

Juzentaihoto Exerts Anti-Allergic Effects by Inhibiting Effector T-Cell Activation and Inducing and/or Activating Regulatory T Cells in a Murine Model of Contact Hypersensitivity

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Keywords

Juzentaihoto · Contact hypersensitivity model · Effector T cell · Regulatory T cell · Kampo medicines

Abstract

Background: Juzentaihoto (JTT) is a Kampo prescription that has been used clinically for treating skin diseases such as atopic dermatitis in Japan. We have previously studied the anti-allergic effects of JTT on 2,4,6-trinitrochlorobenzene (TNCB)-induced contact hypersensitivity (CHS) in mice and demonstrated that it significantly suppresses ear swelling in a dose-dependent manner. However, the mechanism underlying the anti-allergic actions of JTT is obscure. **Methods:** We investigated the mechanism underlying the anti-allergic effects of JTT using a TNCB-induced murine CHS model and adoptive cell transfer experiments. **Results:** We showed that the anti-allergic effects of JTT are due to inhibition of effector T-cell activation and induction and/or activation of regulatory T cells. Furthermore, ex vivo experiments confirmed the effect of JTT on the activation of effector T cells and regulatory T cells, as interferon- γ production decreased, whereas interleukin (IL)-10 production increased, in the cultured lymphocytes obtained from 5% TNCB-sensitized mice treated with anti-CD3 ϵ and anti-CD28 monoclonal antibodies. Flow cytometry showed that the CD4⁺CD25⁺Foxp3⁺,

CD4⁺CD25⁺Foxp3⁺, and CD8⁺CD122⁺ cell population increased after oral administration of JTT. Finally, the anti-allergic effect of JTT by inducing and/or activating regulatory T cells (Tregs) was confirmed to be mediated by IL-10 through in vivo neutralization experiments with anti-IL-10 monoclonal antibodies. **Conclusion:** We suggested that JTT exerts anti-allergic effects by regulating the activation of effector T cells and Tregs involved in murine CHS model.

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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]. AD pathogenesis is complex and involves interplay of skin barrier abnormality, dysregulation of allergic immune response, and pruritus [3].

Topical steroids are the mainstay of current AD treatments, although topical agents with calcineurin inhibitors are also used occasionally. Recently, an anti-interleukin (IL)-4/IL-13 monoclonal antibody (dupilumab), a

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Table 1. Crude drugs in JTT used in this study

Prescription	Yield, g	Crude drug	Original plant source of crude drug	Composition, g	Lot No.	Manufacturer
JTT	9.17±0.15	Astragali radix	Roots of <i>Astragalus membranaceus</i>	3.0	J05801	Tsumura & Co
		Cinnamomi cortex	Bark of <i>Cinnamomum cassia</i>	3.0	J06061	Tsumura & Co
		Rehmanniae radix	Roots of <i>Rehmannia glutinosa</i>	3.0	K32461	Tsumura & Co
		Paeoniae radix	Roots of <i>Paeonia lactiflora</i>	3.0	L29431	Tsumura & Co
		Cnidii rhizoma	Rhizome of <i>Cnidium officinale</i>	3.0	J00391	Tsumura & Co
		Atractylodis rhizoma	Rhizome of <i>Atractylodes japonica</i>	3.0	K06141	Tsumura & Co
		Angelicae radix	Roots of <i>Angelica acutiloba</i>	3.0	F26171	Tsumura & Co
		Ginseng radix	Roots of <i>Panax ginseng</i>	3.0	K03591	Tsumura & Co
		Poria	Sclerotium of <i>Wolfiporia cocos</i>	3.0	P15911	Tsumura & Co
		Glycyrrhizae radix	Roots of <i>Glycyrrhiza uralensis</i>	1.5	F01721	Tsumura & Co

JTT, juzentaihoto.

topical nonsteroidal phosphodiesterase 4 inhibitor (crisaborole), and a Janus kinase inhibitor (delgocitinib) have been developed as new therapeutic agents [4–6]. However, currently, a single treatment for curing the symptoms of AD effectively and completely in all patients is unavailable. Therefore, various therapies, including Kampo medicines, such as juzentaihoto (JTT), are being used to treat AD in Japan.

JTT has been used traditionally to treat fatigue, loss of appetite, and anorexia in patients with various diseases [7, 8]. Various clinical and basic studies have indicated that JTT performs various pharmacological functions. Many studies have revealed that JTT performs immunomodulatory functions in tumors [9–14] and after vaccination [15, 16]. In addition, JTT is also used for treating AD [17], and its anti-allergic properties were evaluated in murine models of allergic dermatitis [18, 19]. However, its mechanism of anti-allergic action remains unclear.

In the present study, we first confirmed the anti-allergic action of JTT in mice showing 2,4,6-trinitrobenzene chloride (TNCB)-induced contact hypersensitivity (CHS). Second, we evaluated the effects of JTT on effector T cell (Teff) activation and induction and/or activation of regulatory T cells (Tregs) using adoptive cell transfer experiments. Third, we investigated cell composition in the draining lymph nodes (dLNs) and cytokine production in lymphocyte culture systems to analyze Tregs induced and/or activated by oral administration of JTT.

Materials and Methods

Materials

The crude drugs for Kampo prescription 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. TNCB and olive oil were purchased from Nacalai Tesque (Kyoto, Japan). High performance liquid chromatography (HPLC)-grade methanol, and other solvents and chemicals were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

Preparation of JTT Extract

A daily dose of crude drugs compounded according to JTT was decocted with 600 mL ion-exchanged and distilled water using an electric heater (HMJ-1000N; HARIO Co. Ltd., Tokyo, Japan) for 60 min. The decoction was filtered and cooled down to approximately 25°C. The filtrate was lyophilized to powder and stored at –20°C until further use. This extract is the daily dose administered to humans, and the yield of the JTT extract was 9.17 ± 0.05 g.

Three-Dimensional HPLC Analysis of the JTT Extract

The lyophilized JTT extract powder was dissolved in distilled water (50 mg/mL) under ultrasonic conditions for few minutes and the solution was filtered with Millex-HP (0.45 µm; Merck Millipore, Ltd., Darmstadt, Germany). The HPLC apparatus was a PU-2089 plus pump and an MD-2018 plus photodiode array detector (JASCO Co., Tokyo, Japan). A TSK gel ODS-80 Ts column (5 µm, 4.6 mm I.D. × 150 mm; Tosoh Co., Tokyo, Japan) was used for analysis. Gradient elution was used in this study and the mobile phase consisted of (a) 50 mM ammonium acetate (pH = 3.6) and (b) acetonitrile. 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 maintained at room temperature. Peaks were identified by comparing with the retention times and UV spectra of authentic compounds. The 3D-HPLC profiles of the JTT used in this study are shown in online suppl. Fig. S1 (for all online suppl. material, see www.karger.com/doi/10.1159/000518448).

Animals

Female BALB/c mice (6 weeks old) were purchased from Japan SLC (Shizuoka, Japan). The mice were housed in standard plastic cages in control of temperature (23 ± 1°C) and humidity (50 ± 5%), environment with availability of food (MM-3; Funabashi Farm Co. Ltd., Chiba, Japan) and water ad libitum. Acclimatization was al-

lowed for at least 7 days before experimentation. All experiments were performed according to the guidelines of the Animal Care Committee of the Faculty of Pharmacy, Meijo University (approval number: 2019PE1).

CHS Model

A hapten-induced CHS model was used to evaluate the anti-allergic effects of JTT. Briefly, the mice were sensitized by painting their shaved abdominal skin with 100 μ L 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 μ L of 1% TNCB in olive oil. Ear thickness was measured before and 24 h after the challenge using a dial thickness gauge (G-1A; Ozaki Mfg. Co., Ltd., Tokyo Japan). Based on body surface area correction, the mouse dose is approximately 12.3 times higher than the human dose [20]; therefore, the JTT dose used in the study was 10 times higher than the human daily dose. JTT (1.5 g/kg; 10 times the human dose) was orally administered once a day for 7 days right after the sensitization.

Adaptive Cell Transfer

The donor mice were sensitized by painting their shaved abdominal skin with 100 μ L of 5% TNCB in acetone, after which JTT 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 dLNs (inguinal and axillary) for use in the subsequent experiments. To study the effect of JTT on the activation of Tregs, lymphocytes (1×10^7 cells/mouse) were injected intravenously into naïve recipient mice, which were challenged with a topical application of 1% TNCB in olive oil after 24 h.

To evaluate the effect of JTT on the induction and/or activation of regulatory Tregs, lymphocytes (1×10^7) were injected intravenously into naïve recipient mice that were sensitized 24 h after injection and challenged after 7 days. Ear thickness was measured before and after the challenge. The vehicle (Hank's buffered salt solution)-injected recipient mice represented the vehicle-negative control group.

Magnetic-Activated Cell Sorting

The donor mice were sensitized by painting their shaved abdominal skin with 100 μ L of 5% TNCB in acetone. The mice were sacrificed 7 days after sensitization, and lymphocyte suspensions were prepared from the dLNs (inguinal and axillary) for use in subsequent experiments. The cells were further sorted on a magnetic-activated cell sorting separation column (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions and separated into CD4-positive and CD4-negative fractions. Furthermore, the cells of the CD4-positive fraction were separated into CD25-positive and -negative fractions. The number of cells adoptively transferred to recipient mice was quantitatively determined by flow cytometry as follows: 1×10^7 cells/mouse for whole cells, 5×10^6 cells/mouse for CD4⁺ cell fraction, 5×10^6 cells/mouse for CD4⁺CD25⁺ cell fraction, and 5×10^5 cells/mouse for CD4⁺CD25⁺ cell fraction.

Flow Cytometry

The mice were sensitized by painting their shaved abdominal skin with 100 μ L of 5% TNCB in acetone and orally administered JTT or water (3–6 mice). Cell suspensions were prepared from mice dLNs on day 7 after sensitization, and 2×10^6 cells were ali-

quoted into each round tube. To determine cell viability, the cells were stained using fixable viability stain 757V (BD Biosciences, Franklin Lakes, NJ, USA) for 15 min at 4°C in the dark. To block Fc receptors and minimize nonspecific binding, the cells were resuspended in staining buffer containing anti-mouse CD16/32 antibody (clone: 2.4G2; TONBO, San Diego, CA, USA) for 10 min at 4°C. For phenotypical characterization of Treg cells, cells were stained with CD4-FITC (clone: GK1.5; BD Biosciences), CD25-PE (clone: PC61; BD Biosciences), and glucocorticoid-induced TNFR-related (GITR)-BV421 (clone: DTA-1; BD Biosciences) in 2% fetal bovine serum/ Hank's buffered salt solution for 30 min at 4°C in the dark. Fixation, permeabilization, and intracellular/nuclear staining of Foxp3-Alexa Fluor® 647 (clone: MF23; BD Biosciences, USA) were performed using the transcription factor buffer set (BD Biosciences) according to the manufacturer's instructions. In addition, CD4-FITC (clone: GK1.5; BD Biosciences), CD49b (clone: DX5; BD Biosciences), and CD223 (clone: C9B7W; BD Biosciences) were used to detect Tr1 cells. Flow cytometry was performed on an LSR Fortessa X20 (BD Biosciences) and each sample, 1×10^6 cells were counted. Data were analyzed using FACS Diva software (BD Biosciences).

Enzyme-Linked Immunosorbent Assay

The mice were sensitized by painting their shaved abdominal skin with 100 μ L of 5% TNCB in acetone and were orally administered JTT or water for 7 days (3–6 mice). Prednisolone (PSL) was also administered for 7 days for the measurement of interferon (IFN)- γ level. Lymphocyte cell suspensions were prepared from the dLNs (inguinal and axillary), and 5×10^5 cells were cultured with anti-CD3 and CD28 antibodies (each 5 μ g/mL) for 48 h in a 96-well plate at 37°C. Cell-free supernatants were collected and stored at -20°C until use. Enzyme-linked immunosorbent assay (ELISA) was performed using the mouse IFN- γ ELISA kit (Diacclone SAS, Besançon, France), mouse IL-10 ELISA Ready-SET-Go!® kit (eBioscience, San Diego, CA, USA), and human/mouse transforming growth factor (TGF)- β (2nd generation) ELISA Ready-SET-Go!® kit (eBioscience) according to the manufacturer's instructions.

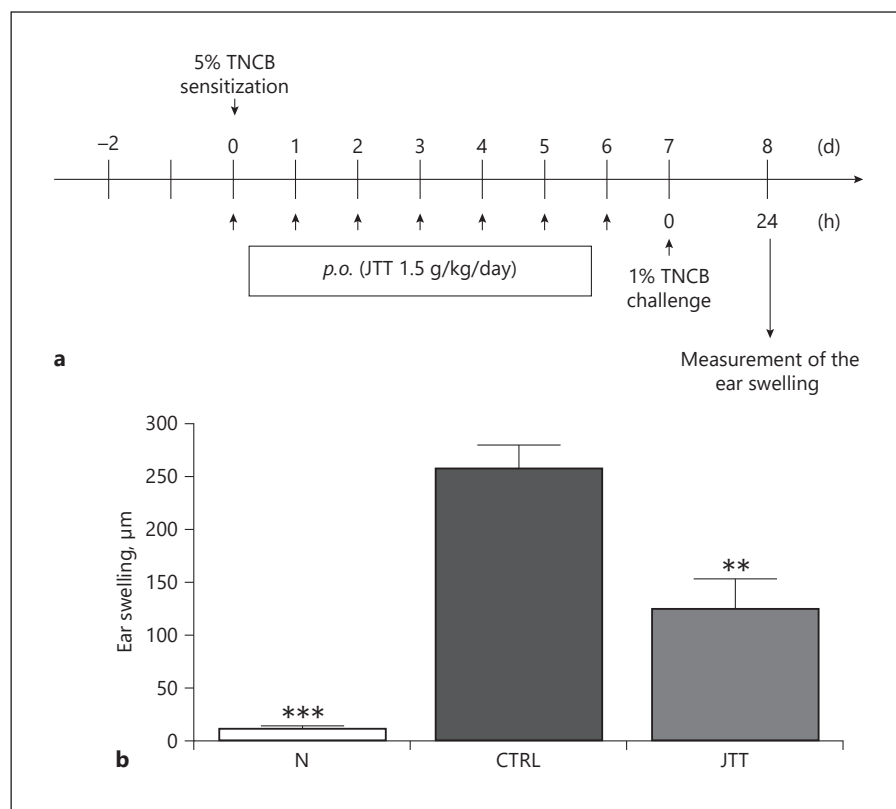
In vivo Neutralization of IL-10

The donor mice were sensitized by painting their shaved abdominal skin with 100 μ L of 5% TNCB in acetone and were orally administered JTT as described above. The mice were sacrificed via cervical dislocation 7 days after sensitization, and lymphocyte cell suspensions were prepared from the dLNs (inguinal and axillary) for subsequent experiments. The cells (1×10^7 cells/mouse) were injected intravenously into naïve recipient mice, which were sensitized 24 h after injection of the cells and challenged after 7 days. Ear thickness was measured before and after the challenge. Recipient mice were injected intraperitoneally with 25 μ g anti-mouse IL-10 monoclonal antibody (R&D Systems) 1 h before sensitization and 1 h before the challenge [7, 8]. Normal, vehicle-treated, and control mice received the same volume of PBS. The IC (isotype control and mouse IgG antibody)-injected recipient mice acted as the IC-negative control group.

Statistical Analysis

Results are represented as mean $\pm$ standard error of mean. Statistical analysis was conducted using analysis of variance with Bonferroni correction for multiple comparisons.

Fig. 1. Anti-allergic effects of JTT on TNCB-induced contact hypersensitivity (CHS) response in mice. **a** Experimental protocol. **b** Ear swelling 24 h after the topical application of 1% TNCB on the ear. Distilled water was administered to the N and CTRL groups. JTT (1.5 g/kg, 10 times the human dose) was orally administered once a day for 7 days. Each column represents the mean $\pm$ S.E.M. of 5 mice. *** $p < 0.001$, ** $p < 0.01$ versus the control group using ANOVA with Bonferroni correction for multiple comparisons. JTT, juzentaihoto; TNCB, 2,4,6-trinitrochlorobenzen; N, normal; CTRL, control; S.E.M, standard error of mean; ANOVA, analysis of variance; CHS, contact hypersensitivity.



Results

The Effect of JTT on TNCB-Induced CHS in Mice

We confirmed the anti-allergic effect of JTT on TNCB-induced CHS response in mice. As shown in Figure 1a and b, oral administration of JTT (1.5 g/kg) significantly suppressed ear swelling 24 h after the topical application of 1% TNCB, as reported previously [18].

Adoptive cell transfer experiments were performed to understand the mechanism of anti-allergic action of JTT in TNCB-induced CHS response. Lymphocyte cell suspensions obtained from dLNs, such as the inguinal and axillary lymph nodes of mice sensitized with 5% TNCB on the abdomen and orally administered JTT or water for 7 days, were injected into naive BALB/c mice. Then, the recipient mice were challenged via topical application of 1% TNCB on the ear after 24 h to study the effect of JTT on Teff activation (Fig. 2a). Compared to that in the vehicle recipient mice in which buffer was injected instead of lymphocytes, ear swelling was observed in control recipient mice administered water, which was indicative of Teff activation (Fig. 2b). In contrast, ear swelling was significantly reduced in mice that received JTT (Fig. 2b).

These results suggested that oral administration of JTT suppressed Teff activation in CHS response.

Similarly, lymphocyte cell suspensions were injected into naive BALB/c recipient mice, which were sensitized with 5% TNCB on their pre-shaved abdomen after 24 h. TNCB (1%) was applied on the right ear 7 days after the sensitization to induce CHS response and investigate the induction or activation of Tregs (Fig. 2a). As shown in Figure 2c, ear swelling was suppressed significantly in the JTT recipient group, suggesting that oral administration of JTT induced and/or activated Tregs. Furthermore, induction and/or activation of Tregs by JTT was not observed without sensitization of donor mice with 1% TNCB (online suppl. Fig. S2). These results suggested the possibility that the Tregs induced and/or activated by JTT could be inducible regulatory T cells (iTregs).

Steroids suppress CHS response, and we have demonstrated the efficacy and mechanism of anti-allergic action of PSL. Oral administration of PSL for 7 days suppressed ear swelling (Fig. 3a), and the adoptive cell transfer experiments revealed that PSL suppressed allergic reaction by inhibiting Teff activation (Fig. 3b), whereas oral administration of PSL did not affect the

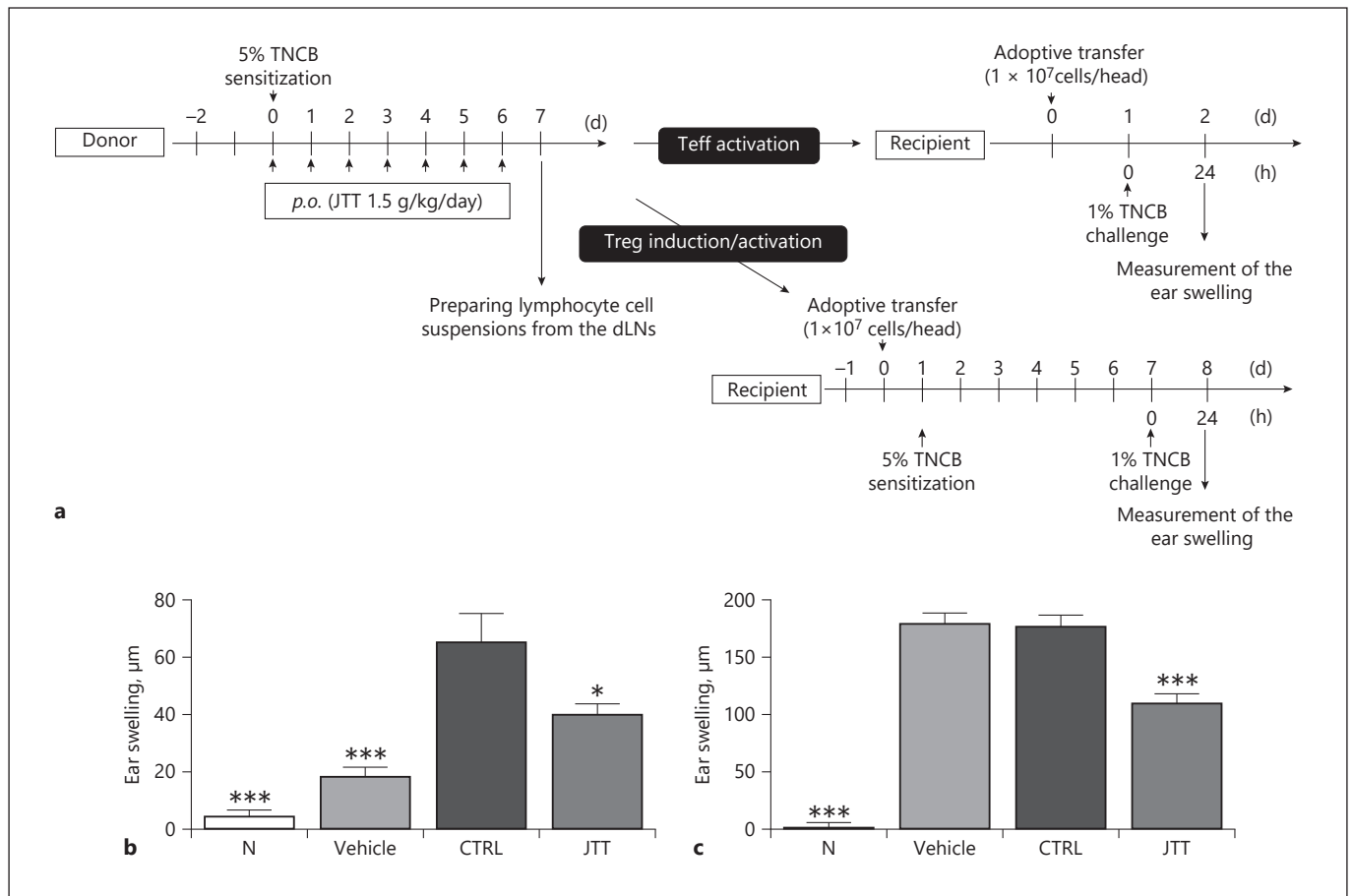


Fig. 2. Effect of JTT on Teff activation and induction/activation of regulatory T cells in TNCB-induced CHS response. **a** Experimental protocol. **b** Ear swelling 24 h after the topical application of 1% TNCB on the ear. In donor mice, JTT (1.5 g/kg, 10 times the human dose) was administered orally once daily to the treated group and distilled water was administered to the CTRL group right after they were sensitized with 5% TNCB. After 7 days, lymphocytes were obtained from the dLNs and injected into naive recipient mice. The lymphocytes obtained from the CTRL donor mice were injected intravenously into the CTRL group, while the lymphocytes obtained from the JTT-treated donor mice were injected intravenously into the JTT group. HBSS alone was injected intravenously in the vehicle-treated mice. All mice, except those in the 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 $\pm$ S.E.M. of 6–7 mice. *** $p < 0.001$, * $p < 0.05$ versus the control group using ANOVA with Bonferroni correction for multiple comparisons. **c** Ear swelling 24 h after the topical application of 1% TNCB on the ear. In donor mice, JTT (1.5 g/kg, 10 times the human dose) was admin-

istered orally once daily to the treated group and distilled water was administered to the CTRL group right after they were sensitized with 5% TNCB. After 7 days, lymphocytes were obtained from the dLNs and then injected into naive recipient mice. The lymphocytes obtained from the CTRL donor mice were injected intravenously into the CTRL group, while the lymphocytes obtained from the JTT-treated donor mice were injected intravenously into the JTT group. HBSS alone was injected intravenously in the vehicle mice. All mice, except those in the N group were sensitized on shaved abdomens with 5% TNCB 24 after cell transfer, and then challenged on their right ears with 1% TNCB after 7 days. Ear swelling was measured 24 h after the challenge. Each column represents the mean $\pm$ S.E.M. of 6–7 mice. *** $p < 0.001$ versus the control group using ANOVA with Bonferroni correction for multiple comparisons. JTT, jumentaihoto; TNCB, 2,4,6-trinitrochlorobenzene; N, normal; CTRL, control; S.E.M, standard error of mean; ANOVA, analysis of variance; dLNs, draining lymph nodes; HBSS, Hank's buffered salt solution; CHS, contact hypersensitivity; Teff, effector T cell; Treg, regulatory T cells.

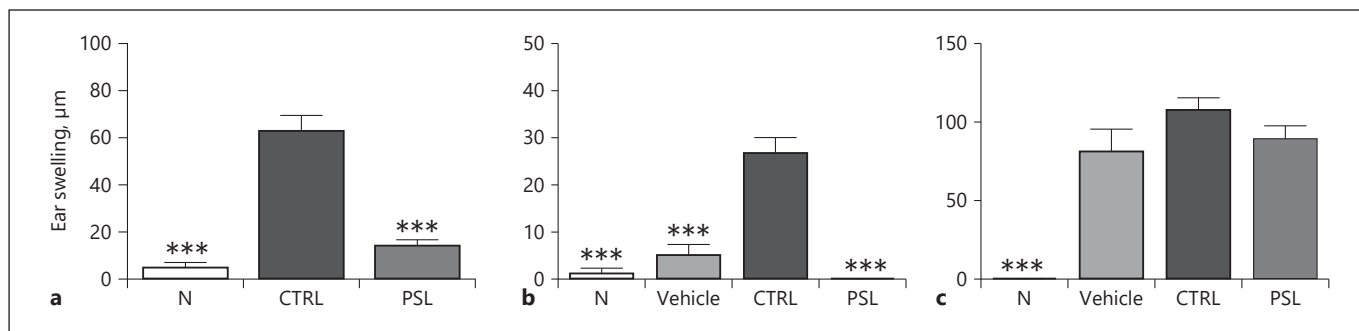


Fig. 3. Anti-allergic properties of PSL on TNCB-induced CHS response in mice. **a** Ear swelling 24 h after the topical application of 1% TNCB on the ear. Distilled water was administered to the N and CTRL groups. PSL (20 mg/kg) was orally administered once a day for 7 days. Each column represents the mean $\pm$ S.E.M. of 5 mice. *** p < 0.001 versus the CTRL group using ANOVA with Bonferroni correction for multiple comparisons. **b** Ear swelling 24 h after the topical application of 1% TNCB on the ear. In donor mice, PSL (20 mg/kg) was administered orally once daily to the treated group and distilled water was administered to the CTRL group right after they were sensitized with 5% TNCB. After 7 days, lymphocytes were obtained from the dLNs and injected into naive recipient mice. The lymphocytes obtained from the CTRL donor mice were injected intravenously into the CTRL group, while the lymphocytes obtained from the PSL-treated donor mice were injected intravenously into the PSL group. HBSS alone was injected intravenously in the vehicle-treated mice. All mice, except those in the N group were then challenged on the right ear with 1% TNCB 24 h after cell transfer, and the ear swelling was measured 24 h after the challenge. Each column represents the mean $\pm$ S.E.M. of 3–5 mice. *** p < 0.001 versus the CTRL group using ANOVA with

Bonferroni correction for multiple comparisons. **c** Ear swelling 24 h after the topical application of 1% TNCB on the ear. In donor mice, PSL (20 mg/kg) was administered orally once daily to the treated group and distilled water was administered to the CTRL group right after they were sensitized with 5% TNCB. After 7 days, lymphocytes were obtained from the dLNs and injected into naive recipient mice. The lymphocytes obtained from the control donor mice were injected intravenously into the CTRL group, while the lymphocytes obtained from the PSL-treated donor mice were injected intravenously into the PSL group. HBSS alone was injected intravenously in the vehicle mice. All mice, except those in the N group were sensitized on the shaved abdomens with 5% TNCB 24 h after cell transfer, and then challenged on their right ears with 1% TNCB after 7 days. Ear swelling was measured 24 h after the challenge. Each column represents the mean $\pm$ S.E.M. of 3–5 mice. *** p < 0.001 versus the CTRL group using ANOVA with Bonferroni correction for multiple comparisons. TNCB, 2,4,6-trinitrochlorobenzene; N, normal; CTRL, control; S.E.M, standard error of mean; ANOVA, analysis of variance; dLNs, draining lymph nodes; HBSS, Hank's buffered salt solution; PSL, prednisolone; CHS, contact hypersensitivity.

induction and activation of Treg (Fig. 3c). It is noteworthy that JTT exerts anti-allergic action via inhibition of Teff activation and induction and activation of Tregs. Hence, we further investigated the properties of Tregs induced and activated by JTT in detail.

Characterization of Tregs Induced and Activated by Oral Administration of JTT

We characterized the Treg subsets induced and activated by oral administration of JTT. First, we fractionated the dLN lymphocytes using magnetic-activated cell sorting to CD4⁺, CD4⁺CD25⁺, and CD4⁺CD25⁺ cell fractions, and then performed adoptive cell transfer experiments (Fig. 4a). The CD4⁺ and CD4⁺CD25⁺ cell fractions suppressed ear swelling, indicating that both fractions contain the Tregs induced and activated by JTT (Fig. 4b). Furthermore, flow cytometry analysis using well-defined Treg markers, such as CD4, CD25, and Foxp3 revealed that oral administration of JTT significantly increased the

percentages of CD4⁺CD25⁺ Foxp3⁺ cells and CD4⁺CD25⁺ Foxp3⁺ cells in CD4⁺ cells (Fig. 5a, b). In addition, we also observed that the ratio of CD8⁺CD122⁺ cells increased in the dLNs of mice treated with JTT (Fig. 5c, d).

Next, we investigated the antigen specificity of Tregs induced and activated by JTT. Therefore, we used TNCB as an antigen for the donor and used another hapten, oxazolone, as an antigen for the recipient. Ear swelling was maximum 48 h after the topical application of FITC (online suppl. Fig. S3). As shown in online suppl. Figure S2, oral administration of JTT did not affect ear swelling. These results suggested that the Tregs induced and activated by JTT worked in an antigen-specific manner.

Next, we performed an ex vivo experiment to confirm the results of the adoptive cell transfer experiments and further investigate the immunosuppressive action of these Tregs induced and activated by JTT. The lymphocytes were prepared from the dLNs of mice sensitized with 5% TNCB and administered orally with JTT for 7 days, and

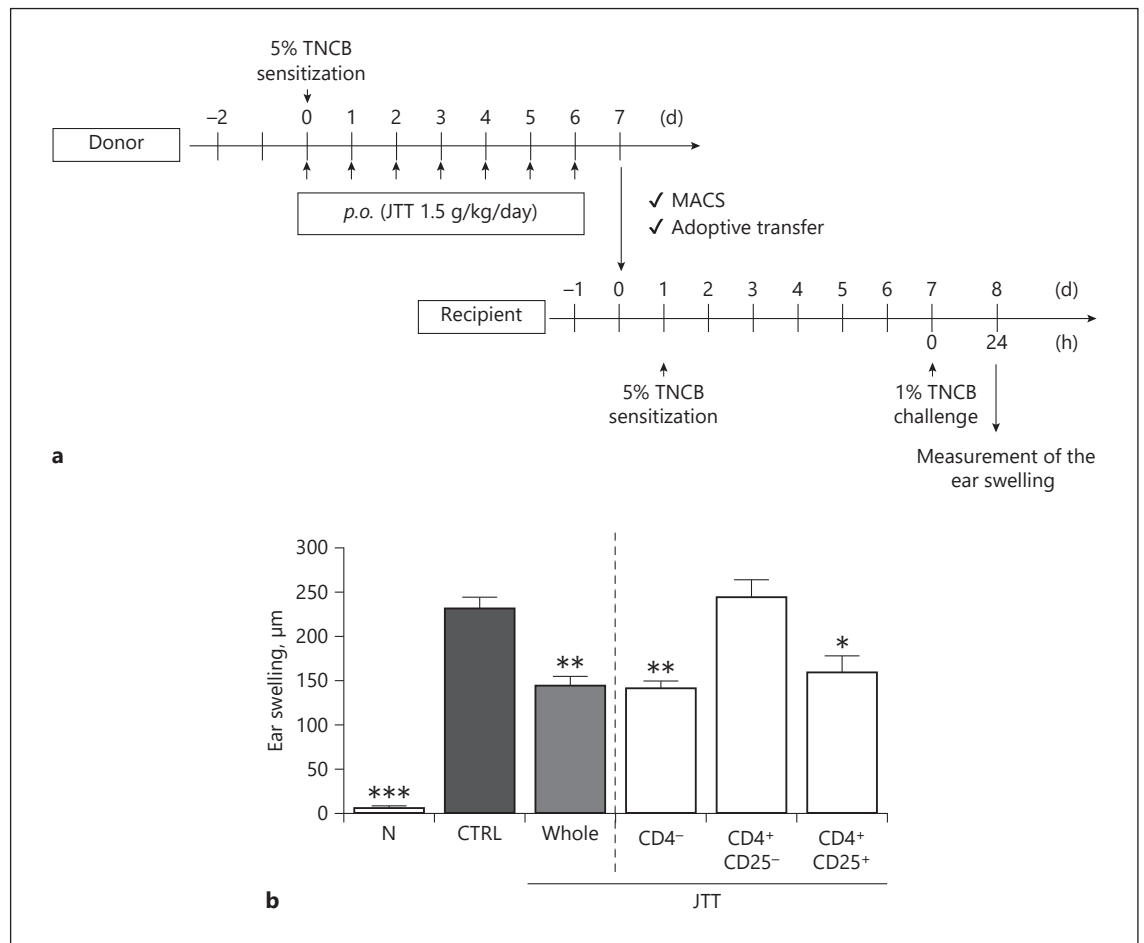


Fig. 4. Characterization of regulatory T cells induced and/or activated by oral administration of JTT using MACS. **a** Experimental protocol. **b** Ear swelling 24 h after the topical application of 1% TNCB on the ear. After sensitization of 5% TNCB, JTT (1.5 g/kg) and distilled water were administered orally once daily to the treated and the CTRL group in donor mice, respectively. After 7 days, lymphocytes, obtained from dLNs of CTRL and JTT-treated donor mice (fractionated into CD4-negative [CD4⁻], CD4-positive CD25-negative [CD4⁺CD25⁻], and CD4-positive CD25-positive

[CD4⁺CD25⁺] cells fractions) were injected intravenously into naive CTRL group and JTT group (whole) of recipient mice, respectively. Each column represents the mean $\pm$ S.E.M. of 3–5 mice. *** p < 0.001, ** p < 0.01, * p < 0.05 versus the CTRL group using ANOVA with Bonferroni correction for multiple comparisons. JTT, juzentaihoto; TNCB, 2,4,6-trinitrochlorobenzene; N, normal; CTRL, control; S.E.M, standard error of mean; ANOVA, analysis of variance; dLNs, draining lymph nodes; MACS, magnetic-activated cell sorting.

then the lymphocytes were cultured after stimulation with anti-CD3 and anti-CD28 antibodies. After 48 h, the culture supernatant was collected, and IFN- γ , IL-10, and TGF- β contents were determined using ELISA (Fig. 6a). Oral administration of JTT and PSL significantly reduced IFN- γ level (Fig. 6b). In addition, IL-10 level increased significantly after the oral administration of JTT (Fig. 6c). Oral administration of JTT with or without T-cell receptor stimulation did not affect TGF- β level (Fig. 6c).

Finally, neutralization with anti-IL-10 monoclonal antibody confirmed the role of IL-10 in the anti-allergic ac-

tion of orally administered JTT via the induction and activation of Tregs. In the adoptive cell transfer experiment, the anti-IL-10 antibody was injected peritoneally immediately before sensitization and challenge with TNCB, and ear thickness was measured 24 h after the challenge (Fig. 7a). We observed that administration of anti-IL-10 antibody eliminated the Treg-mediated anti-allergic effect of JTT (Fig. 7b). Thus, we confirmed that iTregs, induced and activated by JTT, showed immunosuppression via IL-10 production.

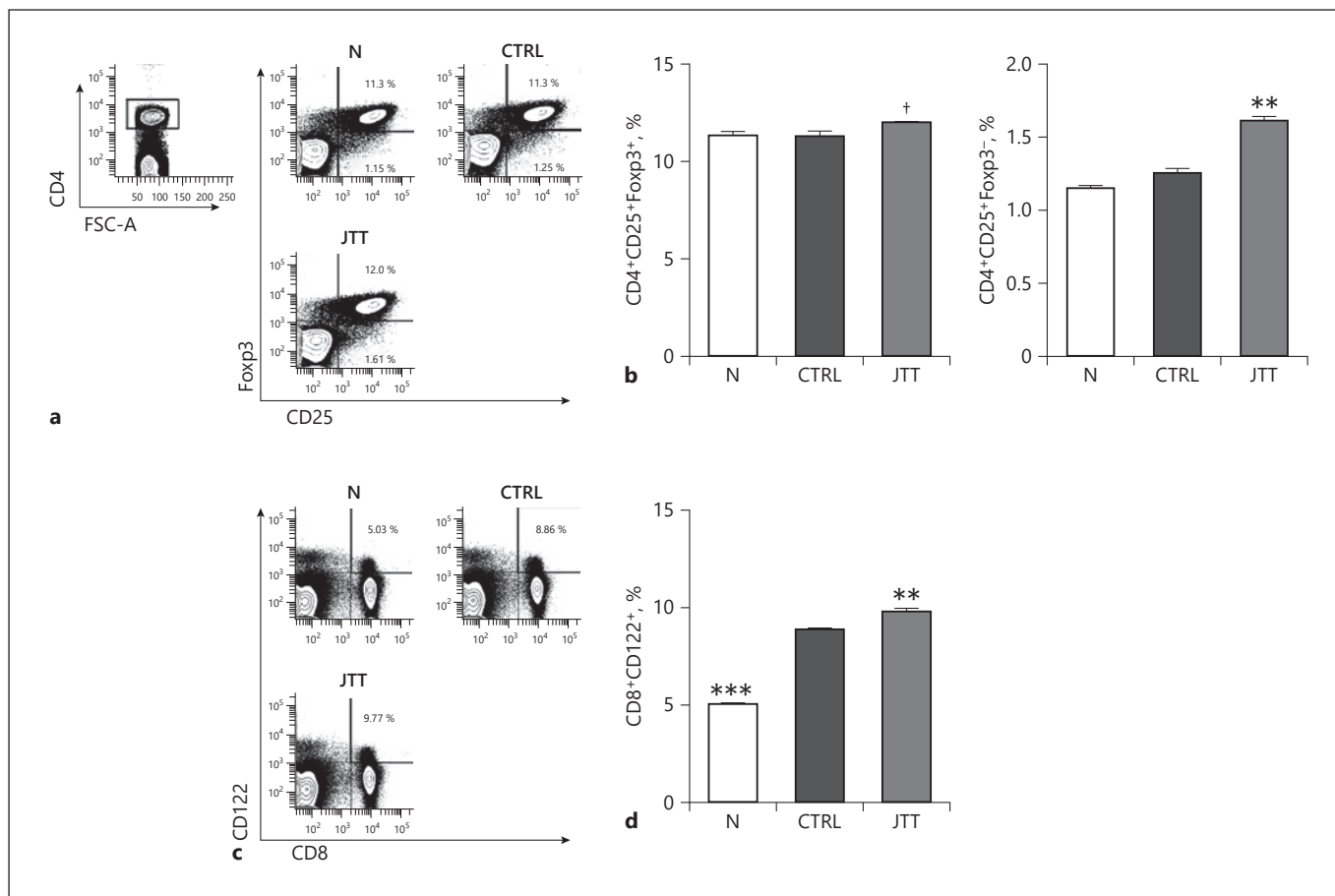


Fig. 5. Characterization of regulatory T cells induced and/or activated by oral administration of JTT by flow cytometry. **a** CD25 and Foxp3 expressions in CD4⁺ cells. **b** Comparison of CD4, CD25, and Foxp3 expression in dLNs obtained from N CTRL, and JTT-treated mice. JTT (1.5 g/kg, 10 times the human dose) and distilled water were administered orally once daily to the treated group and CTRL group, respectively, immediately after sensitization with 5% TNCB. After 7 days, lymphocytes were obtained from the draining lymph nodes. In N mice, distilled water was administered immediately after they were topically applied with vehicle alone on their shaved abdomens. Each column represents the mean $\pm$ S.E.M. of 3 samples. $**p < 0.01$ versus the CTRL group using ANOVA with Bonferroni correction for multiple comparisons and $^{\dagger}p < 0.05$ versus the CTRL group using ANOVA with Bonferroni correction for the selected 2 group comparisons. **c** CD122 expressions in CD8⁺

cells. **d** Comparison of CD8 and CD122 expressions in dLNs obtained from N, CTRL, and JTT-treated mice. JTT (1.5 g/kg, 10 times the human dose) and distilled water were administered orally once daily to the treated group and CTRL group, respectively, immediately after sensitization with 5% TNCB. Seven days later lymphocytes were obtained from the dLNs. In N mice, distilled water was administered immediately after they were topically applied with vehicle alone on their shaved abdomens. Each column represents the mean $\pm$ S.E.M. of 3 samples. $***p < 0.001$, $**p < 0.01$ versus the CTRL group using ANOVA with Bonferroni correction for multiple comparisons. JTT, juzentaihoto; TNCB, 2,4,6-trinitrochlorobenzen; N, normal; CTRL, control; S.E.M, standard error of mean; ANOVA, analysis of variance; dLNs, draining lymph nodes.

Discussion

In this study, we investigated the mechanism underlying the anti-allergic effects of JTT using a TNCB-induced murine CHS model and adoptive cell transfer experiments, and we demonstrated that the anti-allergic effects of JTT were due to inhibition of Teff activation and in-

duction and/or activation of Treg. Mouse models of CHS are frequently used for identifying anti-allergic agents that can be used for treating AD [21–24]. CHS is triggered by low molecular compounds or metal ions, which upon penetrating the skin bind to both extracellular and intracellular proteins or even major histocompatibility complex-bound peptides expressed on the surface of antigen-

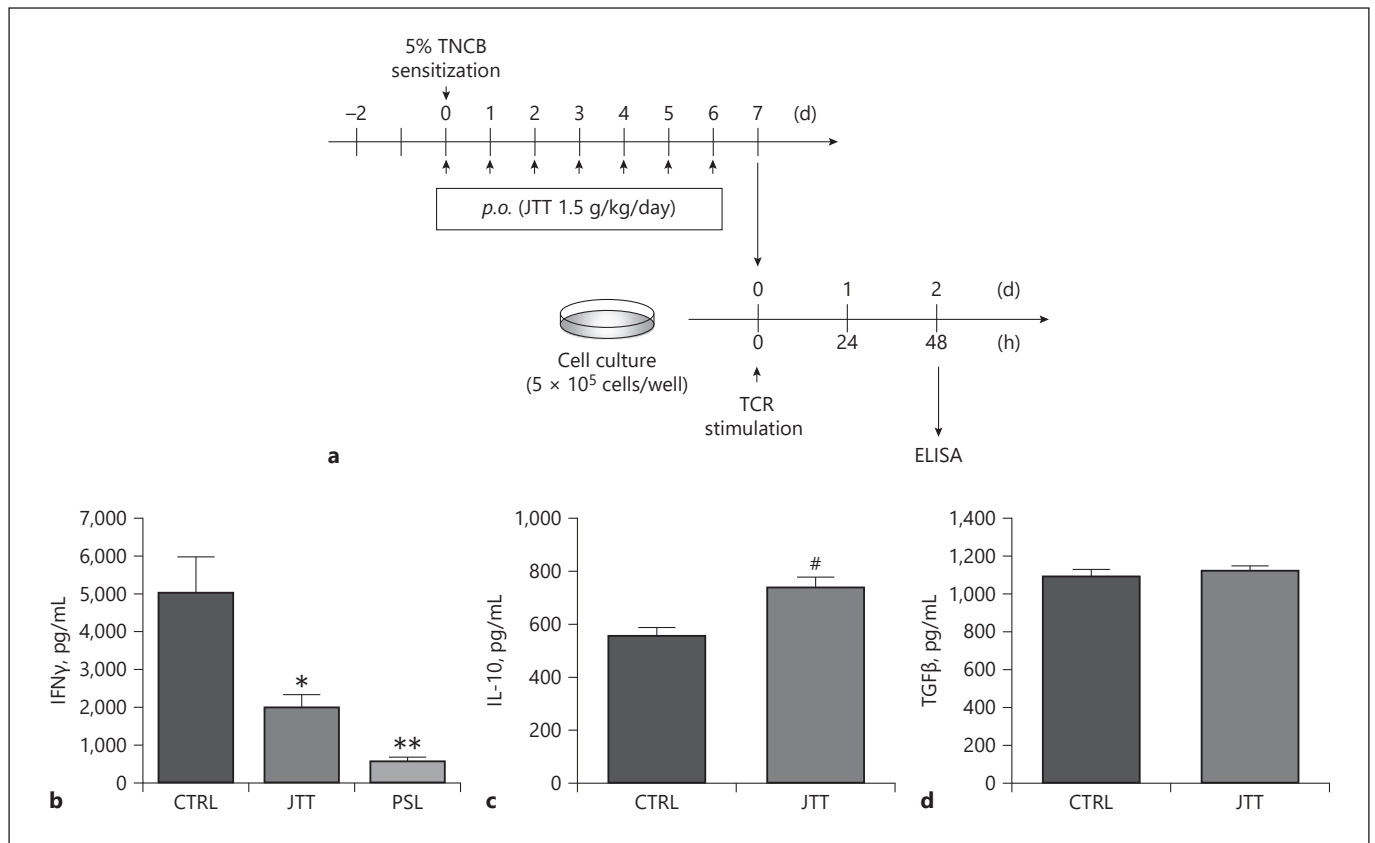


Fig. 6. Effect of JTT on IFN- γ , IL-10, and TGF- β productions in cultured lymphocytes. **a** Experimental protocol. **b** IFN- γ production in the culture supernatant after 48 h incubation with anti-CD3 ϵ and anti-CD28 monoclonal antibodies. **c** IL-10 production in the culture supernatant after 48 h incubation with anti-CD3 ϵ and anti-CD28 monoclonal antibodies. **d** TGF- β production in the culture supernatant after 48 h incubation with anti-CD3 ϵ and anti-CD28 monoclonal antibodies. The mice were sensitized with 5% TNBC and administered orally with JTT, PSL, or water for 7 days. Lymphocyte suspensions were prepared from the draining lymph nodes. Lymphocytes (5×10^6 cells) were stimulated with anti-CD3 ϵ and anti-CD28 monoclonal antibodies (1 μ g/mL of each for

IFN- γ production and 5 μ g/mL for IL-10 production) for 48 h, and the IFN- γ and IL-10 contents of the culture supernatant were measured using ELISA. Each column represents the mean $\pm$ S.E.M. of 3 samples. ** $p < 0.01$, * $p < 0.05$ versus the CTRL group using ANOVA with Bonferroni correction for multiple comparisons. †† $p < 0.01$ versus the CTRL group using the Student's t test. JTT, juzentaihoto; TNBC, 2,4,6-trinitrochlorobenzene; N, normal; CTRL, control; S.E.M, standard error of mean; ANOVA, analysis of variance; dLNs, draining lymph nodes; IFN- γ , interferon- γ ; IL, interleukin; TGF- β , transforming growth factor- β ; PSL, prednisolone; ELISA, enzyme-linked immunosorbent assay; TCR, T-cell receptor.

presenting cells (APCs), such as Langerhans cells (LCs) in the epidermis. These hapten-peptide complexes are processed by LCs and are transported to regional lymph nodes. During this process, LCs undergo maturation, and once in the paracortical region of a lymph node, they present the hapten-modified peptides in association with histocompatibility complex class I and class II molecules to hapten-specific CD8⁺ and CD4⁺ T cells, respectively. Both hapten-specific CD8⁺ and CD4⁺ T cells are activated and then recruited to peripheral tissues as memory Tregs. Upon subsequent reexposure to the same antigen, these Tregs release cytokines that recruit inflammatory cells

such as macrophages and neutrophils to the skin lesion, resulting in inflammation. Simultaneously, CD4⁺ T cells, which perform regulatory function, are generated in the related lymph nodes and skin lesions. Several CD4⁺ T cell subsets with downregulatory activities have been described in mice and human, such as CD4⁺CD25⁺Foxp3⁺ cells, Tr1 cells, Th3 cells, and NKT cells. CD4⁺CD25⁺Foxp3⁺ T cells originating in the thymus are involved in self-antigen tolerance and control of immune responses against pathogens and allergens. It can also be generated at the periphery in tolerogenic conditions of antigen presentation. Many investigators have reported that Tregs play a

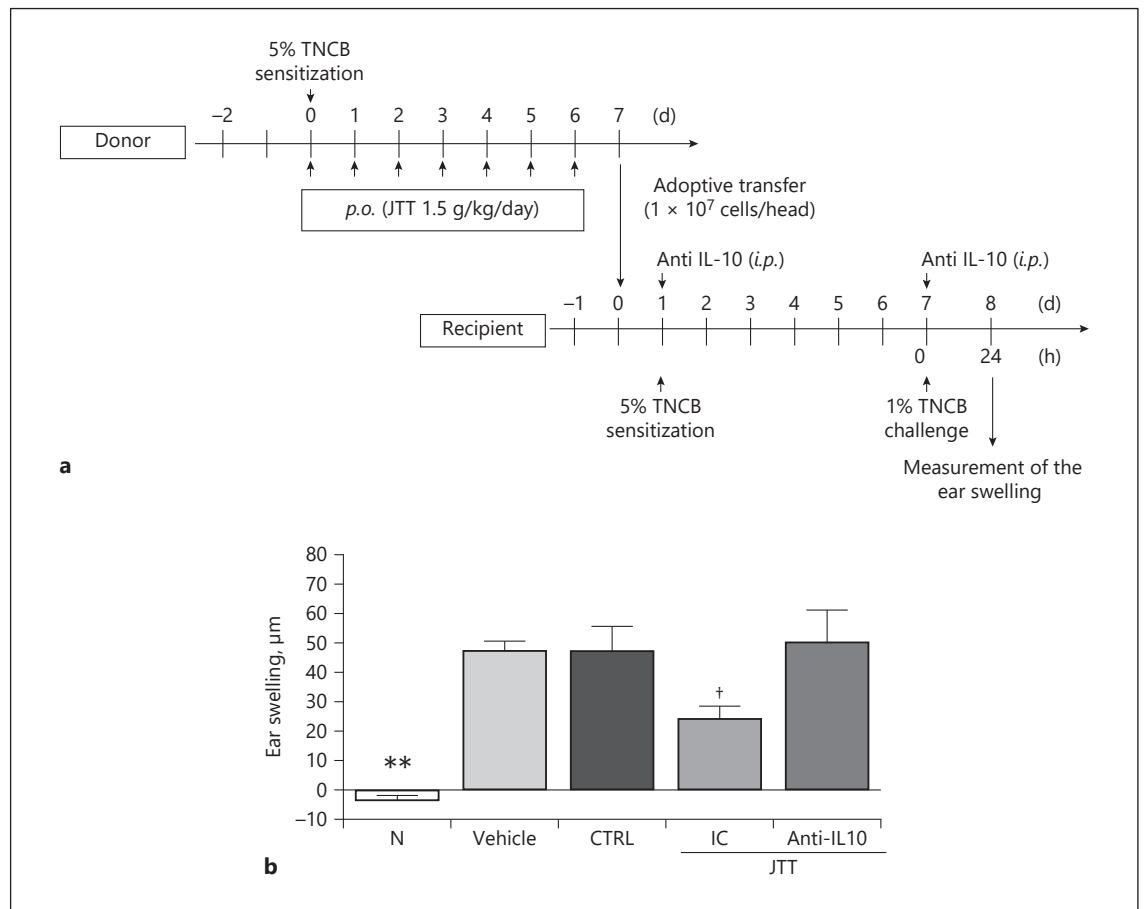


Fig. 7. Effect of anti-IL-10 monoclonal antibody on the anti-allergic action of JTT via the induction and/or activation of regulatory T cells in vivo. **a** Experimental protocol. **b** Ear swelling after 24 h of the topical application of 1% TNCB on the ear. In donor mice, JTT (1.5 g/kg, 10 times the human dose) was administered orally once daily to the treated group and distilled water was administered to the CTRL group immediately after they were sensitized with 5% TNCB. After 7 days, lymphocytes were obtained from the dLNs and injected into naive recipient mice. The lymphocytes obtained from the CTRL donor mice were injected intravenously into the CTRL group, while the lymphocytes obtained from the JTT-treated donor mice were injected intravenously into the JTT group. HBSS alone was injected intravenously in the vehicle mice. All mice, except those in the 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 after 7 days. Ear swelling was measured 24 h after the challenge. In the JTT-treated groups, mouse IgG IC or anti-IL-10 monoclonal antibody (each 100 μg) was injected intravenously prior to the sensitization with 5% TNCB and challenge with 1% TNCB. Each column represents the mean $\pm$ S.E.M. of 3–5 mice. $**p < 0.01$ versus the CTRL group using ANOVA with Bonferroni correction for multiple comparisons. $^{\dagger}p < 0.05$ versus the CTRL group using ANOVA with Bonferroni correction for the selected 2 group comparisons. JTT, juzen-taihoto; TNCB, 2,4,6-trinitrochlorobenzen; N, normal; CTRL, control; S.E.M, standard error of mean; ANOVA, analysis of variance; dLNs, draining lymph nodes; IL, interleukin; dLNs, draining lymph nodes; HBSS, Hank's buffered salt solution; IC, isotype control

critical role in regulating inflammation and facilitating organ repair in CHS. In the sensitization phase of CHS response, Tregs affect APC function and suppress Teff generation in the dLNs. In the elicitation phase of CHS, Tregs infiltrate inflammatory sites and exert a suppressive function against APCs and Teffs. Furthermore, depletion of Tregs prolonged inflammation in skin lesions,

indicating that Tregs may terminate immune response [25, 26].

In the present study, we found that oral administration of JTT inhibited the activation of IFN- γ producing Teff and induced and activated IL-10-producing iTreg, resulting in significant suppression of CHS response in mice. We have shown that JTT is a unique medicine with anti-

allergic properties that suppress the activation of Teff and/or induces and activates Treg. We also confirmed that iTregs can be CD4⁺CD25⁺Foxp3⁺, CD4⁺CD25⁺Foxp3⁻, and CD8⁺CD122⁺ T cells. Some investigators have demonstrated that CD8⁺ T cells are a subset of Tregs [27–29]. Interestingly, we found that the number of CD4⁺GITR⁺CD25⁻Foxp3⁻ cells increased in lymphocytes obtained from JTT-treated donor mice when we used another Treg marker, GITR [30–32], whereas the CD4⁺GITR⁺CD25⁺Foxp3⁺ cell population was not affected by oral administration of JTT (data not shown). We will characterize these subsets in detail, as JTT has been found to induce at least 2 or more types of Tregs to exhibit anti-allergic effects.

It is well known that inducing or activating Tregs is one of the therapeutic strategies for autoimmune and allergic diseases [33, 34]. Rapamycin, an immunosuppressive drug currently used for preventing graft rejection in humans, allows the expansion of naturally occurring Tregs in humans and mice [35]. FTY720, a sphingosine 1-phosphate receptor antagonist, is also used as a novel immunosuppressant to prevent allograft rejection after organ transplantation, as it can increase the number and enhance the functional activity of Foxp3⁺ Tregs in mixed lymphocyte reaction [36]. Leflunomide, an immunosuppressive compound used in the treatment of rheumatoid arthritis, suppressed CHS response by inducing CD8⁺ and CD4⁺ Tregs [37]. Retinoic acid [38] and simvastatin [39] are reported to differentiate naive T cells to Foxp3⁺ Tregs in the presence of TGF- β . Furthermore, a mixture of probiotics upregulated CD4⁺Foxp3⁺ Tregs [40, 41]. The mechanisms via which these agents affected the number and function of Tregs are still under investigation.

This is the first report showing that JTT exerts anti-allergic effects by not only inhibiting Teff activation, but also by inducing and activating Tregs, in a murine model of CHS. In addition, several reports have shown that oral administration of JTT enhanced antitumor immunity in human and rodents [9–14]. A study showed that JTT increased regulatory activities in T cells by decreasing the Foxp3⁺ Treg population in patients with advanced pancreatic cancer [42]. As Tregs are involved in regulating tumor immunity, we need to understand how JTT works in different immunological situations such as in allergy and in tumors.

We are currently investigating which of the 10 constituents of JTT are effective crude drugs through their effect on the murine model of CHS with the adoptive transfer experiment. We have already reported that a hot

water extract of Glycyrrhiza Radix and its major ingredient, glycyrrhizin, shows the anti-allergic effects on a murine model of CHS [43, 44]. Moreover, there are some reports that paeoniflorin, a major ingredient of *Paeoniae Radix*, inhibited inflammatory responses in mice with allergic contact dermatitis induced by DNCB [45–47]. It is also suggested that oral administration of paeoniflorin regulated the balance between inflammatory cytokines, such as IFN- γ and IL-17, and anti-inflammatory cytokines, such as IL-10.

In conclusion, we demonstrated that JTT exerts immunomodulatory effects via both suppression of Teff and induction and/or activation of Tregs. Thus, our observations highlight that existing traditional medicines can be used as ready-to-use medicines for inflammatory diseases, such as AD. We will identify the active ingredients of JTT to understand the immunomodulatory actions of JTT at the molecular level in future.

Statement of Ethics

All experiments were performed according to the guidelines of Animal Care Committee of the Faculty of Pharmacy, Meijo University (approval number: 2019PE1).

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

Funding Sources

There are no funding sources.

Author Contributions

M.N. conceived and designed the study; A.T., S.Y., S.W., Y.K., and M.N. performed the experiments; A.T., S.Y., S.H., and M.N. analyzed data; A.T. and M.N. prepared figures; A.T., S.Y., S.W., Y.K., S.H., and M.N. discussed results; M.N. wrote the main manuscript; A.T., S.H., and M.N. reviewed the manuscript.

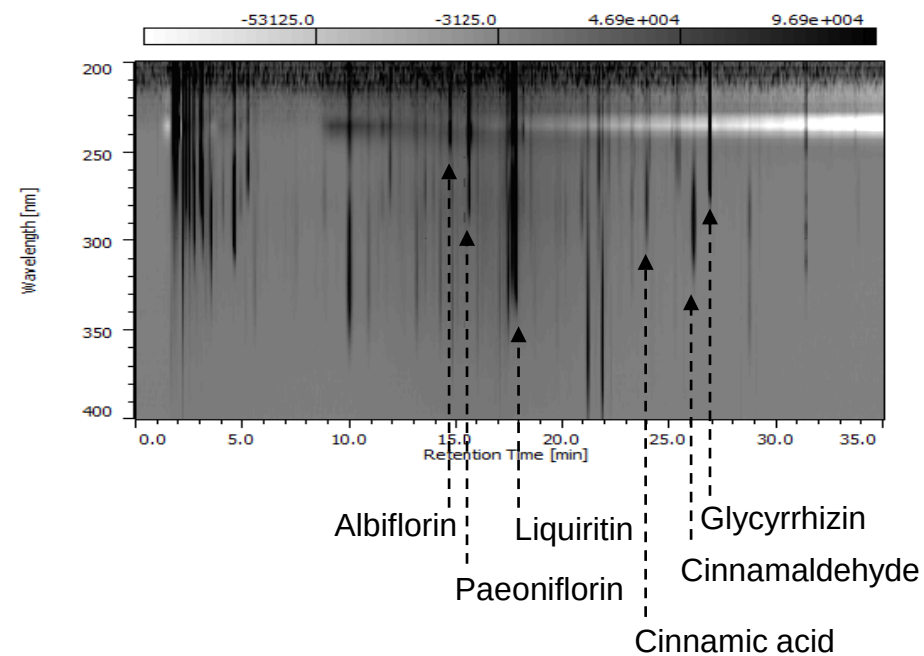
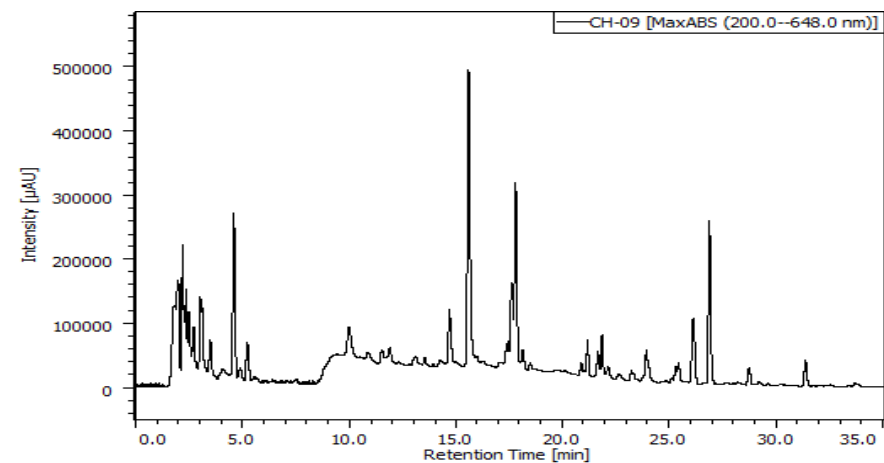
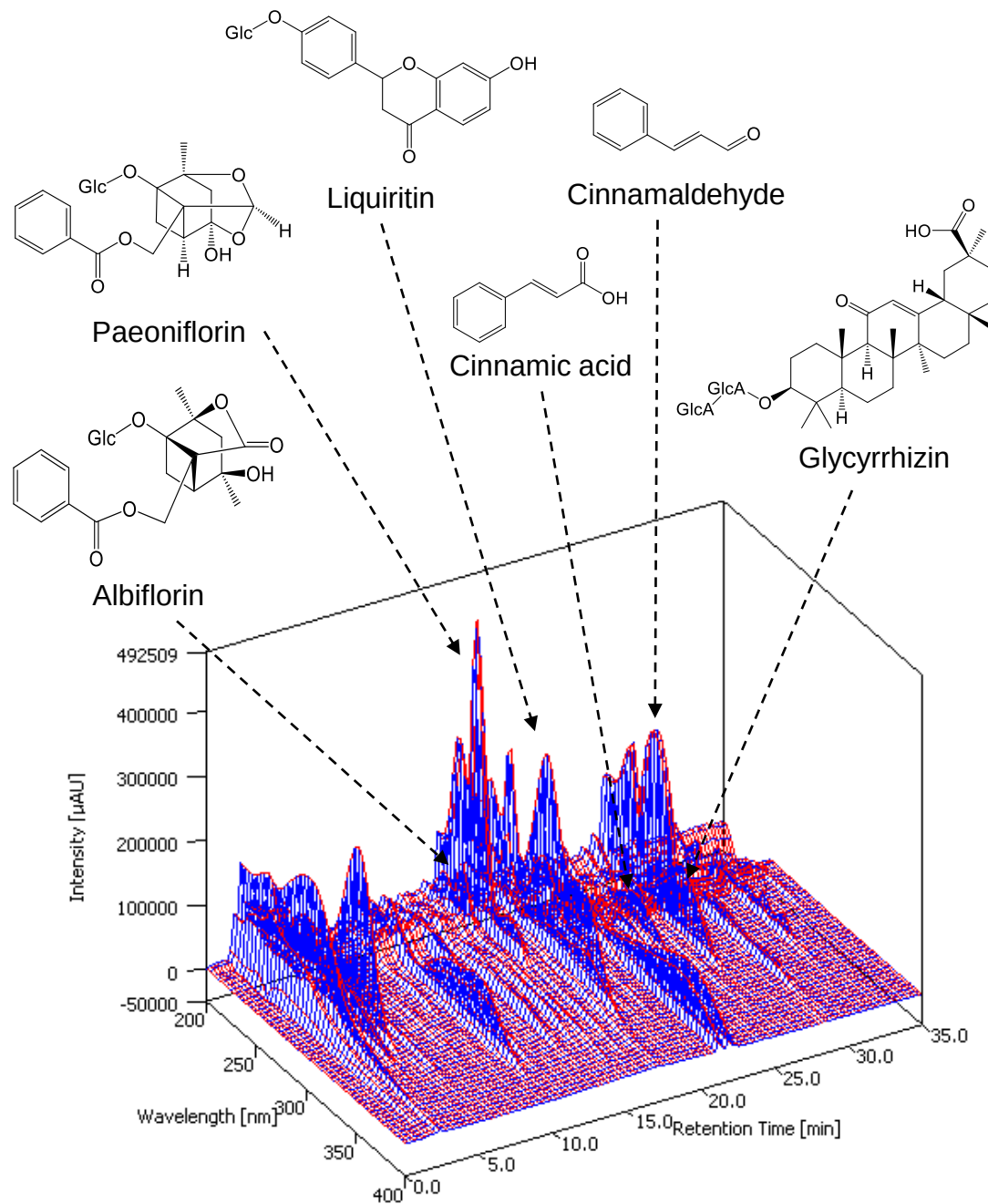
Data Availability

All data generated or analyzed during this study are included in this published article and its online supplementary material files.

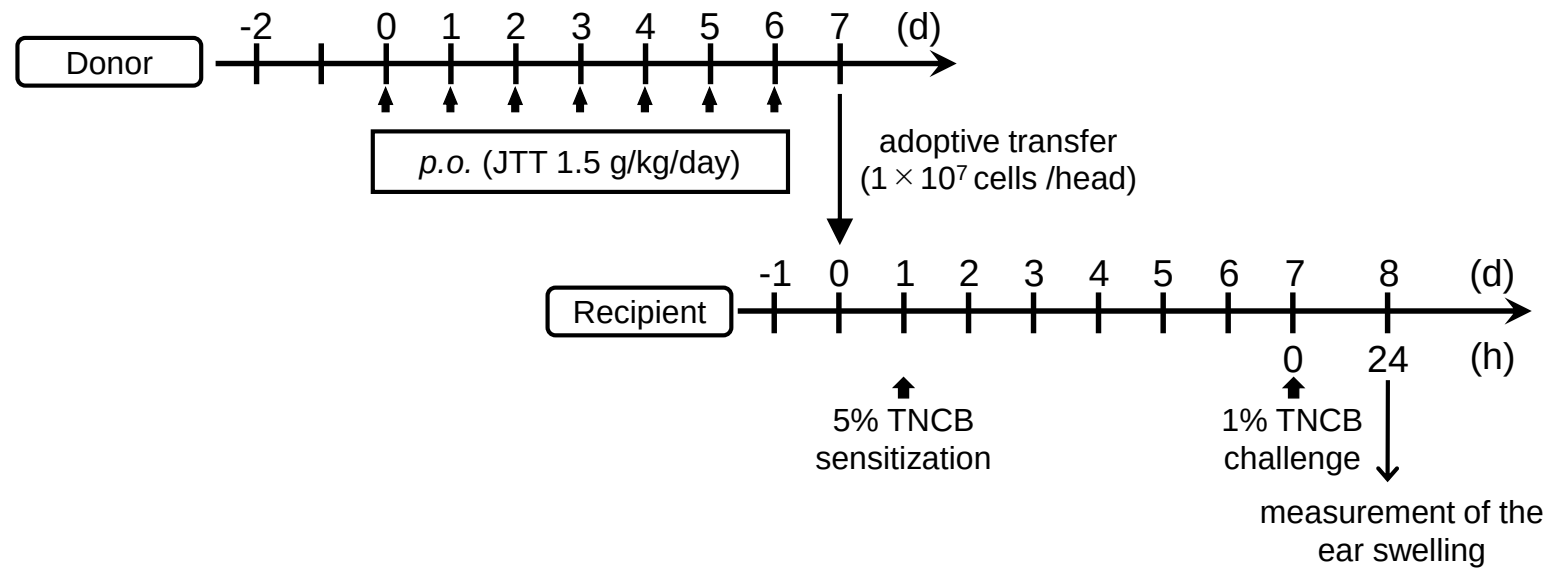
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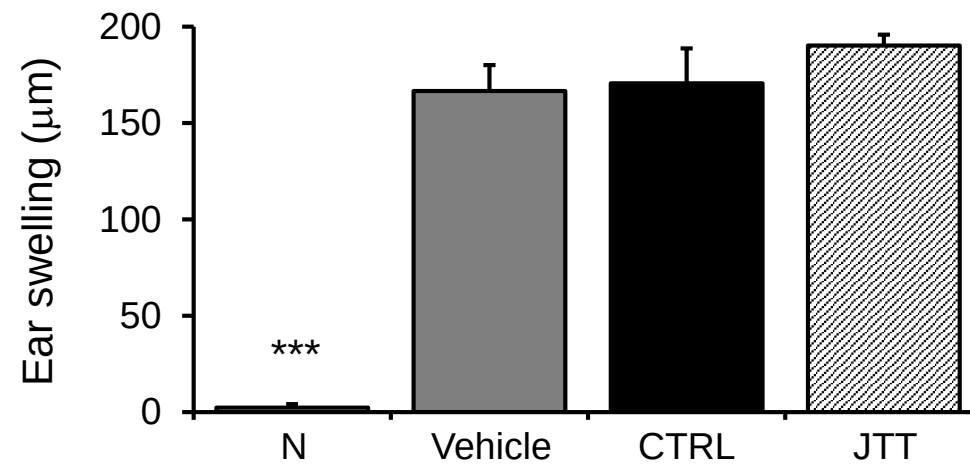
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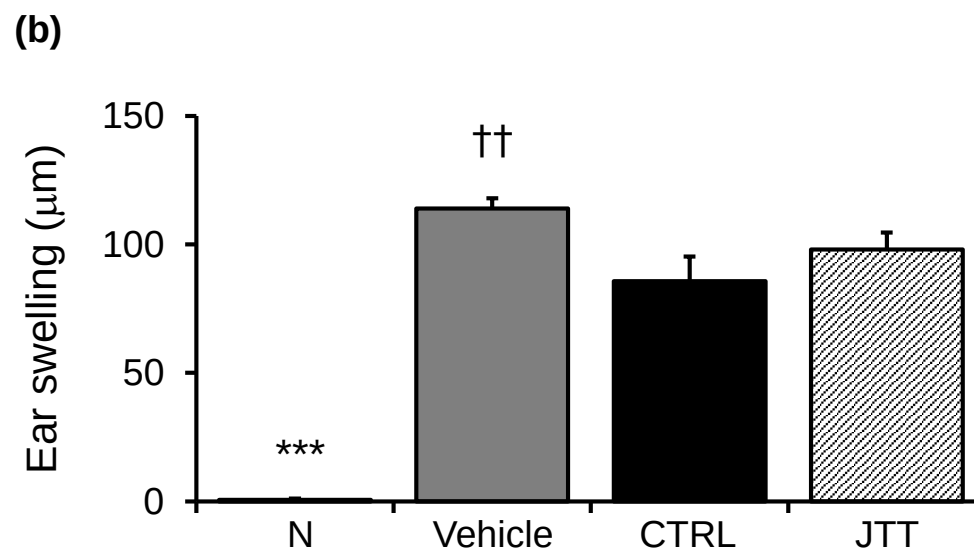
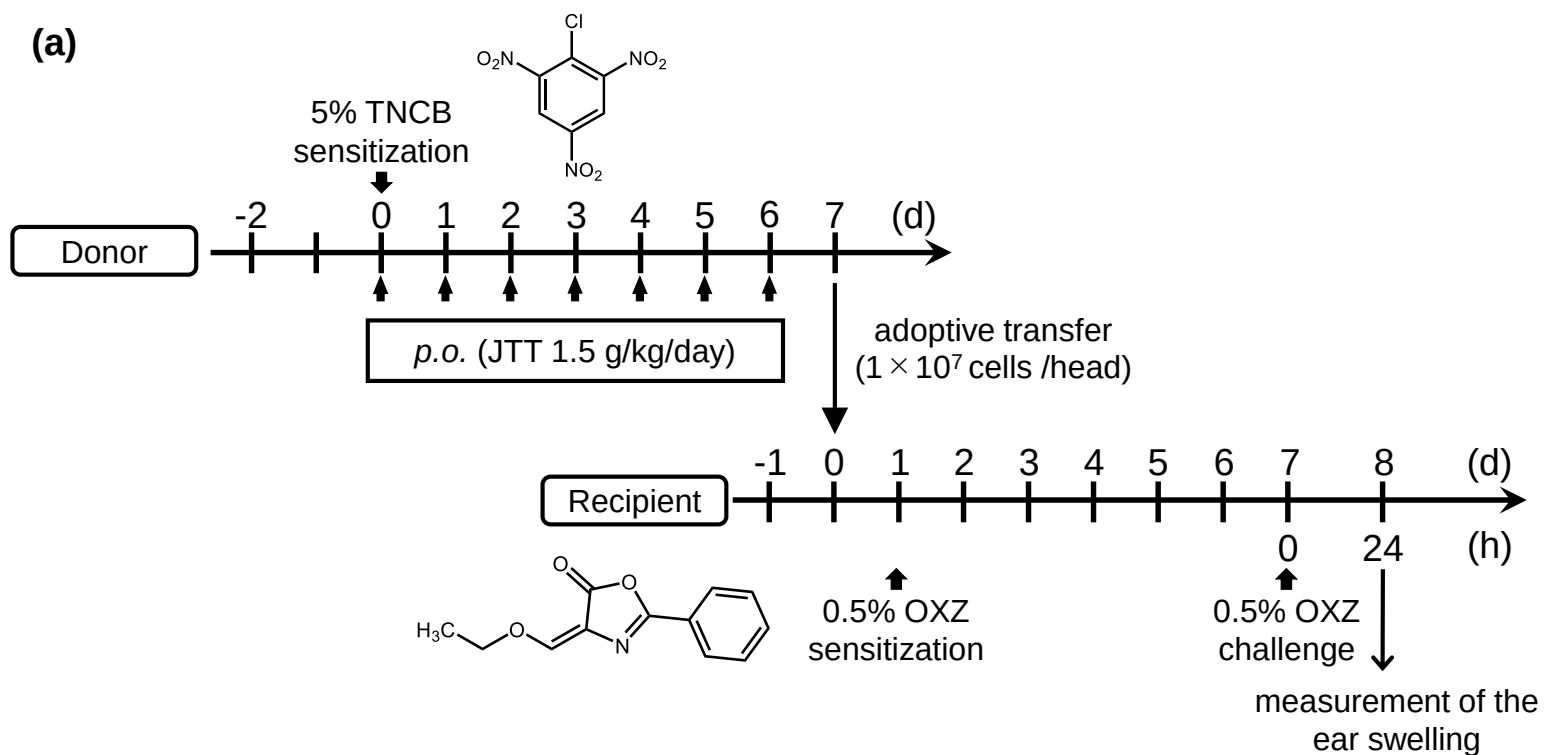


(a)



(b)





Supplementary Materials

*Juzentaihoto Exerts Anti-allergic Effect by Inhibiting Effector T Cell Activation and Inducing
and/or Activating Regulatory T Cells in a Murine Model of Contact Hypersensitivity*

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Short Title: Juzentaihoto exerts anti-allergic effects by regulating effector and regulatory T cells

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Number of Tables: 0

Number of Figures: 3

Word count: 416

Figure S1. Three-dimensional HPLC profile of juzentaihoto (JTT)

Figure S2. Effect of juzentaihoto (JTT) on the induction and/or activation of regulatory T cells without antigen sensitization of donor mice

(a) Experimental protocol. **(b)** Ear swelling 24 h after the topical application of 1% TNCB on the ear. In donor mice, JTT (1.5 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. After 7 days, lymphocytes were obtained from the dLNs and injected into naïve recipient mice. The lymphocytes obtained from the control donor mice were injected intravenously into the CTRL group, while the lymphocytes obtained from the JTT-treated donor mice were injected intravenously into the JTT group. HBSS alone was injected intravenously in the 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 after 7 days. Ear swelling was measured 24 h after the challenge. Each column represents the mean $\pm$ S.E.M. of 6 to 7 mice. *** $p < 0.001$ vs. the control group using ANOVA with Bonferroni correction for multiple comparisons.

Figure S3. Antigen specificity of regulatory T cells induced and/or activated by oral administration of juzentaihoto (JTT)

(a) Experimental protocol. **(b)** Ear swelling 48 h after the topical application of 0.5% oxazolone (OXZ) on the ear. In donor mice, JTT (1.5 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. After 7 days, lymphocytes were obtained from the dLNs and injected into naïve recipient mice. The lymphocytes obtained from the control donor mice were injected intravenously into the CTRL group, while the lymphocytes obtained from the JTT-treated donor mice were injected intravenously into the JTT group. HBSS alone was injected intravenously in the vehicle mice. All mice, except those in

the normal (N) group were sensitized on shaved abdomens with 0.5% OXZ 24 h after cell transfer and then challenged on their right ears with 0.5% OXZ after 7 days. Ear swelling was measured 24 h after the challenge. Each column represents the mean $\pm$ S.E.M. of 6 to 7 mice. *** $p < 0.001$ vs. the control group using ANOVA with Bonferroni correction for multiple comparisons. †† $p < 0.01$ vs. the control group using ANOVA with Bonferroni correction for the selected two group comparisons.