we found that the anti-allergic effects of OGT may be due to the inhibition of effector T cell activation and not the induction and/or activation of regulatory T cells. Flow cytometry analysis revealed that oral administration of OGT suppressed the increase in $CD8^{+}CD44^{high}CD62L^{+}$ cell number in draining lymph nodes (dLNs) of mice sensitized with 5% TNCB. Additionally, ex vivo experiments confirmed the suppressive effect of OGT on the activation of effector T cells, as interferon-γ (IFN-γ) production by cultured lymphocytes obtained from 5% TNCB-sensitized mice and stimulated with anti-CD3ε and anti-CD28 monoclonal antibodies was reduced by OGT administration. In conclusion, our finding suggests that OGT exerts anti-allergic effects by regulating the activation of effector T cells involved in inflammatory skin diseases such as atopic dermatitis.

Keywords Orengedokuto · Contact hypersensitivity · Effector T cell · Interferon-γ

Introduction

Atopic dermatitis (AD) is a chronic inflammatory skin disorder characterized by several features including, pruritus, typical exanthema with characteristic distribution, chronic recurrence, and atopic predisposition [1, 2]. Its pathogenesis is undoubtedly complex and involves an interplay of skin barrier abnormalities, dysregulation of the allergic immune response, and pruritus [3].

Topical steroids are the mainstay of current AD treatments, but topical agents with calcineurin inhibitors are also used occasionally. Recently, an anti-IL-4/IL-13 monoclonal antibody (dupilumab), a topical nonsteroidal phosphodiesterase 4 inhibitor (crisaborole), and a Janus kinase inhibitor (delgocitinib) were developed as new therapeutic agents with great expectation regarding their usefulness [4–6]. However, there is currently no single treatment available that rids all patient of their symptoms effectively and completely. A variety of therapies, including Kampo medicines such as orengedokuto (OGT), have been used to treat AD in Japan.

OGT has been used traditionally to treat inflammation, hypertension, gastrointestinal disorders, and liver and cerebrovascular diseases [7, 8]. Many studies have reported its anti-inflammatory activity, and some of these have demonstrated its anti-allergic properties [9, 10]; however, the detailed mechanisms of action remain unknown.

In the present study, we sought to investigate the mechanism behind its anti-allergic properties. To that aim, we investigated the effect of OGT on T cell activation in a murine model of the 2, 4, 6-trinitrochlorobenznene (TNCB)-induced contact hypersensitivity (CHS).

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Materials and methods

Materials

The crude drugs for OGT were purchased from Tsumura & Co. (Ibaraki, Japan). All crude drugs used in this study were of Japanese Pharmacopeia 17th edition (JP XVII) grade and are listed in Table 1. We purchased TNCB and olive oil from Nacalai Tesque (Kyoto, Japan). Prednisolone (PSL) was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). HPLC-grade methanol, other solvents and chemicals were purchased from Wako Pure Chemical Industries, Ltd.

Preparation of the OGT extract

A daily human dosage of crude drugs compounded according to OGT was decocted with 600 mL ion-exchanged distilled water using an electric heater (HMJ-1000 N; HARIO Co. Ltd., Tokyo, Japan) for 60 min. The decoction was filtered and cooled down to approximately 25 °C. The filtrate was lyophilized to a powder and stored at − 20 ºC until use. This OGT extract was the daily dose for humans, and the yield was 3.32 ± 0.03 g.

Three-dimensional HPLC analysis of the OGT extract

The lyophilized OGT extract powder was dissolved in distilled water (50 mg/mL) under ultrasonic conditions and the solution was filtered with a Millex-HP syringe filter unit (0.45 µm, Merck Millipore, Ltd., Darmstadt, Germany). The HPLC apparatus consisted of a PU-2089 Plus quaternary low pressure gradient pump and an MD-2018 Plus photodiode array detector (JASCO Co., Tokyo, Japan). A TSK gel ODS-80Ts column (5 µm, 4.6 mm I.D. × 150 mm, Tosoh Co., Tokyo, Japan) was used for the analysis. A binary mobile phase consisting of (A) 50 mM ammonium acetate (pH = 3.6) and (B) acetonitrile was used. The gradient elution was programmed as follows: 0–5 min, 5% (B); 5–55 min, 5%–95% (B); and 55–60 min, 95% (B). The flow rate was 1.0 mL/min, and the column temperature was room temperature. Peaks were identified by comparison with the retention times and UV spectra of the authentic compounds. The 3D-HPLC profiles of the OGT extract used in this study are shown in Fig. S1.

Animals

Female BALB/c mice (6 weeks old) were purchased from Japan SLC (Shizuoka, Japan). The mice were housed in standard plastic cages in a temperature- and humidity-controlled environment with food and water available ad libitum. Acclimatization was allowed for at least 7 days before experimentation. All experiments were carried out according to the guidelines of the Animal Care Committee of the Factory of Pharmacy, Meijo University (approval number: 2019PE1).

Contact hypersensitivity (CHS) model

A hapten-induced CHS model was used to evaluate the anti-allergic effects of OGT. Briefly, mice were sensitized by painting their shaved abdominal skin with 100 µL of 5% TNCB in acetone. The CHS response was elicited 7 days later by painting both surfaces of the right ear of each mouse with 10 µL of 1% TNCB in olive oil. Ear thickness was measured before and 24 h after challenge using a dial thickness gauge (G-1A; Ozaki Mfg. Co., Ltd., Tokyo Japan) to calculate ear swelling. Based on body surface area correction, the mouse dose is approximately 12.3 times higher than the human dose [11]; therefore, the OGT dose used in this study was 10 times higher than the human daily dose. OGT (0.55 g/kg) was orally administered once a day for 7 days right after sensitization. In the control mice, mice were sensitized with 5% TNCB, and administered orally with water instead of OGT. In normal group, mice were painted with acetone and administered orally with water for 7 days.

Adaptive cell transfer

Donor mice were sensitized by painting their shaved abdominal skin with 100 µL of 5% TNCB in acetone, after which OGT (0.55 g/kg) was orally administered as described above. Seven days after sensitization, the mice were sacrificed via cervical dislocation, and lymphocyte cell suspensions were prepared from the draining lymph nodes (inguinal

Table 1 Crude drugs of orengedokuto (OGT) used in this study

Prescription	Yield (g)	Crude drug	Original plant source of crude drug	Composition (g)	Lot No	Manufactures
Orengedokuto	3.32 ± 0.03	Scutellaria Root	roots of *Scutellaria baicalensis*	3.0	J21441	Tsumura & Co
		Phellodendron Bark	barks of *Phellodendron amurense*	3.0	AJ7381	Tsumura & Co
		Coptis	Rhizoma rhizomes of *Coptis japonica*	1.5	AD1381	Tsumura & Co
		Gardenia	Fruit fruits of *Gardenia jasminoides*	3.0	AJ8671	Tsumura & Co

and axillary) for use in subsequent experiments. To study the effect of OGT on the activation of effector T cells, lymphocytes (1×10^7 cells/mouse) were injected intravenously into naïve recipient mice and challenged with a topical application of 1% TNCB in olive oil 24 h after adoptive transfer. To evaluate the effect of OGT on the induction and/or activation of regulatory T cells, lymphocytes (1×10^7 cells) were injected into naïve recipient mice, and then mice were sensitized and challenged on day 1 and 7 after injection, respectively. Ear thickness was measured before and after challenge. Vehicle (Hank's buffered salt solution (HBSS))-injected recipient mice represented the negative control group.

Flow cytometry

Mice were sensitized by painting their shaved abdominal skin with 100 µL of 5% TNCB in acetone and orally administered OGT (0.55 g/kg), PSL (20 mg/kg) or distilled water for 7 days. Cell suspensions were prepared from mouse dLNs (inguinal and axillary) on day 7 after sensitization and 2×10^6 cells were aliquoted into each round tube. To determine cell viability, the cells were stained using fixable viability stain 575 V (BD Biosciences, USA) for 15 min at 4 °C in the dark. To block Fc receptors and minimize nonspecific binding, the cells were re-suspended in staining buffer containing anti-mouse CD16/32 antibody (clone: 2.4G2, TOMBO) for 10 min at 4 °C. For phenotypical characterization of effector T cells, cells were stained with CD4-FITC (clone: GK1.5, BD Biosciences), CD8-BUV395 (clone: 53–6.7, BD Biosciences), CD44-PE (clone: IM7, BD Biosciences) and CD62L-BV421 (clone: MEL-14, BD Biosciences) in 2% fetal bovine serum (FBS)/HBSS for 30 min at 4 °C in the dark. Flow cytometry was performed on an LSR Fortessa X20 (BD Biosciences) and 1×10^6 cells were counted for each sample. Gating strategy for analysis was shown in Fig. S2. Data were analyzed using FACS Diva software (BD Biosciences).

Enzyme-linked Immunosorbent assay (ELISA)

Mice were sensitized by painting their shaved abdominal skin with 100 µL of 5% TNCB in acetone and orally administered OGT (0.55 g/kg), PSL (20 mg/kg) or distilled water for 7 days. Lymphocyte cell suspensions were prepared from dLNs (inguinal and axillary) and 5×10^6 cells were cultured with anti-CD3ε and anti-CD28 antibodies (1 µg/mL each) for 48 h in a 96 well plate at 37 °C. Cell-free supernatants were collected and stored at − 80 °C until use. To measure the level of IFN-γ ELISA was performed using a murine IFNγ ELISA kit (Diaclone SAS, France) according to the manufacturer's instructions.

Statistical analysis

Results are represented as mean ± S.E.M., and statistical analysis was conducted using ANOVA with Bonferroni correction for multiple comparisons.

Results

The effect of OGT on the murine model of TNCB-induced CHS

To examine the mechanism whereby OGT exerts an anti-allergic action, we first confirmed the anti-allergic effect of OGT on TNCB-induced CHS response in mice (Fig. 1a). As shown in Fig. 1b, oral administration of OGT (0.55 g/kg) significantly suppressed ear swelling 24 h after the topical application of 1% TNCB, as reported previously [9].

Adoptive cell transfer experiments were performed to understand the mechanism whereby OGT suppresses TNCB-induced CHS. Lymphocyte cell suspensions from mice sensitized with 5% TNCB and orally administered OGT for 7 days (donor mice), were injected into naïve BALB/c mice (recipient mice). Then, the recipient mice were challenged 24 h later via topical application of 1% TNCB on the ear, and ear swelling was measured after a further 24 h (Fig. 2a). Compared to vehicle recipient mice (buffer injected), ear swelling was observed in control recipient mice (administered water), which was indicative of effector T cell activation (Fig. 2b). In contrast, ear swelling was significantly reduced in mice receiving OGT (Fig. 2b). These results suggested that oral administration of OGT suppressed effector T cell activation in CHS.

Similarly, lymphocyte cell suspensions from donor mice were injected into naïve BALB/c recipient mice and the recipient mice were sensitized with 5% TNCB on their pre-shaved abdomen after 24 h. TNCB (1%) was applied on the right ear 7 days after sensitization to induce CHS and investigate the induction and/or activation of regulatory T cells (Fig. 3a). As shown in Fig. 3b, ear swelling was almost the same among the three groups (vehicle, control and OGT). These results suggested that oral administration of OGT did not affect the induction and/or activation of regulatory T cells in CHS.

Taken together, these results suggest that oral administration of OGT exerts anti-allergic effect on a TNCB-induced murine CHS model through the inhibition of effector T cell activation.

The effect of OGT on effector T cell activation in the murine model of TNCB-induced CHS

To confirm the results of the adoptive transfer experiments, we then investigated the effect of OGT on T cell composition

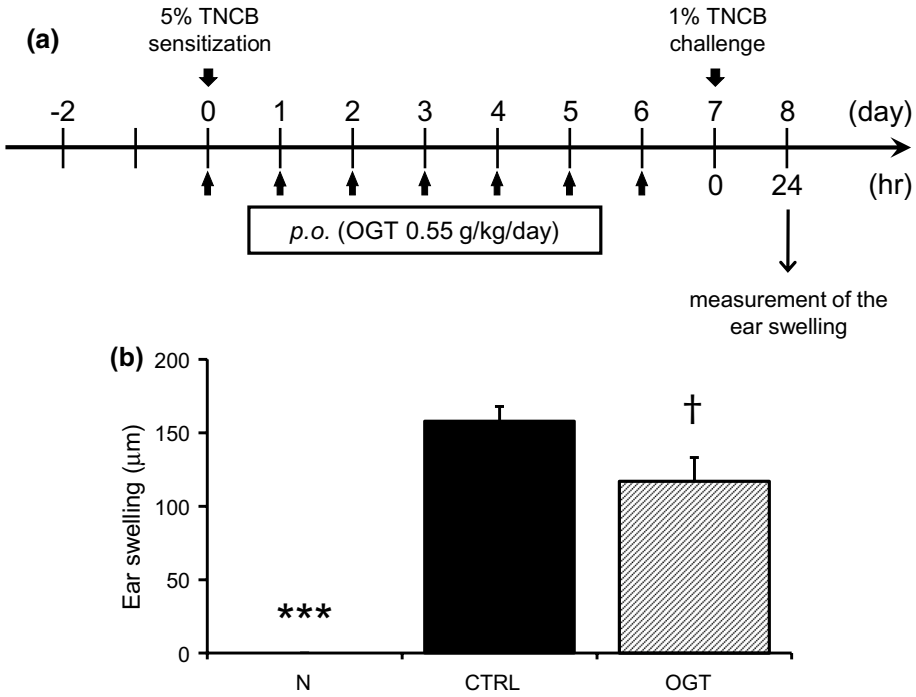

Fig. 1 Effect of orengedokuto (OGT) on the murine model of 2,4,6-trinitrochlorobenzene (TNCB)-induced contact hypersensitivity (CHS). **a** Experimental protocol. **b** Ear swelling 24 h after the topical application of 1% TNCB on the ear. Distilled water was administered to the normal (N) and control (CTRL) groups. OGT (0.55 g/kg, 10 times the human dose) was orally administered once a day for 7 days to mice in the OGT group. Each column represents the mean ± S.E.M. of 3–5 mice. ***$p < 0.001$ *vs.* the control group by ANOVA with Bonferroni correction for multiple comparisons. †$p < 0.05$ *vs.* the control group by ANOVA with Bonferroni correction for two selected comparisons

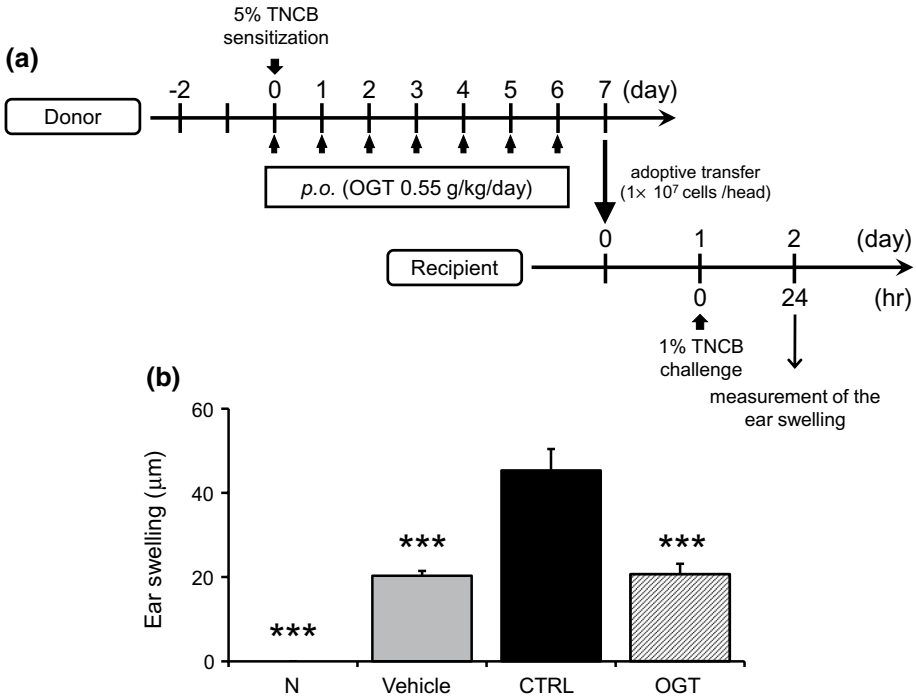

Fig. 2 Effect of orengedokuto (OGT) on effector T cell activation in the murine model of contact hypersensitivity (CHS). **a** Experimental protocol. **b** Ear swelling 24 h after topical application of 1% TNCB on the ear. In donor mice, OGT (0.55 g/kg, 10 times the human dose) was administered orally once daily to the treated group and distilled water was administered to the control (CTRL) group right sensitization with 5% TNCB. After 7 days, lymphocytes were obtained from the dLNs and injected into naïve recipient mice. The lymphocytes obtained from control donor mice were injected intravenously into the CTRL group, while the lymphocytes obtained from OGT-treated donor mice were injected intravenously into the OGT group. HBSS alone was injected intravenously in the vehicle-treated mice. All mice, except those in the normal (N) group were then challenged on the right ear with 1% TNCB 24 h after cell transfer, and ear swelling was measured 24 h after the challenge. Each column represents the mean ± S.E.M. of 3 to 5 mice. ***$p < 0.001$ *vs.* the control group by ANOVA with Bonferroni correction for multiple comparisons

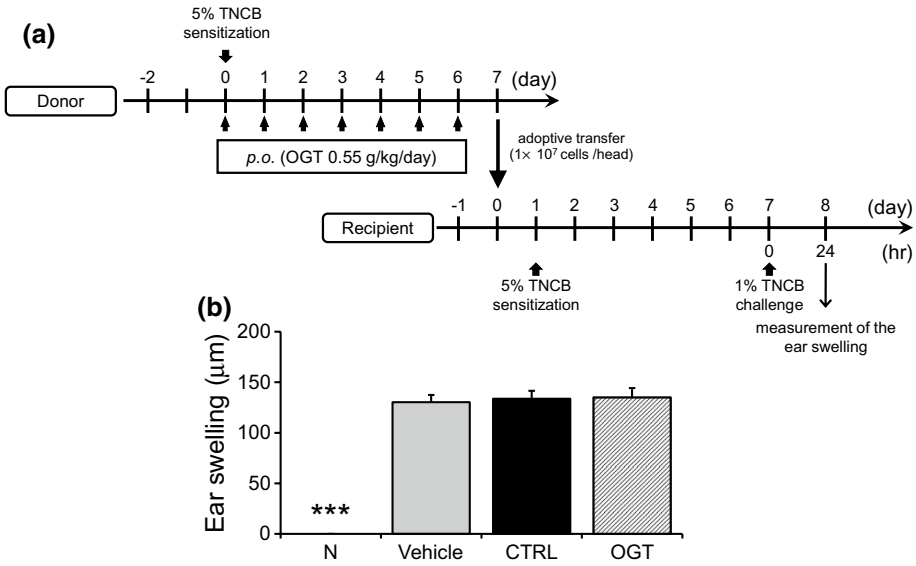

Fig. 3 Effect of orengedokuto (OGT) on the induction and/or activation of regulatory T cells in the murine model of contact hypersensitivity (CHS). **a** Experimental protocol. **b** Ear swelling 24 h after topical application of 1% TNCB on the ear. After sensitization with 5% TNCB, OGT (0.55 g.kg) and distilled water were administered orally once daily to the treated (OGT) and control (CTRL) groups in donor mice, respectively. After 7 days, lymphocytes were obtained from the dLNs and injected into naïve recipient mice. Lymphocytes obtained from the control donor mice were injected intravenously into the CTRL recipient group and lymphocytes obtained from the OGT-treated donor mice were injected intravenously into the OGT recipient group. HBSS alone was injected intravenously into vehicle mice. All mice, except those in the normal (N) group were sensitized on shaved abdomens with 5% TNCB 24 h after cell transfer and then challenged on their right ears with 1% TNCB 7 days later. Ear swelling was measured at 24 h after the challenge. Each column represents the mean ± S.E.M. of 3 to 5 mice. ***$p < 0.001$ *vs.* the control group by ANOVA with Bonferroni correction for multiple comparisons

in the dLNs at the end of the sensitization phase of CHS. Lymphocytes were prepared from dLNs of mice sensitized with 5% TNCB and administered orally with OGT or prednisolone (PSL) for 7 days. It has been suggested that $CD8^+$ T cells are the primary effector T cells in CHS, and it is well known that CD44 is a maker molecule for memory T cells [12–14]. Therefore, we assessed CD44 expression to examine the effect of OGT on the activation of $CD8^+$ T cells.

The percentage of $CD8^+CD44^{high}$ cells was significantly higher in the dLNs of the control group than in those of the normal group, indicating an increase in the levels of memory T cells (Fig. 4a). PSL suppressed the increase to normal levels, and OGT also significantly suppressed the increase (Fig. 4a). Furthermore, the percentage of $CD8^+CD44^{high}CD62L^+$ cells was also increased in the dLNs of mice sensitized with 5% TNCB, and oral administration of PSL reduced the increase to normal levels and OGT significantly reduced the percentage of them (Fig. 4b). Since $CD8^+CD44^{high}CD62L^+$ cells in the dLNs are thought to be central memory T cells at the afferent phase of CHS [15, 16], these results suggested that the oral administration of OGT suppresses the increase in the amount of central memory T cells in the dLNs.

Next, we studied the effect of OGT on effector T cell function in the dLNs of mice during CHS. Lymphocytes were prepared from the dLNs of mice sensitized with 5% TNCB and administered orally with OGT, PSL or distilled water for 7 days. Then, the lymphocyte cell suspensions were stimulated with anti-CD3ε and anti-CD28 antibodies. After 48 h, the culture supernatants were collected, and IFN-γ levels were determined using ELISA (Fig. 5a). Oral administration of either OGT or PSL significantly reduced the IFN-γ levels (Fig. 5b). These results suggested that the oral administration of OGT suppresses the activation of effector T cells both quantitatively and qualitatively.

Discussion

Atopic dermatitis (AD) is a chronic inflammatory skin disorder whose pathogenesis is very complex and for which there is no single effective treatment. OGT has been traditionally used to treat AD in Japan, and its anti-allergic effects have been demonstrated in several studies. However, its mechanism of action remains unknown.

Here, we investigated the mechanism underlying the anti-allergic effects of OGT using a murine model of TNCB-induced CHS and adoptive cell transfer experiments.

Mouse models of CHS are frequently used for identifying anti-allergic agents that can be used for treating AD [17–19]. Our previous study revealed that OGT suppresses ear swelling in a dose-dependent manner [9], but the mechanism

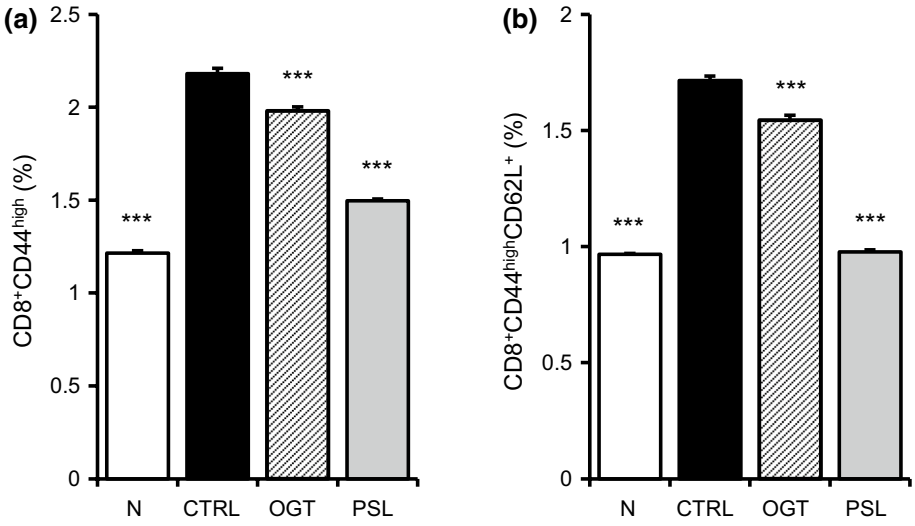

Fig. 4 Effect of orengedokuto (OGT) on effector T cell activation in the draining lymph nodes. **a** Percentage of $CD8^+CD44^{high}$ cells. **b** Percentage of $CD8^+CD44^{high}CD62L^+$ cells. Mice were sensitized with 5% TNCB and administered orally with OGT, PSL, or distilled water for 7 days. Lymphocyte suspensions were prepared from the draining lymph nodes. Lymphocytes (2×10^6 cells) were aliquoted into each tube, and then stained using fixable viability stain 575 V. After Fc blocking with anti-mouse CD16/32 antibody, cells were stained with CD8-BUV395, CD44-PE and CD62L-BV421 in 2% FBS/HBSS. Flow cytometry was performed on an LSR Fortessa X20 and 1×10^6 cells were counted for each sample. Each column represents the mean ± S.E.M. of three samples. ***$p<0.001$ *vs.* the control group by ANOVA with Bonferroni correction for multiple comparisons

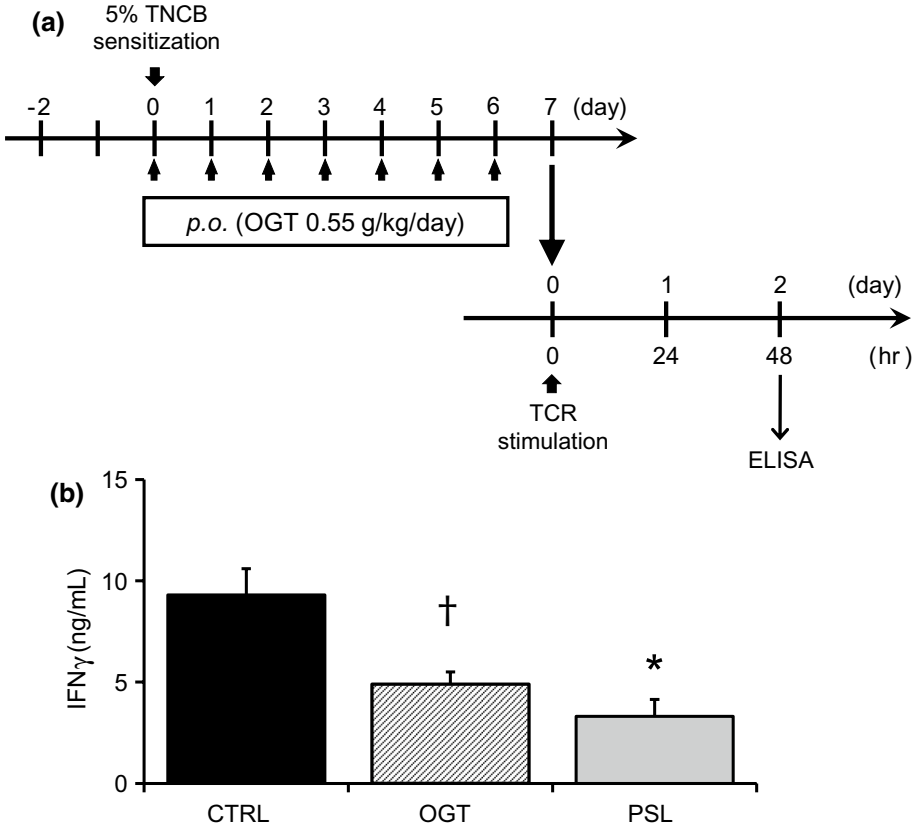

Fig. 5 Effect of orengedokuto (OGT) on interferon-γ (IFN- γ) production in cultured lymphocytes. **a** Experimental protocol. **b** IFN-γ levels in the culture supernatants after incubation with anti-CD3ε and anti-CD28 monoclonal antibodies for 48 h. Mice were sensitized with 5% TNCB and administered orally with OGT, PSL, or distilled water for 7 days. Lymphocyte suspensions were prepared from the draining lymph nodes, and lymphocytes (5×10^6 cells) were stimulated with anti-CD3ε and anti-CD28 monoclonal antibodies (1 μg/mL each) for 48 h. Then, the IFN-γ levels in the culture supernatants were measured by ELISA. Each column represents the mean ± S.E.M. of three samples. *$p<0.05$ *vs.* the control group by ANOVA with Bonferroni correction for multiple comparisons. †$p<0.05$ *vs.* the control group by ANOVA with Bonferroni correction for two selected comparisons

involved remain unknown. In the present study, we found that oral administration of OGT inhibited the activation of IFN-γ-producing effector T cells, resulting in a significant suppression of CHS in mice. Furthermore, oral administration of either OGT or PSL significantly suppressed the increase in the numbers of $CD8^{+}CD44^{high}$ memory T cells elicited in the dLNs of mice sensitized with 5% TNCB. Moreover, oral administration of OGT significantly reduced the increase in the number of $CD8^{+}CD44^{high}CD62L^{+}$ central memory T cells elicited in the dLNs of mice sensitized with 5% TNCB. Thus, we show that the anti-allergic effects of OGT are due to the inhibition of effector T cell activation.

The CHS response is reportedly mediated by IFN-γ-producing $CD8^{+}$ T cells, which are primed in lymphoid organs during the sensitization phase, and are recruited to the skin upon re-exposure to haptens such as TNCB and 2, 4-dinitrofluorobenzene (DNFB) [20–24]. CD44 and CD62L (L-selectin) are well-known adhesion molecules that mediate leukocyte recruitment at inflammatory sites and lymph nodes. CD44 is required for leukocyte extravasation into an inflammatory site such as the skin in murine CHS models [12–14]. In addition, the expression of CD62L divides memory T cells into two functionally distinct subsets [15, 16]. $CD44^{high}CD62L^{+}$ cells are termed central memory cells and preferentially home to lymph nodes, while $CD44^{high}CD62L^{-}$ cells are termed effector memory cells and are preferentially located in non-lymphoid tissues. Here, we confirmed that oral administration of OGT suppresses effector T cell activation in the afferent phase of the TNCB-induced CHS response. Furthermore, we observed that oral administration of OGT reduced the IFN-γproduction from lymphocytes of mice sensitized with 5% TNCB and administered orally with OGT by T cell receptor (TCR)-stimuli. Since the main effector T cells in the murine CHS response are thought to be IFN-γ producing $CD8^{+}$ T cells, we speculated that oral administration of OGT quantitatively and functionally suppressed effector T cell activation in the regional lymph nodes of the skin during CHS response.

Recently, oral administration of OGT was reported to improve the AD-like symptoms caused by multiple application of DNCB in mice [25]. Briefly, female BALB/c mice were sensitized with 0.5% DNCB for 3 days, challenged with 1% DNCB every 3 days, and orally administered with OGT daily from day 14 to day 29 (16 consecutive days). Oral administration of OGT significantly reduced the clinical symptoms by inhibiting eosinophil and mast cell infiltration into the skin lesions. Additionally, oral administration of OGT was reported to reduce the production of IL-4 and TNFα through the suppression of the MAPK and NF-κB pathways [25]. Moreover, OGT could significantly suppress the secretion of nitric oxide, IL-1β, IL-4, MCP-1, and GM-CSF in RAW264.7 cells, and inhibit prostaglandin E2 production in J774.1 cells [26]. However, there have been few reports concerning the immunomodulating effects of OGT on T cell activation. Many studies have reported the anti-inflammatory activities of OGT and its crude drug, Scutellariae Radix, on macrophage-like cells in vitro [27–31], suggesting the possibility that oral administration of OGT may regulate the function of antigen presenting cells (APCs) such as dendritic cells and Langerhans cells in the skin and suppress effector T cell activation in the murine model of CHS. Further studies are needed to examine how OGT suppress effector T cell activation, for example, the effect of oral administration of OGT on antigen uptake by APCs, APC migration into dLNs, and APC and naïve T cell interaction during the sensitization phase of CHS.

In conclusion, our findings suggest that OGT exerts its anti-allergic effects in the murine model of CHS through the inhibition of effector T cell activation. We have previously shown that a hot water extract of Coptidis Rhizoma reduces ear swelling in TNCB-induced CHS [32]; however, further studies are needed to understand the molecular mechanisms whereby specific ingredients in OGT function as anti-allergic agents.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s11418-021-01566-2.

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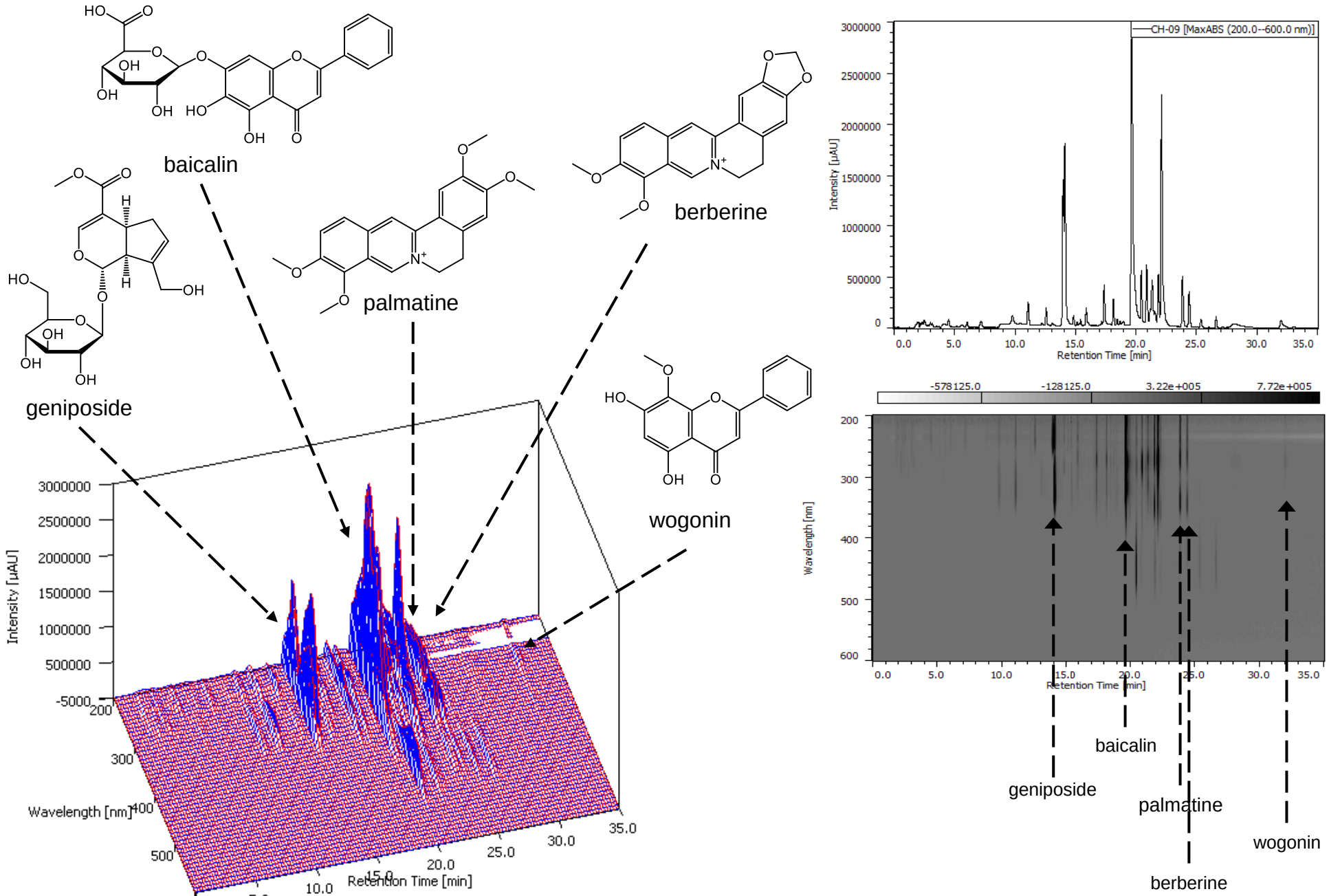
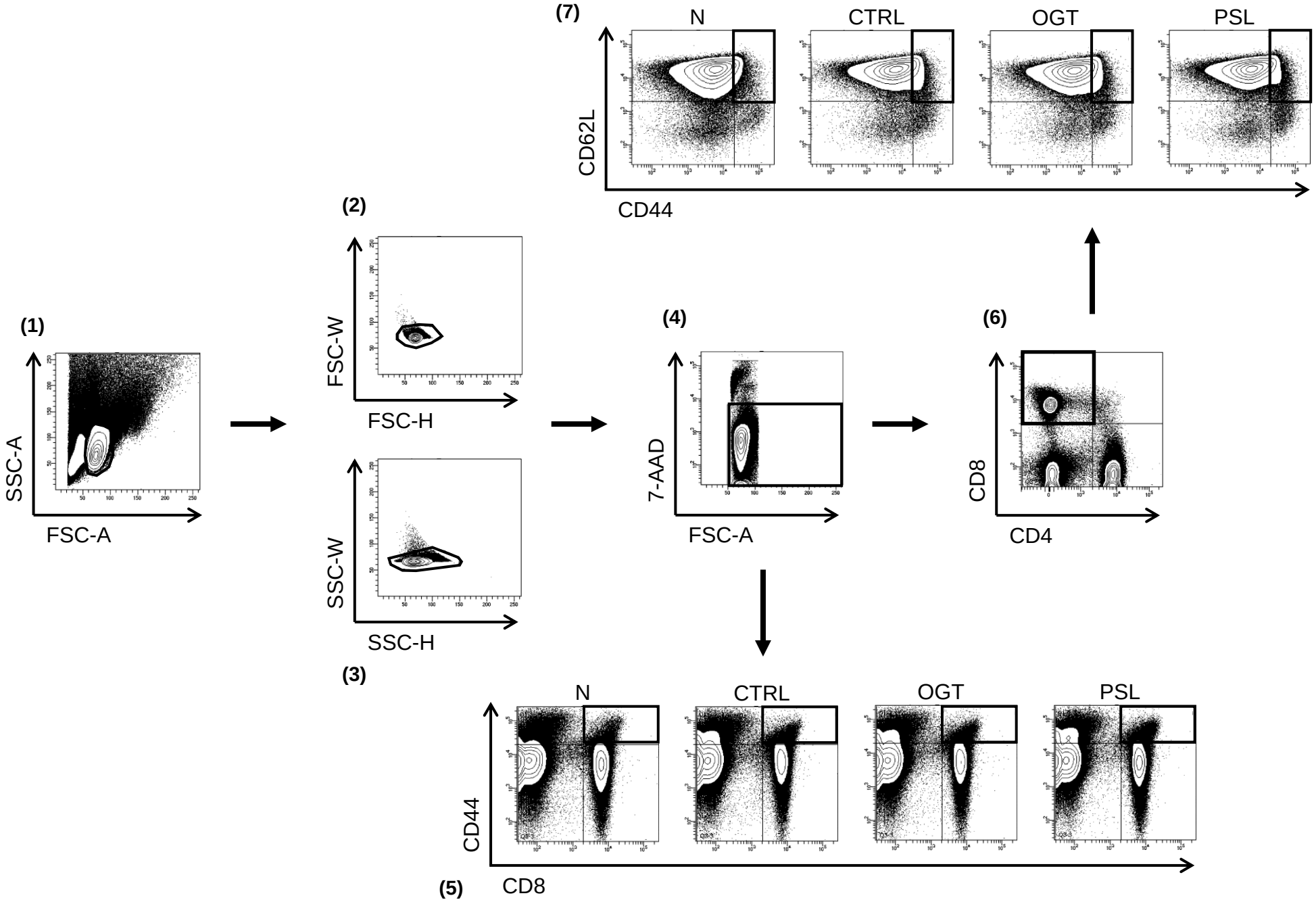


Tsuge et al. Fig. S1



Tsuge et al. Fig. S2

Fig. S1 3D-HPLC profile of orengedokuto (OGT)

Fig. S2 Gating strategy for analysis of $CD8^{+}CD44^{high}$ and $CD8^{+}CD44^{high}CD62L^{+}$ cells in lymphocytes obtained from draining lymph nodes of mice sensitized with TNCB

(1) Whole cells were fractionated by side scatter (SSCs) and forward scatter (FSCs) to lymphocytes. (2, 3) Debris and doublets were discriminated. (4) Single cells were then fractionated by 7-AAD into living cells. (5) Living cells were then fractionated by CD8 and CD44 to analyze the population of $CD8^{+}CD44^{high}$ memory T cells in draining lymph nodes of mice sensitized with TNCB and administered OGT or PSL orally. (6) Living cells were then fractionated by CD4 and CD8. (7) $CD8^{+}$ populations were then fractionated by CD44 and CD62L to analyze the population of $CD44^{high}$ and $CD62L^{+}$ central memory T cells in draining lymph nodes of mice sensitized with TNCB and administered OGT or PSL orally.