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RESEARCH ARTICLE

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Anti-ORAI1 antibody DS-2741a, a specific CRAC channel blocker, shows ideal therapeutic profiles for allergic disease via suppression of aberrant T-cell and mast cell activation

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Abstract

ORAI1 constitutes the pore-forming subunit of the calcium release-activated calcium (CRAC) channel, which is responsible for store-operated calcium entry into lymphocytes. It is known that ORAI1 is essential for the activation of T cells and mast cells and is considered to be a potent therapeutic target for autoimmune and allergic diseases. Here, we obtained a new humanized antibody, DS-2741a, that inhibits ORAI1 function. DS-2741a bound to human-ORAI1 with high affinity and without cross-reactivity to rodent Orai1. DS-2741a demonstrated suppression of CRAC-mediated human and mouse T-cell activation and mast cell degranulation in human ORAI1 knock-in mice. Furthermore, DS-2741a ameliorated house dust mite antigen-induced dermatitis in the human ORAI1 knock-in mouse. Taken together, DS-2741a inhibited T-cell and mast cell functions, thus improving skin inflammation in animal models of atopic dermatitis and reinforcing the need for investigation of DS-2741a for the treatment of allergic diseases in a clinical setting.

Abbreviations: BMMC, bone marrow-derived mast cell; BSA, bovine serum albumin; CRAC, calcium released-activated calcium; Dfb, *Dermatophagoides farinae* bodies; D-PBS, Dulbecco's Phosphate Buffered Saline; DTH, delayed-type hypersensitivity; FBS, fetal bovine serum; HRP, horseradish peroxidase; mBSA, methylated bovine serum albumin; NFAT, nuclear factor activated by T cells; OVA, ovalbumin; PBMC, peripheral blood mononuclear cell; PCA, passive cutaneous anaphylaxis; PMA, phorbol 12-myristate 13-acetate; SCF, stem cell factor; SCID, severe combined immunodeficiency; SEB, *Staphylococcal* enterotoxin B; SOCE, store-operated calcium entry; TCR, T-cell receptor; TMB, 3,3',5,5'-tetramethylbenzidine; TRPA1, transient receptor potential ankyrin 1; TSLP, thymic stromal lymphopoietin.

KEYWORDS

allergy, autoimmune, CRAC channels, cytokine production, store-operated calcium entry

1 | INTRODUCTION

Atopic dermatitis (AD) affects 15-30% of children and 2-10% of adults worldwide.[1] In severe cases, patients suffer from insomnia and anxiety triggered by continuous itching and/or due to changes in appearance, leading to poor quality of life. For patients with mild-to-moderate disease, topical drugs such as corticosteroids, tacrolimus, and moisturizing agents are primarily prescribed.[2] For patients with moderate-to-severe AD, potential treatments include anti-interleukin (IL)-4Ra antibody therapy, dupilumab monotherapy, or systemic administration of immunosuppressants, including cyclosporine A, a powerful immunosuppressant calcineurin inhibitor usually prescribed owing to its strong efficacy in suppressing AD. Widespread unregulated immune activation is characteristic of AD.[3] Cyclosporine A suppresses several T-cell subsets, thereby attenuating AD, but it must be administered cautiously because of toxicity concerns. Intermittent cyclosporine A administration is recommended to avoid kidney toxicity, and blood concentrations must be regularly monitored to avoid emerging toxicity.[4,5] Thus, more effective and safer treatments are needed.

Nuclear factor activated by T cells (NFAT) regulates the transcription of pro-inflammatory T-cell cytokines.[6-9] Upon stimulation of the T-cell receptor (TCR), store-operated calcium entry (SOCE) occurs via the CRAC channel. The elevated calcium activates calcineurin which dephosphorylates and activates NFAT, a pivotal transcription factor in immune cells.[10] Calcium ion (Ca^{2+}) entry from the CRAC channel is also strictly controlled and mediated by IgE-FcεRI signaling upon cross-linking by antigens, leading to degranulation of mast cells.[11] ORAI1, the critical pore subunit of the CRAC channel, is important in immune regulation.[12,13] ORAI1 homogenous deficiency in humans causes severe combined immunodeficiency (SCID), characterized by the absence of or significantly impaired T-cell function.[14] Orai1 knockout mice show reduced T-cell cytokine production and mast cell activation.[15,16] Transcripts of Orai1 are mainly limited to immune cells; Orai2 is mainly found in the brain, lungs, spleen, and small intestine; and Orai3 is abundant in many solid organs.[17,18] Therefore, the specific inhibition of ORAI1 could be a potential mechanism for the treatment of AD and other immune diseases. Here, we generated an anti-human ORAI1 antibody and evaluated the role of ORAI1 in AD pathology using mouse models.

2 | MATERIALS AND METHODS

2.1 | Antibodies and reagents

DS-2741a, a humanized anti-ORAI1 antibody, was generated in rat by DNA immunization based on the method reported previously.[19] For flow cytometric analysis, fluorochrome-conjugated anti-CD3 and anti-CD117 antibodies (Nippon Becton Dickinson Company, Ltd., Tokyo, Japan), anti-FcεRIα antibody (BioLegend Inc., San Diego, CA, USA), biotin conjugated-anti-ORAI1 antibody (SouthernBiotech, Birmingham, AL, USA), and APC-conjugated streptavidin (Thermo Fisher Scientific K.K., Tokyo, Japan) were used.

2.2 | Generation of human ORAI1 knock-in mice (hu-ORAI1 KI mice)

Human ORAI1 knock-in mice in C57BL/6N background were generated by the Institute of Immunology Co., Ltd. (Tokyo, Japan) using a conventional homologous recombination system. In the knock-in mice, an extracellular loop domain of mouse Orai1 [KFLPLKRQAGQPSPTKPPAESVIVANHSDSSGITPGEAAAIASTAI] was replaced by corresponding human ORAI1 sequence [KFLPLKKQPGQPRPTSKPPASGAAANVSTSGITPGQAAAIASTTI]. All animal experimental procedures were performed in accordance with the in-house guidelines of the Institutional Animal Care and Use Committee. We used 7-26 weeks old mice in all experiments.

2.3 | Cell ELISA assay

CHO-K1 cells were cultured in Ham's F-12 Nutrient Mixture supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin and transfected with human ORAI1 expression vector using Lipofectamine 2000 (Thermo Fisher Scientific K.K., Tokyo, Japan). The transfected cells were seeded onto a 96-well plate, fixed with 10% formalin neutral buffer solution, and washed gently with Dulbecco's Phosphate Buffered Saline (D-PBS) (Thermo Fisher Scientific K.K.). For binding assay, the fixed cells were incubated with blocking buffer (D-PBS containing 2% bovine serum albumin (BSA)) for 1 hour at room temperature, washed with D-PBS, and then incubated with indicated concentrations of DS-2741a for

1 hour at room temperature, and again washed with D-PBS. The amount of DS-2741a bound to the cells was detected using horseradish peroxidase (HRP)-labeled secondary antibody and 3,3',5,5'-tetramethylbenzidine (TMB) substrate.

For competitive inhibition assay, cells were incubated with blocking buffer and washed similarly as described above. Cells were then incubated with 2 nmol/L DS-2741a and indicated competitive peptides (several types of human ORAI1 family and ORAI1 ortholog extracellular domain peptides (human/cynomolgus monkey ORAI1 Loop2 peptide, human ORAI1 Loop1 peptide, mouse Orai1 Loop2 peptide, rat Orai1 Loop2 peptide, human ORAI12 Loop2 peptide, and human ORAI13 Loop2 peptide: all peptides were synthesized by Sigma-Aldrich Co. LLC.) for 1 hour at room temperature, and subsequently washed. The amount of DS-2741a antibody bound to the cells was detected by HRP-labeled secondary antibody and TMB substrate.

2.4 | Fluorescence-based T-cell intracellular Ca^{2+} assay

Jurkat cells were cultured in RPMI-1640 supplemented with 10% FBS and 1% penicillin-streptomycin (RPMI culture medium). Cells in suspension were labeled with 3 μmol/L Fura 2-AM (DOJINDO, Tokyo, Japan) at room temperature in the dark for 30 minutes with rotation. The labeled cells were centrifuged, and the supernatant was discarded. The cells were suspended in 10 mL HEPES assay buffer supplemented with D-(+)-glucose (SIGMA-Aldrich, Tokyo, Japan), 1.2 mmol/L $MgCl_2$, and 0.1% BSA fraction IV (SIGMA) and seeded in a 96-well microtiter plate containing indicated concentrations of DS-2741a or isotype control IgG_1 (Eureka Therapeutics, Inc., Emeryville, CA, USA); this was followed by incubation for 1 hour at room temperature, stimulation with 1 μmol/L thapsigargin and re-incubation for 10 minutes at room temperature in the dark. $CaCl_2$ (2 mmol/L) was added to the stimulated cells and incubated for 15 minutes at room temperature avoiding light exposure. Fura-2 emission was detected at 510 nm with excitation at 340 and 380 nm using Spectra Max M5e (Molecular Devices Japan K.K., Tokyo, Japan), and Ca^{2+} levels were determined by the emission ratio (340/380) at every 5-second interval after background subtraction.

2.5 | T-cell cytokine release assay

Human PBMCs were purchased from Cellular Technology Ltd. (OH, USA). Mouse $CD3^+$ T cells were isolated from mouse spleen by using EasySep mouse T-cell isolation kit (STEMCELL, Vancouver, Canada) according to the manufacturer's protocol.

Jurkat cells, human PBMCs, or $CD3^+$ T cells of hu-ORAI1 KI mouse were cultured in 96-wells microtiter plates containing the indicated concentrations of DS-2741a or the isotype control IgG_1 (Eureka Therapeutics, Inc) diluted in RPMI culture media, and were incubated for 1 hour at 37°C in a humidified incubator containing 5% CO_2. The cells were stimulated by 61.7 nmol/L PMA (Phorbol 12-myristate 13-acetate, SIGMA) and 141 nmol/L A23187 (SIGMA) or anti-CD3/28 beads (Thermo Fisher Scientific K.K.) at a bead-to-cell ratio of 1:1 for 18 to 24 hours at 37°C in a humidified incubator containing 5% CO_2. The supernatants were then collected, and IL-2 concentration was determined using an ELISA kit (R&D systems, Minneapolis, MN, USA).

2.6 | Differentiation and activation of effector T-cell subsets

Human PBMCs were purchased from Cellular Technology Ltd.. Naïve $CD4^+$ T cells were isolated from PBMCs using a human Naïve CD4 isolation kit (Miltenyi Biotec K.K., Tokyo, Japan) according to the manufacturer's instructions. To initiate differentiation into Th1, Th2, Th22, and Treg cells, the naïve $CD4^+$ T cells (5×10^4/well) were treated with anti-CD3/28 beads (Thermo Fisher Scientific K.K.) and relevant cytokines (listed below) for 5 days. DS-2741a or FK-506 was added 1 hour before the differentiation procedure commenced.

Th1: 5 ng/mL IL-2 (Peprotech Inc., NJ, USA) and 10 ng/mL IL-12 p70 (Peprotech Inc.)

Th2: 10 ng/mL IL-4 (Peprotech Inc.)

Th22: 100 ng/mL TNFα (R&D systems) and 20 ng/mL IL-6 (Peprotech Inc.)

Treg: 20 ng/mL IL-2 and 10 ng/mL TGFβ (R&D systems).

To evaluate the inhibitory effects on cytokine production using DS-2741a or FK-506, the levels of several cytokines (IFNγ, IL-13, IL-31, and IL-22) were measured using ELISA after treatment with 61.7 nmol/L PMA and 141 nmol/L A23187 for 24 hours (IFNγ, PerkinElmer, Inc. MA, USA; IL-13 and IL-31, Thermo Fisher Scientific K.K.; IL-22, R&D systems). For the evaluation of Treg differentiation, the number of $Foxp3^+CD4^+$ cells was determined by flow cytometry.

2.7 | Induction of delayed-type hypersensitivity (DTH) reaction

DTH was induced in hu-ORAI1 KI mice as previously described.[20] Briefly, 50 μL of emulsified 5 mg/mL methylated bovine serum albumin (mBSA, SIGMA) with equal volume of Freund's Complete Adjuvant was subcutaneously injected to both sides of the flank of mice at day 0, followed by an intradermal injection of 25 μg of mBSA to the left hind footpad at day 7. After 0, 6, 24, and 48 hours of antigen injection, footpad thickness and foot volume were measured by dial

thickness gauge (G-0.4N, OZAKI MFG. CO., LTD., Tokyo, Japan) and by a paw volumeter (Unicom K.K., model number: TK-101), respectively. DS-2741a (3-60 mg/kg) or vehicle was administered by tail vein injection at day 1 and day 6. Increased footpad thickness (Δ footpad thickness) or foot volume (Δ foot volume) at each time point was calculated according to the following formula:

Δfootpad thickness (10^{-2} mm) = left footpad thickness (10^{-2} mm) at each measurement point after induction − left footpad thickness (10^{-2} mm) just before induction.

Δfoot volume (mL) = left foot volume (mL) at each measurement point after induction − left foot volume (mL) just before induction.

2.8 | Mast cell degranulation assay

Bone marrow–derived mast cells (BMMCs) were obtained as described previously[21] using 10 ng/mL recombinant mouse IL-3 (rmIL-3) (R&D Systems). The obtained BMMCs were co-cultured in the presence of 100 ng/mL stem cell factor (SCF, PeproTech Inc., Rocky Hill, NJ, USA) with Swiss 3T3 fibroblasts treated with 3 μg/mL mitomycin C (SIGMA). An equal volume of fresh medium containing 100 ng/mL SCF was added 2 days later and further cultured for 2 more days. BMMCs were restimulated with new feeder cells and fresh culture medium containing 100 ng/mL SCF and 10 ng/mL rmIL-3 every 4-7 days. DS-2741a or control IgG1 (Eureka Therapeutics, Inc.) was incubated for 1 hour before thapsigargin treatment. The degree of mast cell degranulation was determined by measuring β-hexosaminidase release.

2.9 | Induction of passive cutaneous anaphylaxis (PCA) reaction

PCA was induced in hu-ORAI1 KI mice as previously described.[22] Briefly, 0.5 μg ovalbumin (OVA)-specific IgE (Mouse Monoclonal IgE Antibody (E-C1), Chondrex Inc., Redmond, WA, USA) was intradermally administered to the ears of mice, followed by an intravenous injection of 10 mg/kg of antigen (OVA, SIGMA) after 24 hours. Twenty-six hours prior to the antigen injection, 60 mg/kg DS-2741a or control IgG (Jackson ImmunoResearch Laboratories, Inc., Pennsylvania 19390 USA) was administered to the mice. The anaphylaxis responses were studied by means of assessing the leakage of Evans blue dye.

2.10 | Induction of dermatitis

Dermatitis was induced in hu-ORAI1 KI mice by repeated paint of house dust mite antigen, *Dermatophagoides farinae* bodies (Dfb) as previously described.[23] The design of this study has been summarized in Figure S1. One hundred milligrams of Dfb (Dfb ointment Biostir® AD, Biostir Inc., Kobe, Japan) and 10 μg/mL *Staphylococcal* enterotoxin B (SEB) (Toxin Technology, Inc., Sarasota, FL, USA) diluted with 0.5% Tween® 20 (SIGMA)/D-PBS were applied onto both ears, shaved postauricular region, and back of skin (n = 7). From the second induction and thereafter, 4% sodium dodecyl sulfate (SDS) (Wako Pure Chemical Industries, Ltd., Osaka, Japan) was applied onto the primary treated region, followed by Dfb and SEB ointment treatment every 2 or 3 days for 21 days. After dermatitis was fully established, 10 mg/kg of DS-2741a was intravenously injected at 2 weeks after induction (day 0), and 30 mg/kg of cyclosporine A (Novartis Pharma Co., Ltd.) was orally administered daily from day 0 to day 5 (Figure S1). Ear thickness was measured using dial thickness gauge (G-0.4N, OZAKI MFG. CO., LTD.) and increased ear thickness was calculated using the following formula Δ (10^{-2} mm) = ear thickness at 30 minutes after induction (10^{-2} mm) − ear thickness just before induction (10^{-2} mm).

3 | RESULTS

3.1 | Binding specificity of anti-ORAI1 antibody, DS-2741a

DS-2741a bound to human ORAI1 overexpressing CHO-K1 cells in a concentration-dependent manner but not to control (mock vector transfected) cells as determined by cell-based enzyme-linked immunosorbent assay (ELISA) (Figure 1A). The obtained EC_{50} value by cell ELISA was defined as the Kd value. The Kd value for the binding of DS-2741a to the human ORAI1 was 0.791 nmol/L. To determine binding selectivity of DS-2741a, competitive ELISA was performed. DS-2741a was incubated with human ORAI1 expressing CHO-K1 cells in the presence of various concentrations of competitors (extracellular domain peptides of ORAI1 orthologs and an ORAI1 homologue, human ORAI2). The amino acid sequence of human ORAI1 loop2 peptide is identical to the amino acid sequence in cynomolgus monkey, and the human/cynomolgus ORAI1 loop2 peptide showed competitive inhibitory activity against DS-2741a binding to full-length human ORAI1, in a concentration-dependent manner (Figure 1B). In contrast, human ORAI1 loop1 peptide did not inhibit DS-2741a binding (Figure 1B), which indicated that the binding domain of DS-2741a is within loop2. We therefore focused on loop2, and further competition assays were conducted using ortholog and homologue peptides. Mouse and rat Orai1 loop2 peptides did not change DS-2741a binding to full-length human ORAI1. Similar results were observed with human ORAI2 and ORAI3 loop2 region (Figure 1B). These results suggested that DS-2741a

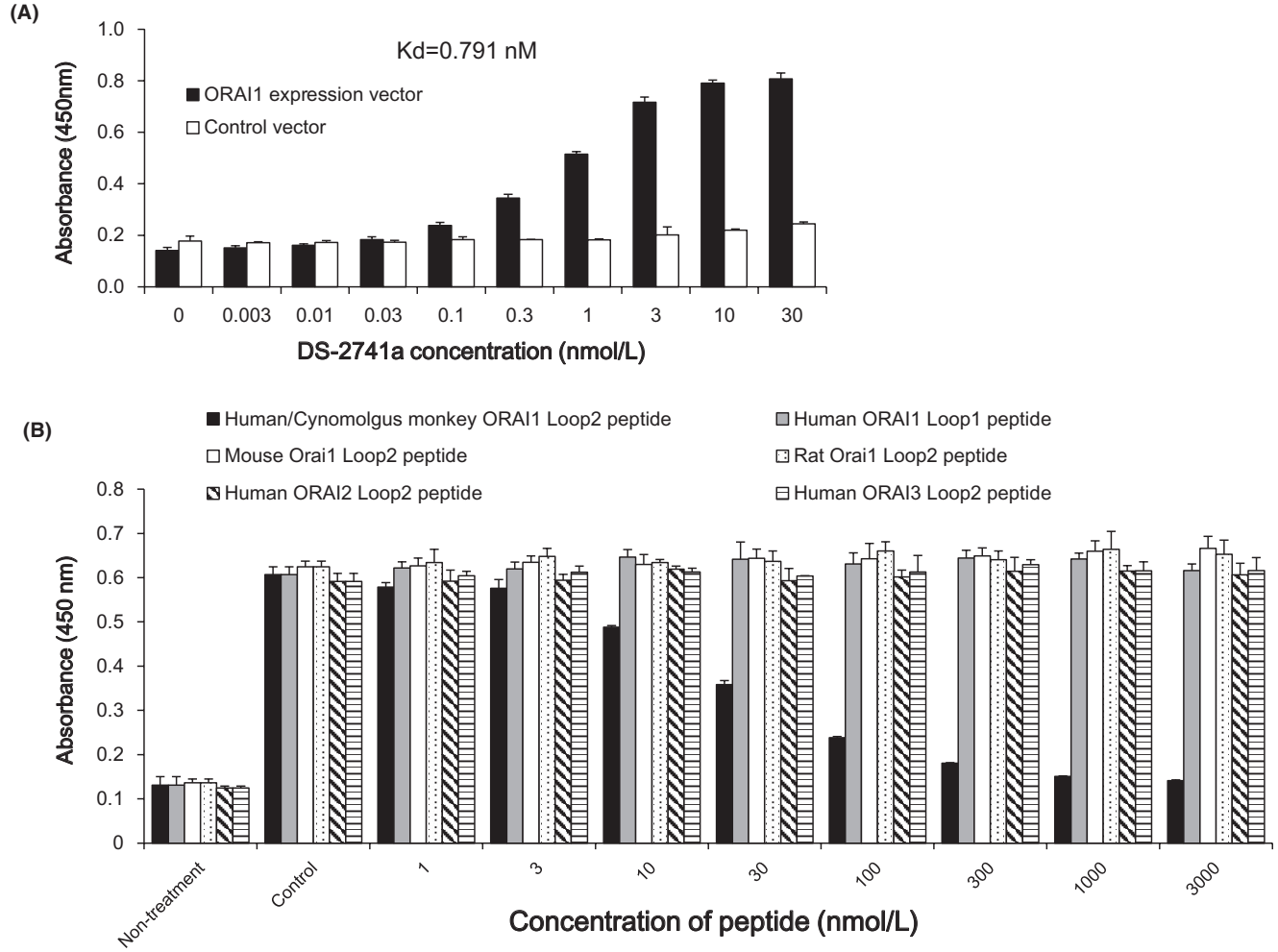

FIGURE 1 Binding specificity of an anti-ORAI1 antibody, DS-2741a. A, The humanized anti-human ORAI1 antibody, DS-2741a, bound to human ORAI1-transfected CHO-K1 cells, but not to control mock vector transfected cells. B, Competitive binding assays were performed to examine the binding epitope, specifically to ORAI1, and cross-reactivity with orthologs. DS-2741a binding to human ORAI1-expressing CHO-K1 cells competed with peptides. Graphs show the means and standard errors in triplicate

specifically binds to the extracellular loop2 domain of human and cynomolgus monkey ORAI1.

3.2 | DS-2741a inhibited Ca^{2+} influx and cytokine release

Since ORAI1 is a critical component of CRAC, and CRAC mediates TCR signaling,[13,24] functional impact of DS-2741a on store-operated Ca^{2+} influx and T-cell cytokine production was examined. After stimulation of a human T-cell line (Jurkat cells) with thapsigargin, Ca^{2+} entry was increased as determined by Fra2-AM, and the increase was inhibited by DS-2741a (Figure 2A). In addition, DS-2741a also inhibited PMA/Iono-induced IL-2 production (Figure 2B). We also confirmed an inhibitory effect of DS-2741a on IL-2 production in PMA/Iono- or CD3/28-stimulated human peripheral blood peripheral blood mononuclear cells (PBMCs) (Figure 2C and Figure S2). These results indicate that DS-2741a inhibits T-cell activation.

3.3 | Characterization of human ORAI1 knock-in mice

Since DS-2741a did not show cross-reactivity to rodent Orai1 (Figure 1B), we generated human ORAI1 knock-in mouse for in vivo evaluation of DS-2741a. To confirm the extracellular expression of human ORAI1, $CD3^+$ T cells from splenocytes of human ORAI1 knock-in mice were examined by flow cytometry. DS-2741a bound to $CD3^+$ T cells from the knock-in mice but not to cells from wild-type control mice (Figure 3A). DS-2741a also inhibited PMA/Iono-induced IL-2 production to a similar extent as in human T cells (Figure 2B, Figure 3B). These results indicate that the human ORAI1 knock-in mice could be useful to conduct further examination of DS-2741a in vivo.

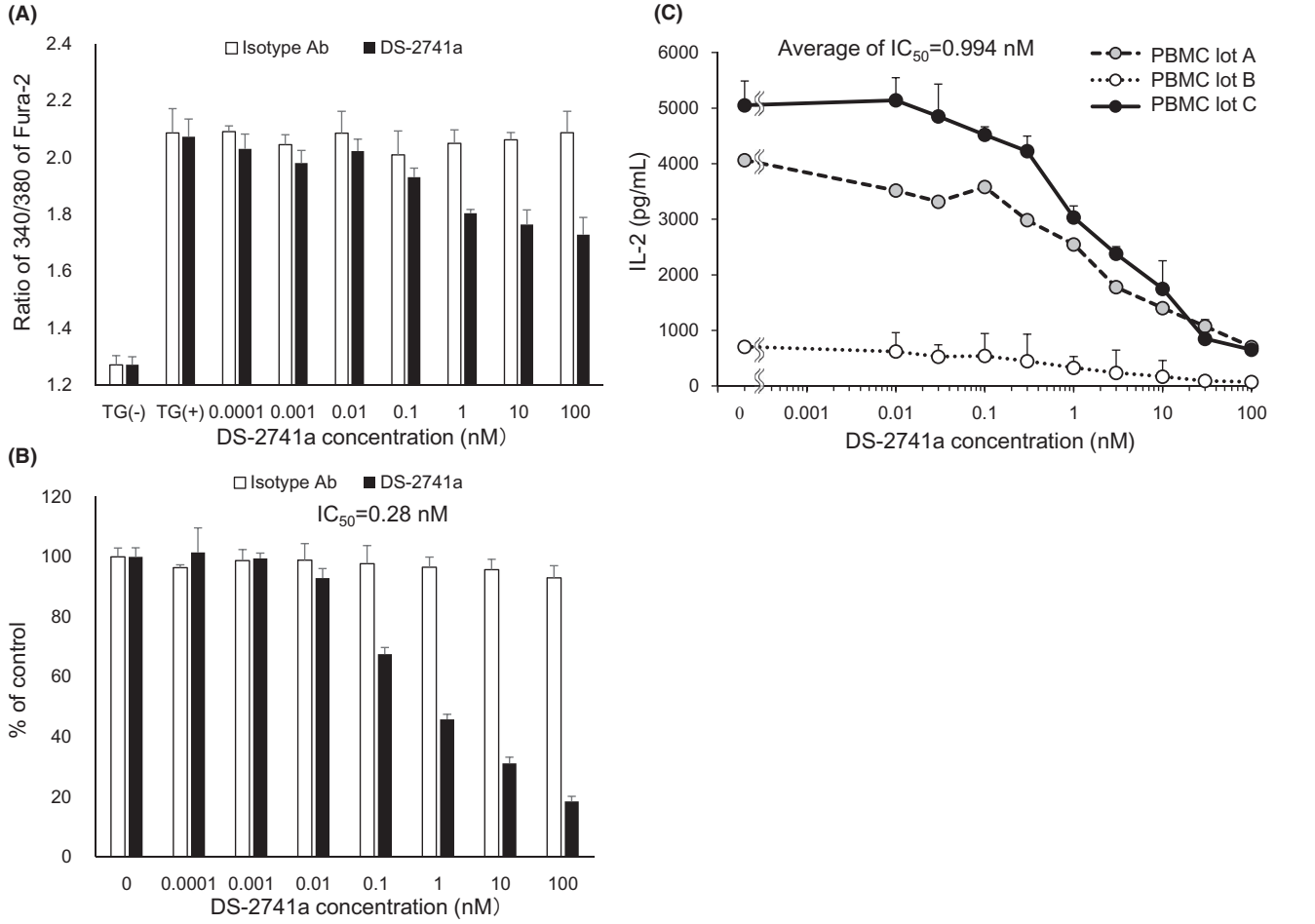

FIGURE 2 Inhibitory activity of DS-2741a anti-ORAI1 antibody against T-cell signaling. A, Jurkat cells were stimulated with PMA and A23187. Inhibitory effect of DS-2741a on Ca^{2+} influx was determined by Fura-2. B, Inhibitory effect of DS-2741a on IL-2 production was determined by ELISA. C, Effect of DS-2741a on three batches of human primary T cells stimulated with PMA and A23187. IL-2 levels were determined by ELISA. Open squares denote isotype control IgG and filled squares denote DS-2741a. Graphs show the means and standard errors in triplicate. IC_{50} was defined as the estimated concentration of DS-2741a at half the total Emin and Emax by the sigmoid Emax model

3.4 | Suppression of T-cell function in a mouse DTH model

Since DS-2741a suppressed Ca^{2+} influx and IL-2 production in T cells, we examined the efficacy of DS-2741a on DTH reaction in human ORAI1 knock-in mice. DS-2741a showed dose-dependent and continuous inhibition of DTH response as judged by footpad thickness (Figure 4A) and foot volume (Figure 4B), which suggested that DS-2741a had an inhibitory effect on T-cell activation in both in vitro (Figure 3B) and in vivo models.

3.5 | Inhibition of mast cell degranulation by anti-ORAI1 antibody

Since store-operated Ca^{2+} entry (SOCE) from CRAC is also crucial for mast cell function, we examined the effect of DS-2741a on mast cells. To illustrate the cutaneous mast cell response, we isolated connective tissue-type mast cells from bone marrow–derived mast cells (BMMCs) of human ORAI1 knock-in mice and confirmed that 93% of cells expressed the typical mast cell markers, c-kit$^+$ and FcεRI$^+$, after 8 weeks of culture (Figure 5A). DS-2741a showed inhibition of thapsigargin-induced mast cell degranulation compared to control IgG as judged by β-hexosaminidase release (Figure 5B).

3.6 | Suppression of mast cell function (PCA reaction) in vivo

We further investigated whether DS-2741a could inhibit in vivo mast cell degranulation (PCA reaction) in human ORAI1 knock-in mice. DS-2741a inhibited PCA reaction significantly (Figure 5C). Since PCA reaction reflects the IgE-mediated mast cell degranulation in vivo, these data

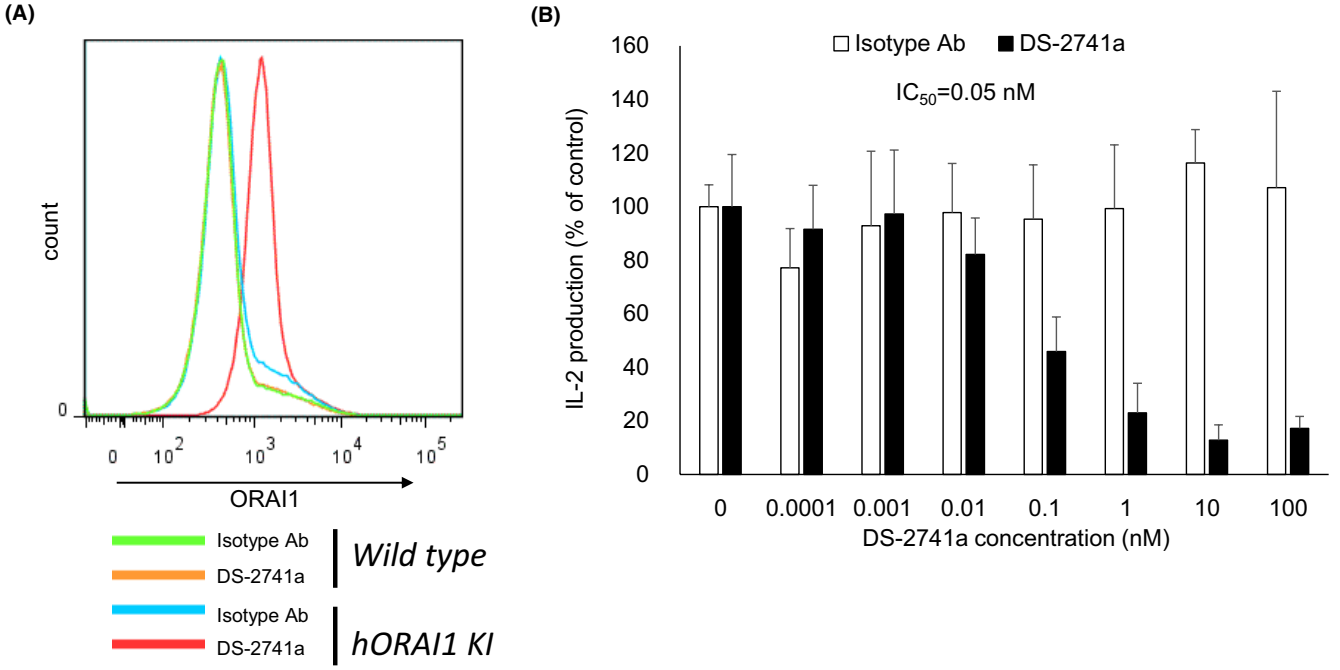

FIGURE 3 Inhibition of T cells from human ORAI1 knock-in mice by DS-2741a. A, $CD3^+$ T cells were isolated from the spleen of human ORAI1 knock-in mice, and the expression of human ORAI1 was examined by flow cytometry. B, Splenic $CD3^+$ T cells from the human ORAI1 knock-in mice were stimulated with PMA and A23187. The inhibitory effect of DS-2741a on IL-2 production was determined by ELISA. Graph shows the means and standard errors (n = 3). IC_{50} was defined as the estimated concentration of DS-2741a at half the total Emin and Emax by the sigmoid Emax model

support the substantial effect of DS-2741a in mast cell regulation under physiological conditions.

3.7 | Amelioration of dermatitis in a mouse model of mite antigen-induced atopic dermatitis

Finally, we investigated whether DS-2741a improved mite-induced chronic dermatitis which has been recognized as the most relevant model to study atopic dermatitis pathology.[25,26] Since dermatitis in C57/BL6 background mice is less severe than that in NC/Tnd mice, which are often used as an AD model, human ORAI1 knock-in mice were frequently treated with mite antigen and *Staphylococcal* enterotoxin B (SEB). Two weeks after initial mite antigen treatment, ear swelling to twice the size of that prior to the induction of dermatitis and extensive lesions of dermatitis on the dorsal skin of mice were observed. As a positive control, from week 2, the mice with dermatitis were administered cyclosporine A, a therapeutic agent for AD. Dermatitis was ameliorated in these mice (Figure 6), which suggests that this model was suitable to determine pharmacological action of agents against atopic dermatitis. Therapeutic administration of DS-2741a also significantly ameliorated dermatitis to a similar extent as cyclosporine A (Figure 6). Of note, DS-2741a inhibited ear swelling, even on the first day following its administration.

4 | DISCUSSION

DS-2741a demonstrated in vitro concentration-dependent suppression of IL-2 production in Jurkat cells and in primary human T cells (Figure 2A,B). DS-2741a specifically bound to human and cynomolgus monkey ORAI1 but not to its homolog proteins ORAI2 and ORAI3 or to its mouse and rat orthologs (Figure 1B). Although other anti-ORAI1 antibodies that do not elicit cross-reactivity with mice Orai1 have been reported previously, the effect of the antibody was studied in a T-cell-dependent antibody response monkey model. The antibodies also bind only to ORAI1 in humans and monkeys. The study conducted on monkeys aimed to compare its efficacy with that of FK-506; however, it focused solely on safety evaluation and was not a disease model in particular.[27] Hence, specific indications could not be discussed.

In the present study, the effects exerted by DS-2741a in vivo can be evaluated based on the inherent expression pattern of ORAI1 after knocking in the human ORAI1 in mice. We believe that multiple T-cell subsets, mast cells, and other cells contribute concomitantly to the development of dermatitis, and that the effects of targeting ORAI1 could be evaluated comprehensively. DS-2741a suppressed

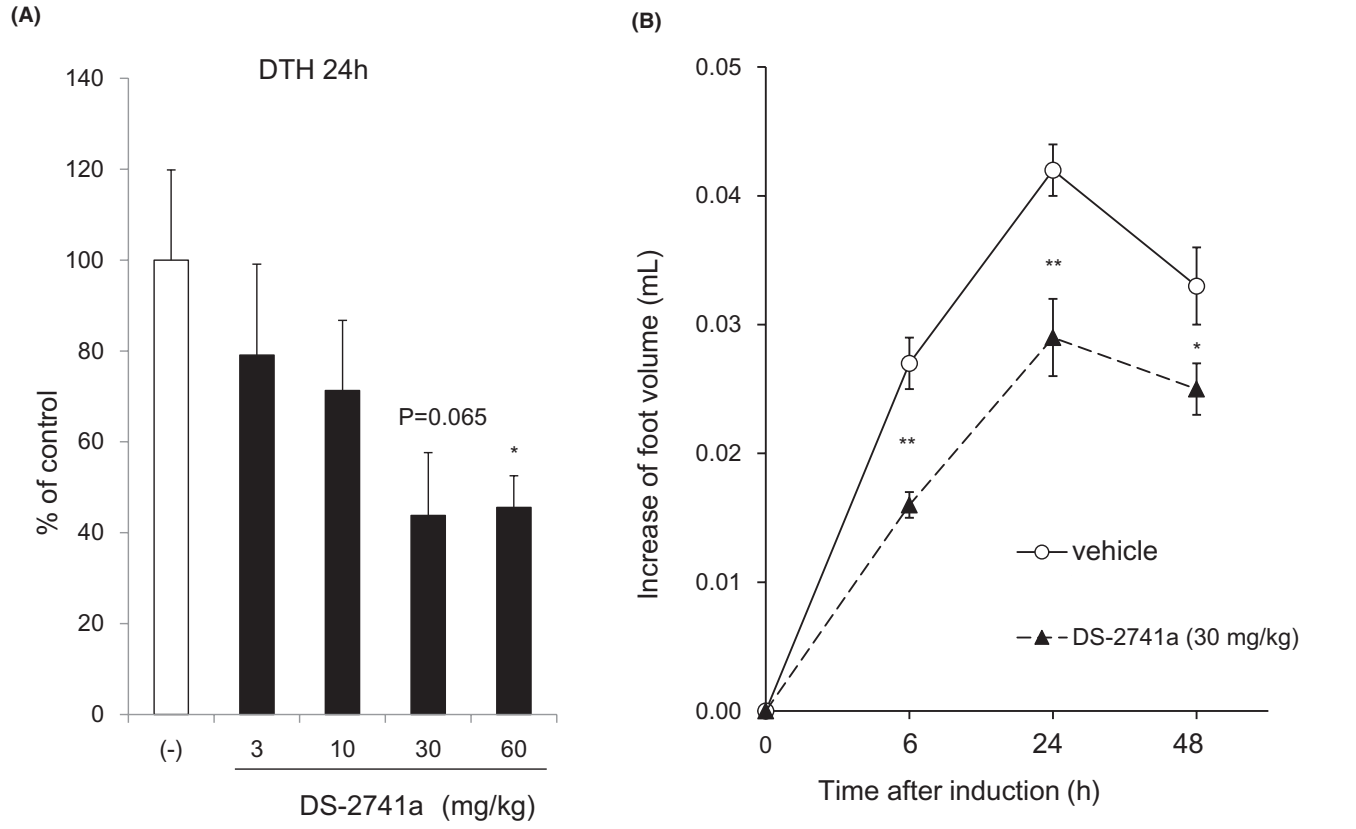

FIGURE 4 DS-2741a inhibited DTH response. Efficacy of DS-2741a on DTH reaction in human ORAI1 knock-in mice was examined. On the day before immunization (day-7) and on the day of induction (day 0), DS-2741a was administered to the mice (at days −8 and −1; 30 mg/kg, intravenously). A, Measurement of footpad thickness 24 h after DS-2741a administration (n = 5 or 6). B, Footpad volume measured at various time points (n = 10). Graph shows the means and standard errors. Student's *t* test was performed for comparison between control and DS-2741a. **P* < .05, ***P* < .01

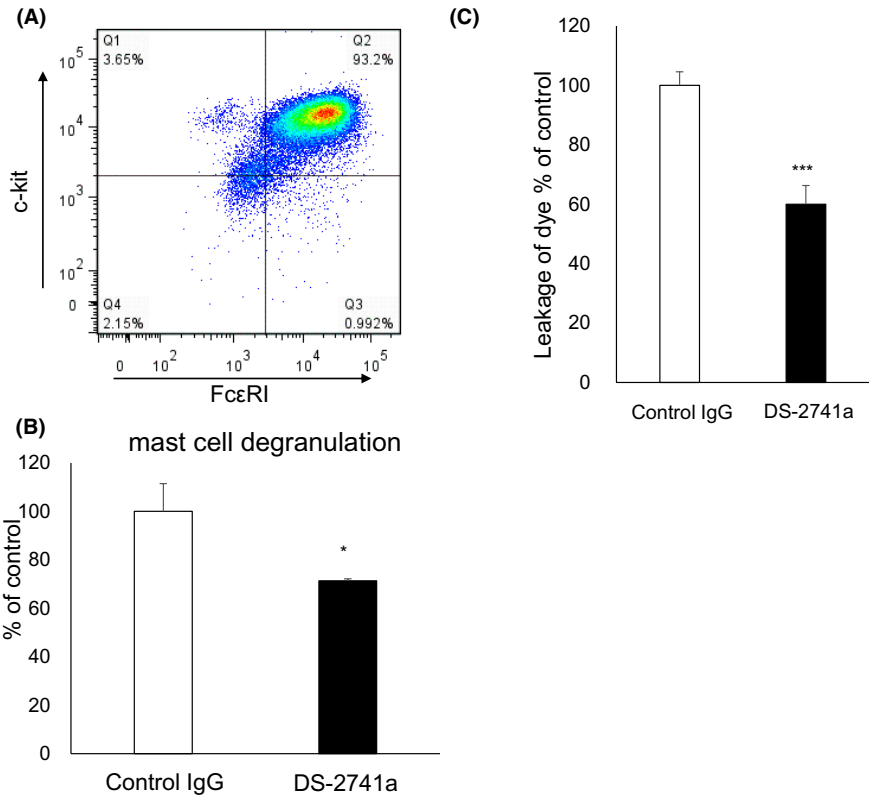

FIGURE 5 Inhibition of mast cell degranulation and PCA reaction by DS-2741a. A, Expression of mast cell markers, c-Kit and FcεRI. Representative data are shown. B, Effect of DS-2741a on thapsigargin-induced degranulation of mast cells in human ORAI1 knock-in mice was examined. The mast cells were treated with 60 nmol/L DS-2741a or control IgG_1 1 h before the thapsigargin treatment. Graph shows the means and standard deviation in triplicate. C, The PCA reaction was inhibited by DS-2741a in human ORAI1 knock-in mice. DS-2741a was administered to the mice on the day of IgE sensitization, at 60 mg/kg, intravenously (n = 4). Graph shows the means and standard errors. Student's *t* test was performed for comparison between control and DS-2741a. **P* < .05, ****P* < .001

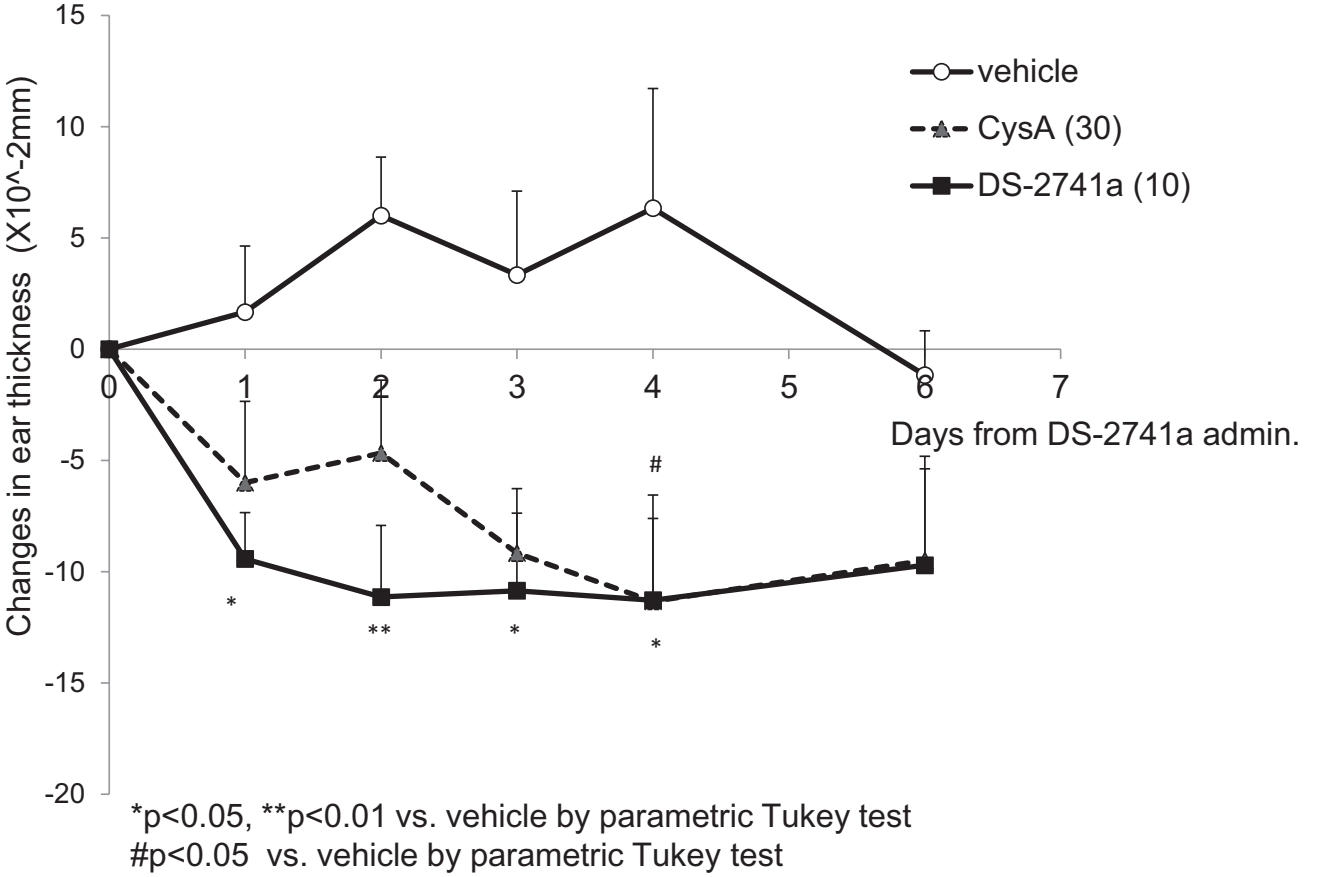

FIGURE 6 Amelioration of house dust mite-induced dermatitis by DS-2741a. Dermatitis was induced in human ORAI1 knock-in mice by repeated painting of house dust mite antigen on mouse skin. The change in ear thickness was determined. DS-2741a was administered once intravenously (10 mg/kg) and cyclosporine A was administered (30 mg/kg, p.o. once a day, 6 times) from day 0 (n = 7). Graph shows the means and standard errors. Parametric Tukey's test was performed for comparison between control and DS-2741a and between control and cyclosporine A. $*P < .05$, $**P < .01$, $^{\#}P < .05$

T-cell–mediated DTH reaction in ORAI1 knock-in mice (Figure 4A,B). DS-2741a also attenuated BMMC degranulation in vitro and the PCA reaction in vivo (Figure 5B,C). DS-2741a was effective in a mouse model of dermatitis induced by house dust mites (Figure 6). These results clearly indicate that ORAI1 is an important target for the treatment of diseases associated with T- and/or mast cell activation and that DS-2741a efficiently inhibits ORAI1, resulting in improved inflammatory responses in mouse models of DTH, PCA, and AD.

In TCR-induced NFAT activation in T cells, ORAI1, the critical component of CRAC, allows Ca^{2+} to enter into cells, inducing activation of the calcineurin-NFAT axis followed by subsequent cytokine production.[14] CRAC-induced T-cell activation was significantly suppressed by DS-2741a. In contrast to T cells, the ORAI1-CRAC signal contributes to mast cell degranulation through a pathway distinct from NFAT signaling.[28,29] In mast cells, CRAC mediates SOCE, which induces degranulation without gene transcription.[30] Consistent with previously published data,[16] DS-2741a inhibited mast cell degranulation. The multidimensional effect (T- and mast cell inhibition) of DS-2741a would provide potential efficacy in the treatment of allergic diseases. Therefore, we evaluated the effect of DS-2741a and cyclosporine A as a standard therapy for moderate-to-severe AD in a mouse model of dermatitis induced by mite antigens (Figure 6). Cyclosporine A substantially inhibited dermatitis, and DS-2741a also significantly inhibited dermatitis, even 1 day after administration. This suggests that DS-2741a shows similar or better efficacy than cyclosporine A. Cyclosporine A has been the standard choice for severe AD treatment.[31] Despite its efficacy, it must be administered cautiously owing to its adverse effects, such as cyclosporine nephropathy.[32] Cyclosporine A exhibits several drug-drug interactions that make it more difficult to maintain effective and safe blood concentrations.[33] ORAI1-deficient patients do not show obvious kidney defects, although ORAI1 regulates upstream of the calcineurin pathway.[14] Owing to its pharmacological action and efficacy, the anti-ORAI1 antibody DS-2741a is expected to be a new therapeutic choice for AD.

The Th2 cytokines IL-4 and IL-13 are recognized as key drivers of AD, especially in the acute phase.[24,34] Dupilumab, a blocking antibody for IL-4Ra that prevents IL-4/IL-13 signaling, has recently been approved for the treatment of AD and produces marked effects.[35,36] Although the importance of the IL-4/IL-13 cytokine pathway has been confirmed in humans, AD is heterogeneous and also involves other T-helper cell subsets, including Th17, Th22, and Th1 cytokine pathways.[37,38] Inhibition of pathway(s) other than Th2 cytokine signaling would provide improved treatment efficacy. As previously confirmed, ORAI1-regulated T-cell signaling is common across T-helper cell subsets.[39,40] DS-2741a was also shown to suppress the release of a wide range of cytokines that are triggered by SOCE in a variety of T-helper subsets except Treg (Table S1). Thus, DS-2741a is expected to provide a different outcome than dupilumab. The wide range of action of DS-2741a across T cells may raise concerns as to whether ORAI1 would suppress Treg function and thus promote unwanted immune activation. However, ORAI1 does not control Treg function.[41]

ORAI1-NFAT-calcium signaling enhances the secretion of thymic stromal lymphopoietin via PAR2 from keratinocytes, stimulating a subset of TRPA1-positive sensory neurons to trigger robust itching behavior.[42] Moreover, itching is caused by mast cells, which accumulate and are activated in the diseased area of AD.[43-45] Furthermore, ORAI1 inhibition attenuates mast cell activity (Figures 5and 6).[16] Hence, ORAI1 is likely involved in inflammation and itching. Further studies could elucidate whether DS-2741a affects cytokines and mediators that are associated with itching.

In summary, the anti-human ORAI1 antibody DS-2741a showed significant T- and mast cell inhibitory activity and attenuated dermatitis in mouse models. These results support further investigation of DS-2741a for the treatment of allergic diseases.

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AUTHOR CONTRIBUTIONS

A Aki and T Komai performed pharmacological experiments and analyzed the data; T Komai, K Tanaka, N Nagaoka, T Kimura, D Baba, and Y Onodera generated antibody against ORAI1; A Aki, T Komai, and T Wada drafted the manuscript. All authors corrected and approved the manuscript.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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