



JAK inhibitor JTE-052 regulates contact hypersensitivity by downmodulating T cell activation and differentiation

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ABSTRACT

Background: Using JAK inhibitors to inhibit cytokine signaling is presumed to be a possible means of treating skin inflammatory disorders such as contact dermatitis.

Objective: To clarify the action site of JAK inhibitors in skin inflammatory disorders.

Methods: We analyzed the mechanism of action of the JAK inhibitor JTE-052 using murine skin inflammation models, including contact hypersensitivity (CHS) and irritant contact dermatitis. Cells isolated from ear tissue or lymph node (LN) were analyzed by flow cytometry. The amounts of cytokines in the culture medium were measured by ELISA or bead array system. Proliferation of LN cells was evaluated by measurement of tritiated thymidine incorporation.

Results: Oral administration of JTE-052 during both sensitization and elicitation phase attenuated CHS, but did not affect croton oil-induced irritant contact dermatitis. JTE-052 potently inhibited T cell proliferation and activation by antigen presentation in vitro, and attenuated skin inflammation in a sensitized-lymphocyte transfer model without suppressing T cell migration. JTE-052 did not affect hapten-induced cutaneous dendritic cell migration into draining lymph nodes or their costimulatory molecule expressions.

Conclusion: The JAK inhibitor JTE-052 exerts an inhibitory effect on antigen-specific T cell activation and subsequent inflammation in acquired skin immunity, such as CHS.

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1. Introduction

Allergic contact dermatitis (ACD), one of the most prevalent occupational skin diseases in humans, is characterized by allergic delayed-type hypersensitivity to environmental, occupational, and nutritional allergens [1,2]. In contrast to irritant contact dermatitis (ICD), which is induced by keratinocyte damage and local inflammation as a result of direct toxic effects of physical or chemical agents [3], ACD is dependent on acquired skin immunity [1,2,4].

In the sensitization phase of ACD, epicutaneous invasion of allergens induces an innate inflammatory immune response that occurs in association with the formation of immunogenic self-proteins [1,2]. In addition, antigen-presenting cells (APCs) such as

dendritic cells (DCs) ingest and process antigens. APCs express major histocompatibility complex (MHC) class I/II and co-stimulatory molecules such as CD86 and migrate to the skin-draining lymph nodes (dLN), where they present antigens to naïve T cells, thus generating antigen-specific memory T cells [5].

In the elicitation phase, a previously presented allergen triggers an infiltration of immune cells, such as memory T cells and neutrophils, to the skin lesion. In particular, CD4⁺ T-helper (Th)1 and CD8⁺ T-cytotoxic (Tc)1 cells, which produce interferon (IFN)- γ , have been considered to be crucial in the pathogenesis of ACD [6].

Cytokines play an important role in the development of ACD [1,2]. Cytokines exert biological effects by binding to their cognate receptors, which activate Janus kinases (JAKs), a small family of cytoplasmic protein tyrosine kinases comprising JAK1, JAK2, JAK3, and tyrosine kinase 2 (Tyk2) [7]. JAK family members then activate signal transducers and activators of transcription (STAT) proteins to induce specific gene expression. The JAK-STAT pathway is activated by over 40 cytokines, including interleukin (IL)-2, IFN- γ

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and IL-6 [8]. Therefore, the inhibition of JAK activities is expected to be a therapeutic strategy for immune and inflammatory disorders. Indeed, inhibitors of the JAK family kinases have demonstrated clinical efficacy against rheumatoid arthritis and other inflammatory disorders [9]. It has been reported that the JAK inhibitor tofacitinib inhibits mouse and human T cell differentiation [10] and cytokine production of CD3-stimulated T cells in vitro [11], while another JAK inhibitor, ruxolitinib, impairs the phenotype and function of monocyte-derived DCs that are stimulated with lipopolysaccharide (LPS) in vitro [12]. It has been suggested that JAK1/2 inhibitors may attenuate murine contact hypersensitivity (CHS), a model of ACD, by suppressing STAT phosphorylation at inflammatory skin lesions [13], but the action site of JAK inhibitors in CHS remains unclear.

Here we report that the JAK inhibitor JTE-052 ameliorated a hapten-induced murine CHS model. The JAK inhibitor potently attenuated skin-homing antigen-specific T cell activation without affecting DC functions or croton oil-induced ICD.

2. Materials and methods

2.1. Animals

C57BL/6J (B6) mice were purchased from Japan SLC (Shizuoka, Japan) and maintained on a 12-h light/dark cycle at a temperature of 24 °C and humidity of 50% ± 10% under specific pathogen-free (SPF) conditions at Kyoto University Graduate School of Medicine. All experimental procedures were approved by the Institutional Animal Care and Use Committee of Kyoto University Graduate School of Medicine.

2.2. Drugs and reagents

The JAK inhibitor JTE-052 was synthesized at Japan Tobacco Inc. (Osaka, Japan). JTE-052 inhibits JAK1, JAK2, JAK3 and Tyk2 enzymatic activity with 50% inhibitory concentrations of 2.8, 2.6, 13 and 58 nmol/L, respectively, and inhibits STAT phosphorylation [14,15]. For in-vitro studies, test compounds were dissolved in dimethyl sulfoxide (DMSO) and added at a final DMSO concentration of less than 0.1%. For in-vivo studies, JTE-052 was suspended in 0.5% methylcellulose (Wako, Japan) and was orally administered to mice once a day.

We purchased dinitrofluorobenzene (DNFB) from Nacalai Tesque (Kyoto, Japan), dinitrobenzene sulfonic acid (DNBS) from Alfa Aesar (Ward Hill, MA, USA) and carboxyfluorescein diacetate succinimidyl ester (CFSE) from Invitrogen (Carlsbad, CA, USA). Fluorescent (fluorescein isothiocyanate (FITC), PE, PE-Cy5, and PE-Cy7)-labeled anti-CD4, CD8, B220, CD11c, CD80, CD86, MHC class II, CD62L, CD44, CD45, Gr-1 and CD11b monoclonal antibodies were purchased from eBioscience (San Diego, CA, USA).

2.3. CHS and ICD

For CHS, shaved abdominal skin of 8-week-old female mice was sensitized with 25 µL 0.5% (w/v) DNFB in acetone/olive oil (4:1). Five days after sensitization, the ears were challenged with an application of 20 µL 0.3% DNFB. Ear thickness was measured for each mouse before, 24 h after and 48 h after elicitation at a predetermined site with a spring-loaded micrometer (Mitsutoyo, Kanagawa, Japan). The difference was referred to as ear swelling change. Test drug or vehicle was given orally once daily during sensitization or elicitation phase.

For ICD, either 20 µL 0.3% DNFB or 20 µL 1% (v/v) croton oil (Sigma-Aldrich, St Louis, MO, USA) in acetone was applied to the ears without prior sensitization. Ear thickness was measured at different time points as indicated.

2.4. Flow cytometry

Cells isolated from ear tissue were analyzed by flow cytometry. Briefly, an ear tissue sample was punched out with an 8-mm trepan (Kai Industries, Gifu, Japan) and cut into small pieces, then incubated for 25 min at 37 °C with a solution of RPMI containing 100 µg/mL DNase (Sigma-Aldrich) and 50 U/mL collagenase type IV (Worthington Biochemical, Freehold, NJ, USA) to obtain a homogeneous cell suspension.

Cells stained with each antibody were analyzed using the FACS LSR Fortessa flow cytometric system (BD Biosciences, San Diego, CA, USA) and FlowJo software (TreeStar, Ashland, OR, USA). Dead cells or debris were eliminated by gating out a PI-positive fraction.

2.5. FITC-induced cutaneous DC migration assay

The shaved abdominal skin of B6 mice was painted with 100 µL of 2% FITC (Sigma-Aldrich) in acetone/dibutyl phthalate (1:1). Twenty-four hours after painting, draining axillary LNs were collected and incubated for 25 min at 37 °C with a solution of RPMI containing 100 µg/mL DNase and 50 U/mL collagenase type II (Worthington Biochemical) to obtain a homogeneous cell suspension. The isolated LN cells were taken for flow cytometric analysis.

2.6. Quantitative RT-PCR analysis

All primers were obtained from Greiner Japan (Tokyo, Japan). Quantitative RT-PCR (qPCR) analysis was performed to quantify the level of mRNA as described previously [16]. The primer sequences were *Ifng*, 5'-GAA CTG GCA AAA GGA TGG TGA-3' (forward), 5'-TGT GGG TTG TTG ACC TCA AAC-3' (reverse); *Cxcl9*, 5'-ATT GTG TCT CAG AGA TGG TGC TAA TG-3' (forward), 5'-TGA AAT CCC ATG GTC TCG AAA G-3' (reverse); *Cxcl10*, 5'-CCA AGT GCT GCC GTC ATT TTC-3' (forward), 5'-GGC TCG CAG GGA TGA TTT CAA-3' (reverse); *Il8*, 5'-ATG GCT GGG ATT CAC CTC AA-3' (forward), 5'-AAG CCT CGC GAC CAT TCT T-3' (reverse); *Icam1*, 5'-AGG GCT GGC ATT GTT CTC TA-3' (forward), 5'-CTT CAG AGG CAG GAA ACA GG-3' (reverse); *Il1b*, 5'-GAA ATG CCA CCT TTT GAC AGT G-3' (forward), 5'-CTG GAT GCT CTC ATC AGG ACA-3' (reverse). Results were normalized to those of the "housekeeping" glyceraldehyde-3-phosphate dehydrogenase mRNA.

2.7. Cell culture and proliferation assay

The complete RPMI (cRPMI) culture medium consisting of RPMI 1640 (Invitrogen) containing 10% heat-inactivated fetal calf serum, 5×10^{-5} mol/L 2-mercaptoethanol, 2 mM L-glutamine, 25 mM N-2-hydroxyethylpiperazine-N 9 –2-ethanesulfonic acid, 1 mmol/L nonessential amino acids, 1 mmol/L sodium pyruvate, 100 units/mL penicillin, and 100 mg/mL streptomycin was used unless otherwise indicated.

For DNBS-dependent proliferation, single-cell suspensions were prepared from skin draining axillary LNs of mice five days after sensitization with DNFB on the abdomen. One million LN cells were cultured with or without 100 µg/mL DNBS sodium for 72 h, pulsed with 0.5 µCi [³H] thymidine for the last 24 h, and subjected to liquid scintillation counting. For measurement of cytokine production, culture supernatants were collected 72 h post-incubation.

2.8. Enzyme-linked immunosorbent assay (ELISA) and beads array

The amounts of IFN-γ in the culture medium were measured by ELISA (BD Biosciences). The amounts of IL-13 and IL-17 were measured using a cytometric bead array system (BD Biosciences) according to the manufacturer's instructions.

2.9. Adoptive transfer

For adoptive transfer, LN cells were collected from the draining LNs (axillary, inguinal, brachial and cervical) of B6 mice five days after sensitization with 0.5% DNFB and labeled with CFSE. LN cells were then transferred intravenously into one naïve B6 mouse (1×10^7 /head). Twenty-four hours after transfer, mice were challenged with 20 μ L of 0.5% DNFB applied to each side of both ears, and ear swelling was measured 24 h after the challenge.

2.10. Statistical analysis

Unless otherwise indicated, data are presented as means $\pm$ standard deviation (SD) and each data point is representative of at least three independent experiments. Differences between two groups were analyzed by means of one-way ANOVA, and *P* values were calculated with the two-tailed *t* test. For multiple comparisons, statistical significance was assessed with the Dunnett test (for homoscedastic data) or the Steel test (for heteroscedastic data) after homoscedasticity analysis with the Bartlett test.

3. Results

3.1. Oral administration of JTE-052 attenuates CHS response

First, we evaluated the effects of JTE-052 on a murine CHS model. B6 mice were administered either vehicle or JTE-052 (3 or 30 mg/kg) orally once a day, starting at the time of sensitization until the day of elicitation with DNFB. Oral administration of JTE-052 inhibited ear swelling (Fig. 1A). Forty-eight hours after the ear challenge, FACS analysis revealed that the numbers of CD4⁺ and CD8⁺ T cells infiltrated into the ear had been reduced by JTE-052 (Fig. 1B). In addition, we applied the continuous infusion by means of osmotic pump to obtain the maximal effect of JTE-052. JTE-052 significantly inhibited ear swelling and the number of draining LN cells compared to the vehicle-treated group (Fig. S1A and B). Next, to evaluate the effects of JTE-052 on skin inflammation that is independent of acquired immunity, we used a murine model of ICD induced by application of croton oil to the ears of naïve B6 mice [17]. JTE-052 did not show a significant inhibitory effect on croton oil-induced ear swelling (Fig. 1C) or mRNA expression levels of *Il6*,

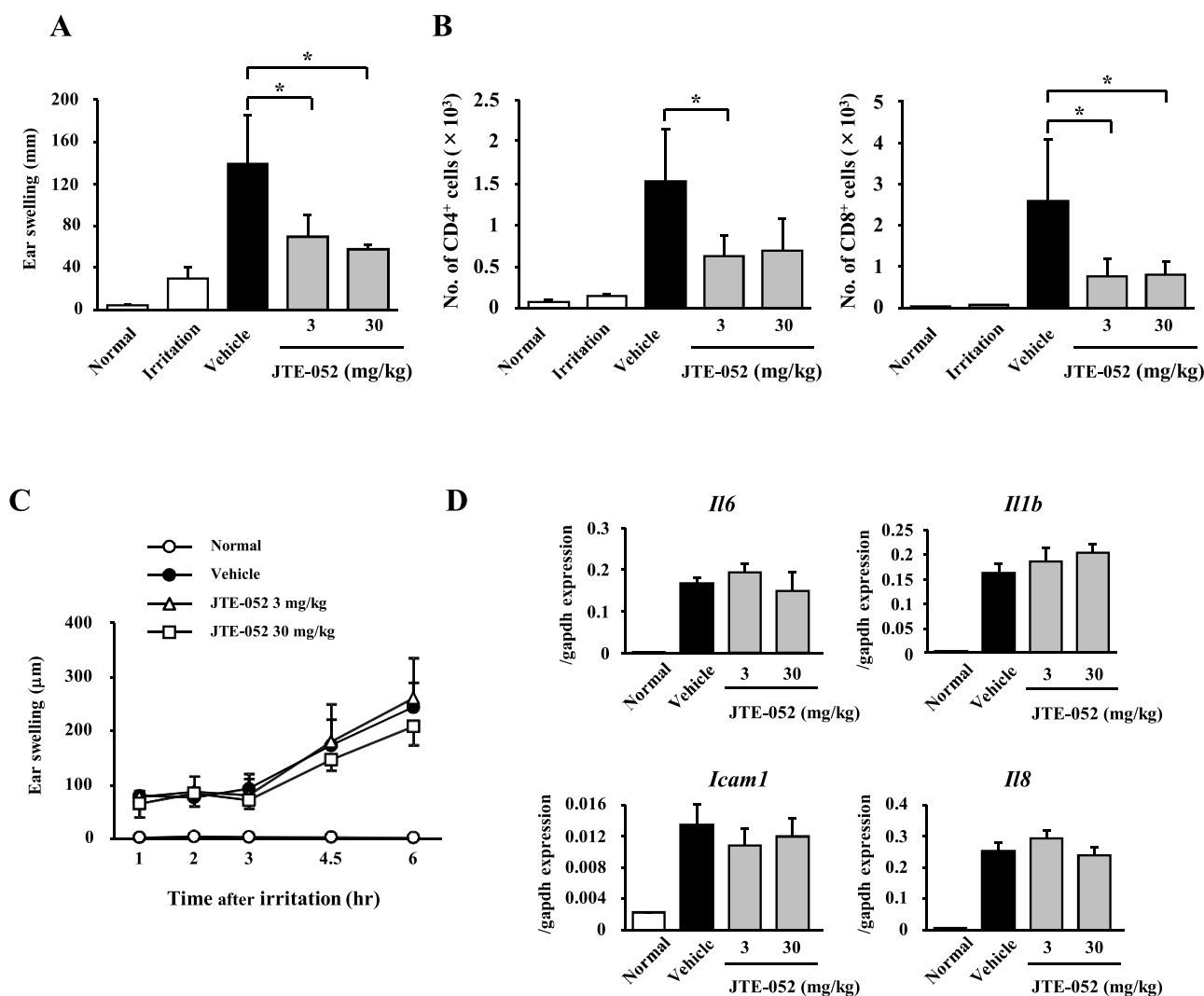
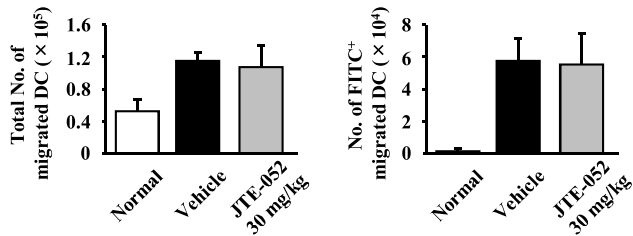


Fig. 1. Effects of JTE-052 on DNFB-induced CHS model and croton oil-induced ICD model.

Ears of B6 mice (*N* = 5 per group) were challenged with DNFB five days after sensitization. JTE-052 was orally administered once a day after sensitization. (A). 48 h after DNFB challenge, both ears were collected and the number of cells that had infiltrated into each ear (B) was counted by FACS. For ICD, 1% croton oil was applied to the ears of B6 mice (*N* = 5), and ear swelling was measured (C). Six hours after croton oil treatment, total RNA was extracted and *Il6*, *Il1b*, and *Icam1* mRNA expression levels were measured by qPCR (D). **P* < 0.05 versus vehicle-treated mice (Dunnett test).

A



B

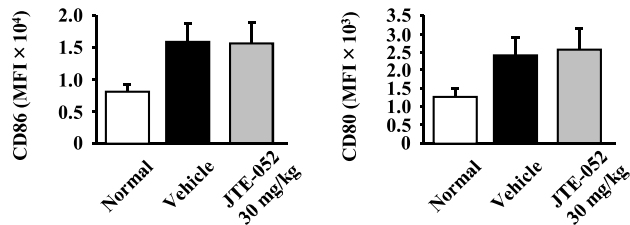


Fig. 2. JTE-052 had no effect on FITC-induced DC migration.

For FITC migration assay, the numbers of CD11c⁺ MHC class II⁺ DCs (left) and FITC⁺ CD11c⁺ MHC class II⁺ DCs (right) in the draining axillary LNs of B6 mice treated with vehicle or JTE-052 (N = 5) were analyzed by flow cytometry 24 h after application of 100 μ L of 2% FITC or vehicle on the abdomen (A). MFI of CD86 or CD80 in the migrated DCs was analyzed (B).

intercellular adhesion molecule 1 (*Icam1*) and *Il8* in the ear (Fig. 1D). These data suggest that JTE-052 works on acquired immune responses rather than innate immune activation in this model.

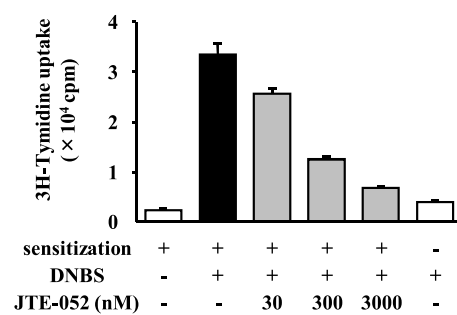
3.2. JTE-052 had no effect on DC migration and activation

It has been reported that a JAK inhibitor regulates bone marrow-derived DC (BMDC) or monocyte-derived DC function [12,18]. We next evaluated the effect of JTE-052 on the migration and activation of cutaneous DCs during the sensitization phase in vivo. We painted FITC on the shaved abdominal skin of B6 mice and evaluated DC migration into the skin draining LNs 24 h after application. The number of CD11c⁺ MHC class II⁺ DCs (Fig. 2A, left) and FITC⁺ CD11c⁺ MHC class II⁺ DCs that had migrated from the skin into the draining LNs (Fig. 2A, right) were not inhibited by oral administration of JTE-052. In addition, the mean fluorescence intensity (MFI) of CD86 and CD80, a costimulatory molecule found in migrated DCs, were not affected by JTE-052 (Fig. 2B). These data indicate that oral administration of JTE-052 does not modulate the migration and activation of cutaneous DCs.

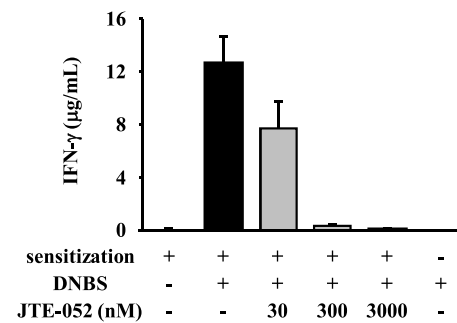
3.3. JTE-052 inhibited IFN- γ , IL-13, and IL-17A production by antigen-specific T cells

We next evaluated the effect of JTE-052 on the activation of antigen-specific T cells. Skin draining LN cells were collected from B6 mice that had been sensitized with DNFB five days previously. LN cells were then re-stimulated with DNBS, a soluble form of DNFB, with or without JTE-052 in vitro. Forty-eight hours after stimulation, cell proliferation was observed in the LN cells from sensitized B6 mice, but not in those from naïve B6 mice. JTE-052 inhibited proliferation in a dose-dependent manner (Fig. 3A). In addition, the production of IFN- γ , IL-13, and IL-17A protein induced by the DNBS challenge in vitro were also inhibited by JTE-052 (Fig. 3B and C).

A



B



C

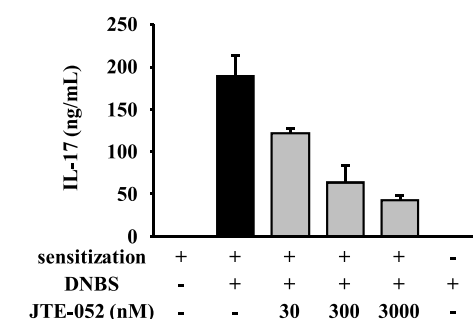
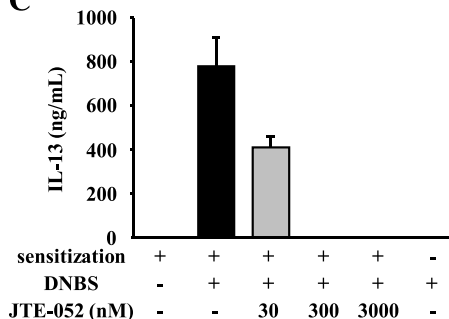


Fig. 3. Inhibitory effects of JTE-052 on antigen specific T cell activation.

Cells were collected from the draining LNs of B6 mice five days after DNFB application and cultured for 48 h with or without 100 μ g/mL DNBS and JTE-052. Cell proliferation was measured by [³H]thymidine incorporation (A). The amount of IFN- γ in the culture medium was measured by ELISA (B), and IL-13 and IL-17 were measured by CBA (C).

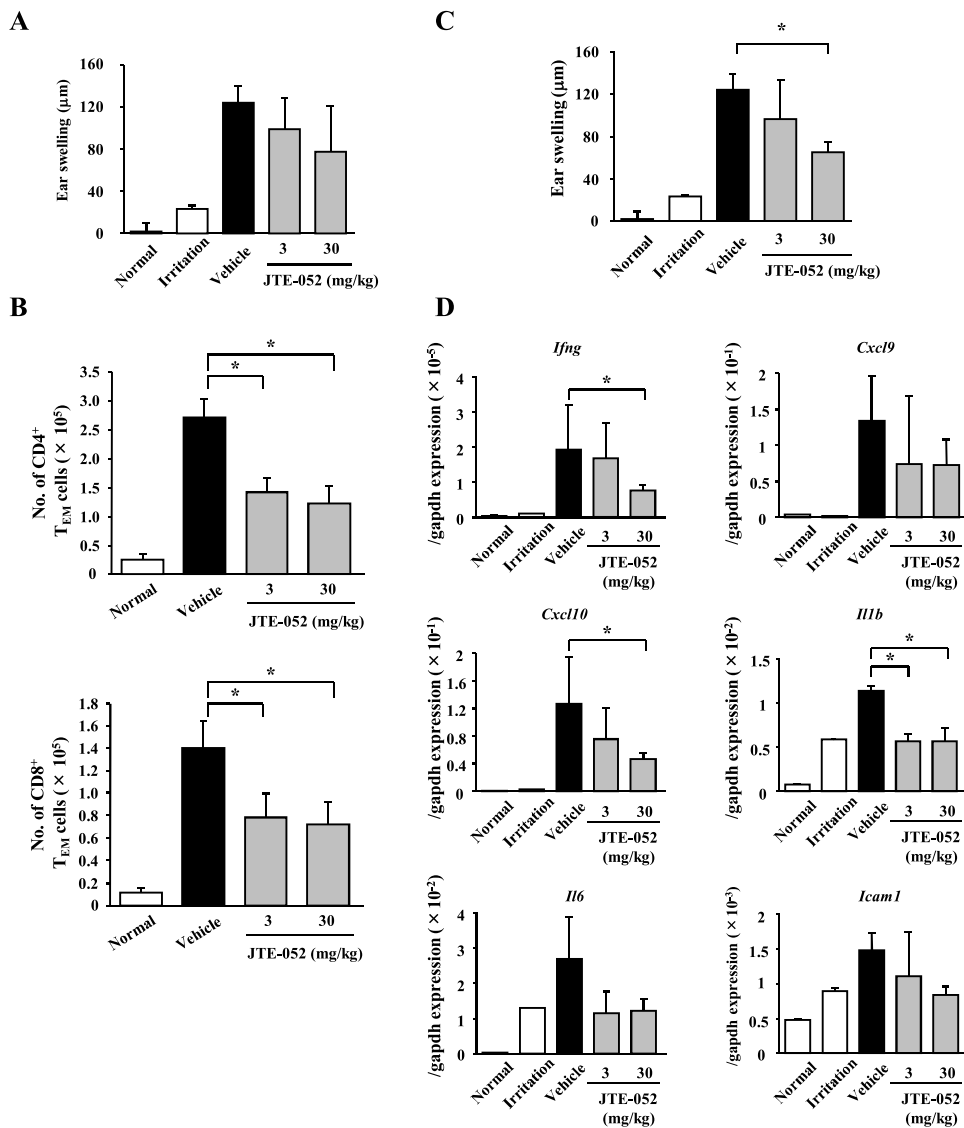


Fig. 4. Effects of JTE-052 during either sensitization or challenge phase.

JTE-052 was orally administered once a day during the sensitization phase only (A and B). Ear swelling was measured 24 h after DNFB challenge (N=4) (A). The draining LNs were collected five days after sensitization and the T cell subset was analyzed by FACS (B). JTE-052 was orally administered once before DNFB challenge (C and D). Ear swelling was measured 24 h after DNFB challenge (N=4) (C). Ears were collected and *Ifng*, *Cxcl9*, *Il6*, *Il1b*, and *Icam1* mRNA expression levels (12 h after challenge) were measured by qPCR (D). *P < 0.05 versus vehicle-treated mice (Dunnett or steel test).

3.4. JTE-052 inhibited CHS response by inhibiting T cell proliferation and activation

Based upon the above findings, it is considered that JTE-052 ameliorates ear swelling by suppressing T cell proliferation and activation in the CHS response. Next, to analyze the mechanism of action of JTE-052 in more detail, we separately evaluated the effect of JTE-052 administered during either sensitization or elicitation phase. To evaluate the effect of JTE-052 on sensitization phase, JTE-052 was administered once a day during sensitization phase only (starting at the time of sensitization until one day before elicitation). Ear swelling measured 24 h after the challenge tended to be suppressed by oral administration of JTE-052, but statistically not significant (Fig. 4A). FACS analysis revealed that JTE-052 significantly decreased the numbers of LN cells, CD3⁺ CD4⁺ cells, and CD3⁺ CD8⁺ cells in the draining LN (Fig. S1C). In addition, CD4⁺ CD62L⁻ CD44⁺ effector memory T cells (CD4⁺ T_{EM} cells) and CD8⁺ CD62L⁻ CD44⁺ effector memory T cells (CD8⁺ T_{EM} cells) in the draining lymph nodes were significantly decreased by oral

administration of JTE-052 for five days after sensitization (Fig. 4B). These data suggest that JTE-052 inhibited differentiation and proliferation of T_{EM} in the draining lymph nodes during sensitization phase.

To evaluate the effect of JTE-052 on elicitation phase, mice sensitized with DNFB were given either vehicle or JTE-052 orally one hour before challenge with DNFB, and ear swelling was measured 24 h after the challenge. JTE-052 treatment resulted in suppression of ear swelling (Fig. 4C). Twelve hours after DNFB challenge, mice were sacrificed and both ears were collected for analysis of mRNA expression. Expression levels of Th1-related genes (*Ifng*, *C-X-C-motif ligand 9* (*Cxcl9*) and *Cxcl10* [6]) were inhibited by JTE-052. In addition, *Il6* and *Il1b* mRNA expressions were also inhibited by JTE-052, though they were not suppressed in croton oil-induced ICD (Fig. 4D). On the other hand, single administration of JTE-052 did not show an inhibitory effect on the numbers of CD4⁺ and CD8⁺ T cells or the number of IFN-γ⁺ T cells at the site of DNFB challenge (Fig. S2A and B). These data suggest that JTE-052 inhibited T cell activation and subsequent cytokine and

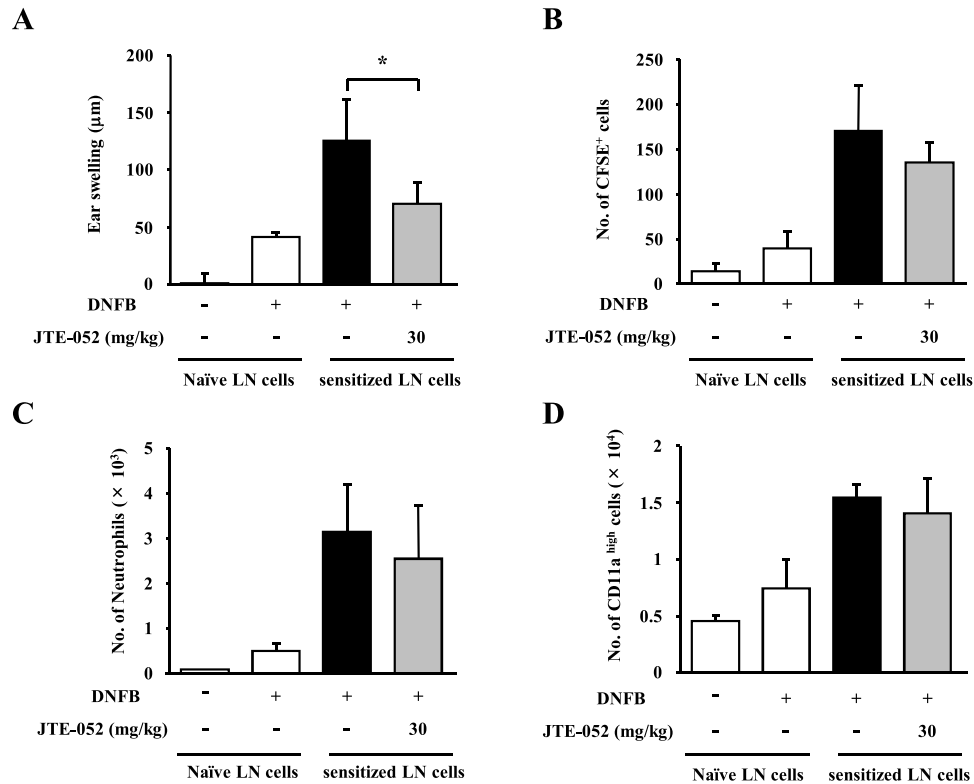


Fig. 5. Effects of JTE-052 on T cell response in adoptive transfer.

Five days after sensitization with DNFB, cells from the skin dLNs of B6 mice were labeled with CFSE and intravenously injected into naïve B6 recipient mice (1×10^7 cells/head). Twenty-four hours after injection, mice were given JTE-052 orally and challenged on the ear with DNFB ($N = 4$). The swelling reaction was measured 24 h after DNFB challenge (A). After measurement of ear swelling, ears were collected and the numbers of cells infiltrated into the ears were counted by FACS (B–D). * $P < 0.05$ versus vehicle treatment (t test).

chemokine production without affecting T cell migration to the site of challenge in the elicitation phase.

3.5. JTE-052 inhibited CHS response during challenge phase without altering T cell migration to the site of challenge

To clarify the effect of JTE-052 on T cell and non-T cell migration, the effect of JTE-052 on CHS response was assessed through adoptive transfer of sensitized T cells. Mice injected with CFSE-labeled LN cells following DNFB sensitization exhibited a CHS response after DNFB challenge of the ear skin. Oral administration of JTE-052 prior to DNFB challenge inhibited ear swelling (Fig. 5A). FACS analysis revealed that the number of CFSE⁺ cells migrating into the ear was not altered by JTE-052 24 h after challenge (Fig. 5B). In addition, there was no significant difference in the number of neutrophils or in the number of subsets highly positive for CD11a (an adhesion molecule expressed on leukocytes) between vehicle- and JTE-052-treated mice (Fig. 5C and D). These data indicate that JTE-052 dominantly inhibited antigen-specific T cell responses in the challenge phase of CHS without affecting the expression of adhesion molecules or T cell migration into the site of challenge.

4. Discussion

In this study, we have demonstrated that oral administration of the JAK inhibitor JTE-052 attenuated CHS. Oral administration of JTE-052 did not alter the migratory function of DCs, nor did it affect croton oil-induced ICD, but it did potentially inhibit antigen-specific T cell proliferation and cytokine production. In addition, single administration of JTE-052 inhibited contact hypersensitivity by

downmodulating T cell activation without suppressing T cell migration to the site of challenge. Taken together, oral administration of JTE-052 attenuates CHS responses by suppressing antigen-specific T cell responses both in draining LN and at the local site of challenge. These findings suggest that JTE-052 has a potent inhibitory effect on T_{EM}-induced acquired skin immune response (Fig. 6).

It has been demonstrated that the JAK-STAT pathway regulates multiple immune cell functions [8]. Hence, inhibition of JAK activities may be a promising therapeutic strategy for immune and inflammatory disorders including ACD. Because the JAK-STAT pathway involves a variety of cytokines and growth factors, it is difficult to identify the mechanism of action of the JAK inhibitors *in vivo*. Although several studies have reported the inhibitory effects of JAK inhibitors on skin inflammation, insights regarding the mechanisms responsible for immune modulation by the JAK inhibitors are limited [11,19]. In this report, we used a murine CHS model to elucidate the mechanism of action of the JAK inhibitor JTE-052 on a cutaneous immune response.

We demonstrated that oral administration of JTE-052 ameliorated CHS response although it did not affect croton oil-induced ICD or FITC-induced DC migration into the draining LNs (Fig. 2). It should be noted, however, that the present study only analyzed croton oil-induced swelling and mRNA expression levels in mouse ears, and that, accordingly, these results may not be assumed to represent the therapeutic effect of JTE-052 on human ICD. A previous report suggested that inhibition of JAK2 activity is necessary for DC development and maturation [20], but in the present study there were no significant changes in the number of migrated DCs or expression levels of CD86 and CD80 on DCs between vehicle- and JTE-052-treated mice (Fig. 2). We previously

Contact Hypersensitivity (CHS)

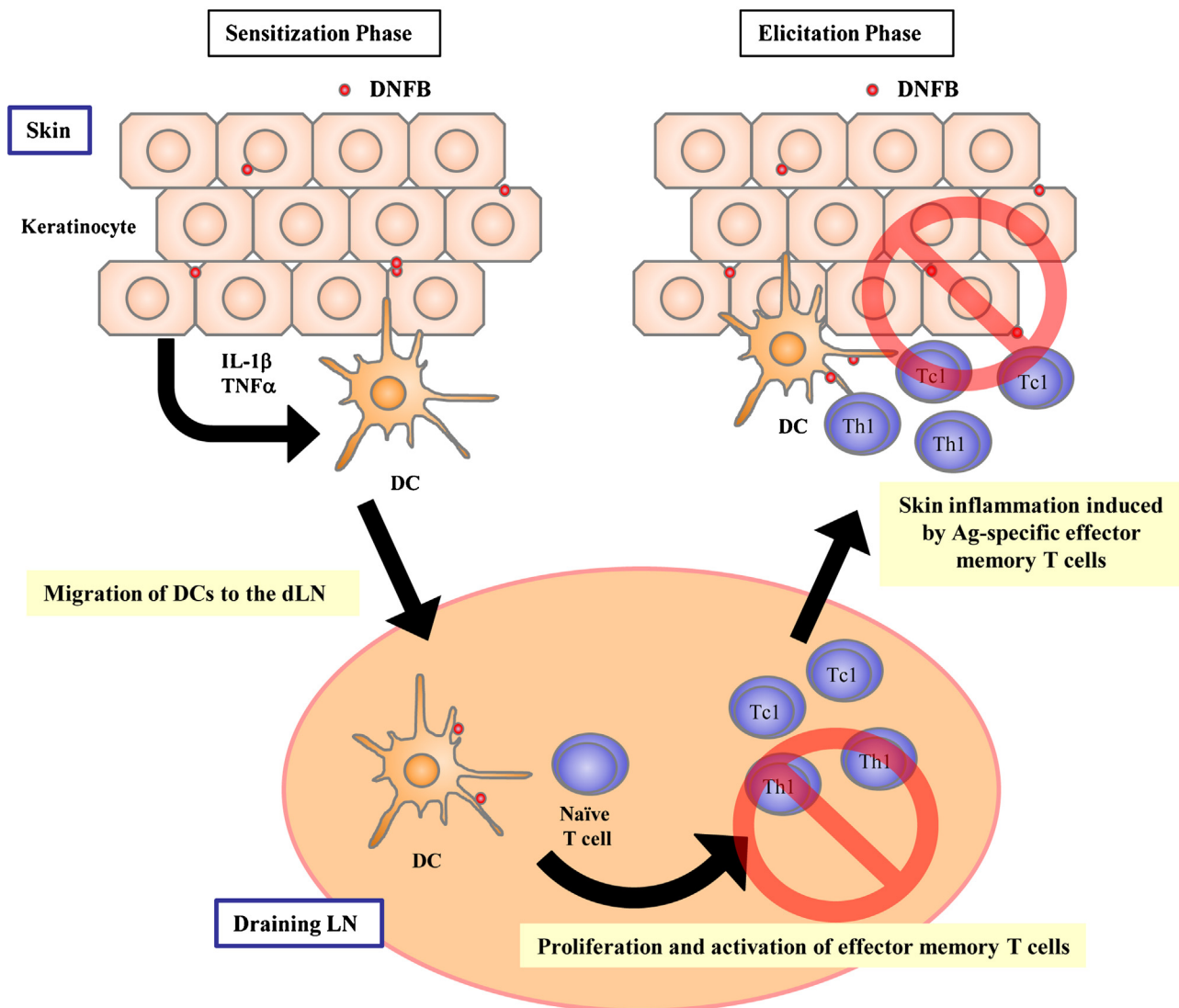


Fig. 6. JTE-052 regulates contact hypersensitivity by inhibiting T cell differentiation and activation in vivo.

JTE-052 affected neither croton oil-induced ICD nor DC migration in the sensitization phase. It did inhibit T cell proliferation and differentiation during the sensitization phase, and it inhibited activation of T_{EM} stimulated by antigen re-stimulation during the elicitation phase without disrupting T cell migration to the site of challenge.

reported that JTE-052 inhibited IL-2 and IL-6 signaling more potently than GM-CSF signaling did [14]. These data suggest that JTE-052 could suppress the maturation of DCs in vitro, but oral administration of JTE-052 may not have a sufficient effect to cause observable suppression of DC function in this murine CHS model.

In the sensitization phase, naïve T cells proliferate and differentiate to T_{EM} upon antigen presentation by DCs in draining LN. This study demonstrated that continuous infusion or consecutive oral administration of JTE-052 decreased the number of LN cells. In addition, JTE-052 decreased the number of CD4⁺ and CD8⁺ T_{EM} in the draining LN during the sensitization phase. Moreover, JTE-052 dose-dependently inhibited the proliferation and cytokine production by antigen-restimulated LN cells in vitro. These findings suggest that JTE-052 inhibits T cell proliferation induced by APCs in the draining LN.

In the elicitation phase, T_{EM} migrated rapidly to the site of challenge and were then activated locally by hapten-carrying APCs [1,21–23]. It has been shown elsewhere that re-activated T_{EM} produce IFN- γ which up-regulates the production of chemokines

(e.g., CXCL-9, CXCL-10 and IL-8) from keratinocytes and APCs that support the cytotoxic effects of CD8⁺ T cells [6,24,25]. We identified IFN- γ ⁺ CD4⁺ and CD8⁺ T cells at the site of challenge by FACS analysis, and JTE-052 did not affect these numbers in the elicitation phase unexpectedly (Fig. S2B). This result seems to have occurred due to the lack of an inhibitory effect of JTE-052 on T cell migration, and an enforced activation of ear-infiltrated all T_{EM} cells by PMA/ionomycin during FACS procedure. Intriguingly, we demonstrated that oral administration of JTE-052 during the elicitation phase inhibited the mRNA expression of *Ifng*, *Cxcl9*, *Cxcl10*, *Il6*, and *Il1b* (Fig. 4D), suggesting that JTE-052 attenuated hapten-induced skin inflammation by inhibiting migrated-T_{EM} activation.

Two adhesion molecules, lymphocyte function-associated antigen-1 (LFA-1) and ICAM-1, are believed to play critical roles in this migration process [26]. LFA-1 is expressed on T_{EM} while ICAM-1 is expressed on vascular endothelial cells at sites of inflammation as well as on keratinocytes in a variety of T cell-mediated disorders [27,28]. The induction of *Icam1* mRNA expression by croton oil (Fig. 1D) and the number of cells positive

for CD11a, a subunit of LFA-1, in the cell transfer model (Fig. 5C) were not affected by JTE-052. These data indicate that JTE-052 did not affect T cell migration directly but rather inhibited T cell activation by APCs and subsequent inflammation at the site of challenge.

Taken together, the evidence suggests that JTE-052 exerts an inhibitory effect on antigen-specific T cell activation and subsequent inflammation, especially at the site of challenge, in a murine CHS model. It has been reported that the JAK inhibitor R348 attenuates skin inflammation while reducing CD4⁺, CD8⁺, and CD25⁺ T cell infiltration and ameliorating total clinical score in a murine T cell-dependent psoriasis model [29], and that JAK inhibitors can be effective treatment for psoriasis [30]. Additionally, it has been reported that another JAK inhibitor promotes hair growth in alopecia areata, which results from damage to the hair follicle by cytotoxic T cells [31]. These data suggest that JAK inhibitors have the advantage of being able to suppress T cell activation in an efficient manner. T cells are known to be involved in several skin diseases such as atopic dermatitis, psoriasis and others [32–34]. Our data suggest that JTE-052 could be a unique therapeutic reagent against exaggerated T cell response to antigens, and that the topical application of this JAK inhibitor to lesions may decrease inflammation in an effective manner.

Conflict of interest

W.A., Y.Y., A.T. and M.M. are employees of Japan Tobacco Inc.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.jdermsci.2016.09.007>.

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ONLINE METHODS

Implantation of osmotic pumps

Osmotic mini-pumps (Alzet Model 1007D, 0.5 µl/h, 7 d; Durect Co, CA, USA) were used to deliver a dose of 30 mg/kg/day of JTE-052 or vehicle alone (PEG300). For implantation of osmotic pumps, mice were anesthetized and their dorsal surface was shaved. A 1-cm incision was made on the back to create an s.c. pocket and insert the pump twenty-four hours before sensitization.

SUPPLEMENTAL FIGURE LEGENDS

Figure S1

B6 mice (N=3 per group) were sensitized with DNFB for five days. Ear swelling was measured twenty-four hours after the DNFB challenge in the mice receiving continuous administration of JTE-052 (30 mg/kg/day) or vehicle via osmotic pump (A). For irritation, mice were challenged with DNFB without sensitization. Number of cells in draining LN was counted by FACS forty-eight hours after the DNFB challenge (B). (C) JTE-052 was orally administered once a day during the sensitization phase only. The draining LNs were collected five days after sensitization and the T cell subset was analyzed by FACS (N=5). #P < .05 versus vehicle-treated mice (t-test). *P < .05 versus

19 vehicle-treated mice (Dunnett).

20

21 **Figure S2**

22 JTE-052 was orally administered once before DNFB challenge. Twenty-four hours after

23 DNFB challenge, both ears were collected and the number of CD4⁺ or CD8⁺ T cells (A)

24 and INF γ ⁺ CD4⁺ or INF γ ⁺ CD8⁺ T cells (B) were analyzed by FACS (N=4-8).

Figure S1

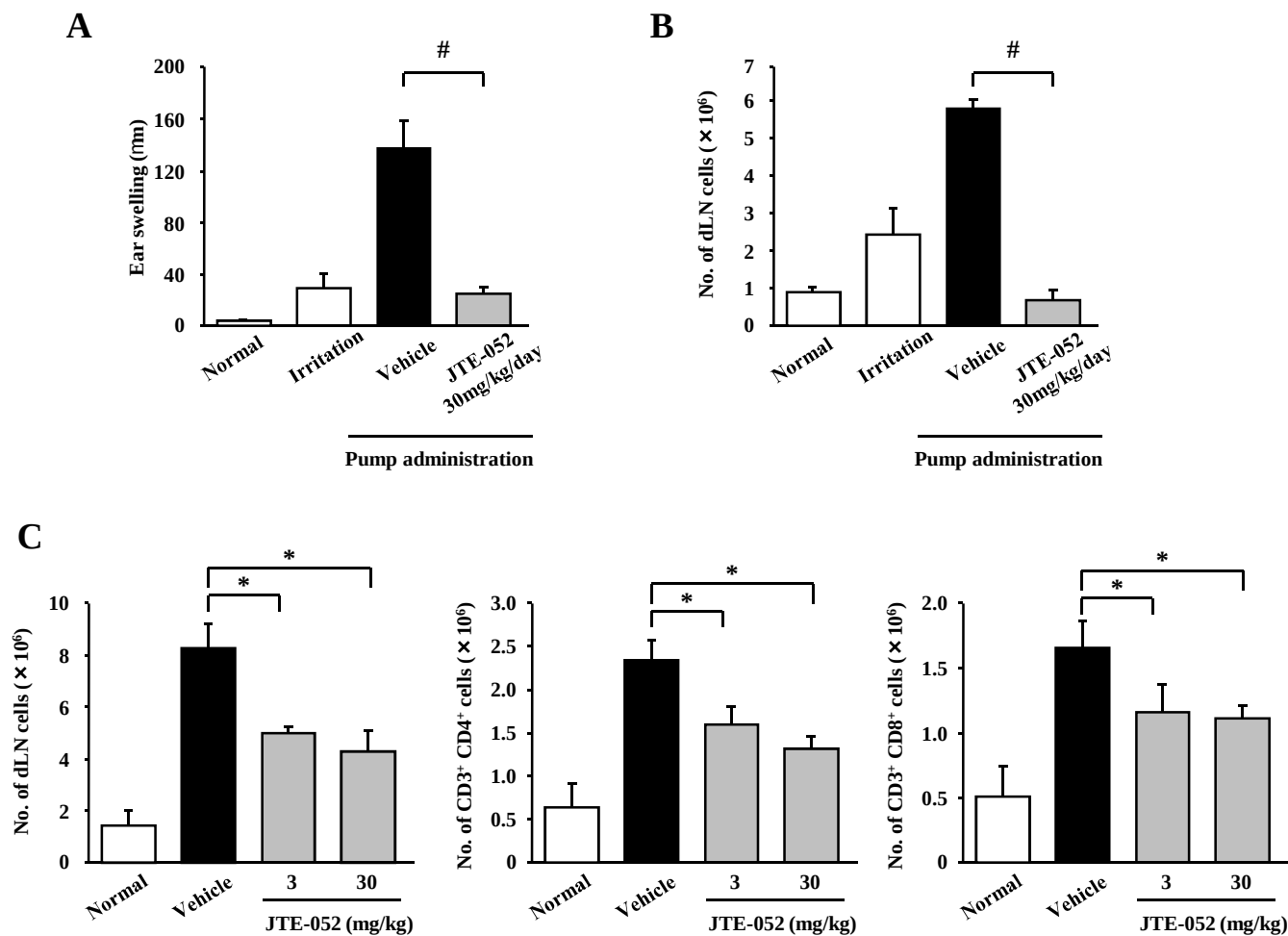
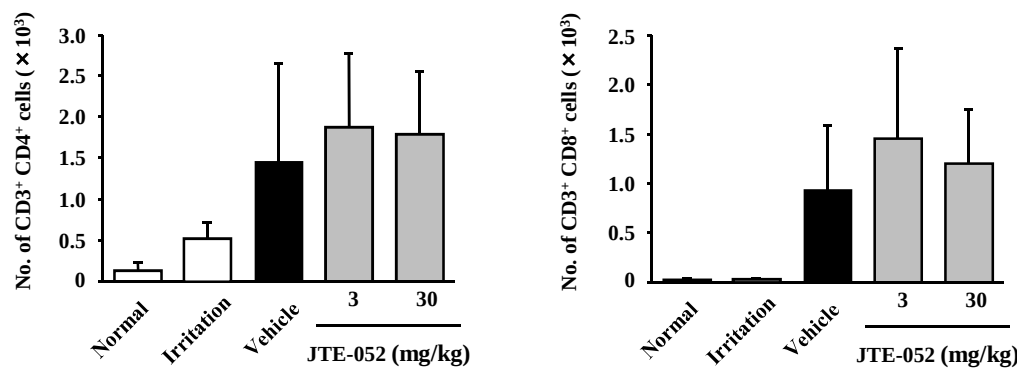


Figure S2

A



B

