



Immunopharmacology and Inflammation

Novel CC chemokine receptor 4 antagonist RS-1154 inhibits ovalbumin-induced ear swelling in mice

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ABSTRACT

CC chemokine ligand 17 (CCL17/thymus and activation-regulated chemokine: TARC) and CCL22 (macrophage-derived chemokine: MDC) selectively bind to CC chemokine receptor 4 (CCR4). The CCR4 system is considered to be responsible for the pathology of allergic diseases such as atopic dermatitis. To find and develop potential medicines against allergic diseases, we screened an in-house library to search for compounds having a profile as a CCR4 antagonist. From among the screening hits, we focused on 3-[2-[(2R)-2-phenyl-4-(4-pyridin-4-ylbenzyl)morpholin-2-yl]ethyl]quinazoline-2,4(1H,3H)-dione (named RS-1154), which had been newly synthesized in our laboratory. This compound inhibited the binding of [¹²⁵I]CCL17 to human CCR4-expressing CHO cells with an IC₅₀ value of 27.7 nM and moreover inhibited CCL17-induced migration of DO11.10 mice-derived T helper 2 cells with an IC₅₀ value of 1.5 nM *in vitro*. We then examined the effect of RS-1154 in an ovalbumin-induced ear swelling assay. The ear thickness was decreased by intravenous administration of anti-CCL17 or anti-CCL22 antibodies, suggesting that the CCR4 system is involved in the ear swelling. Though partially, the oral administration of RS-1154 also significantly ameliorated the ear swelling at the doses of 30 and 100 mg/kg. Furthermore, the serum level of interleukin-4 decreased after the administration of RS-1154. In this study, we succeeded in obtaining a newly-synthesized compound, RS-1154, which has a potential to inhibit the chemotaxis of T helper 2 cells *in vitro* and to ameliorate ovalbumin-induced ear swelling *in vivo*. These results raise the possibility that RS-1154 or one of derivatives might become a therapeutic agent for atopic dermatitis patients.

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1. Introduction

Chemokines and their receptors elaborately regulate chemotactic migration of leukocytes by making and recognizing a chemoattractant gradient. Breakdown of the system leads to the occurrence and exaggeration of many allergic diseases such as atopic dermatitis, asthma, allergic rhinitis etc. (Luster, 2001). Therefore, chemokine antagonists are an attractive target to develop a medicine against the above diseases. Indeed, many experiments have demonstrated such possibility using anti-chemokine antibodies and chemokine receptor antagonists (Luster, 2001; Schwarz and Wells, 2002).

CC chemokine ligand 17 (CCL17/thymus and activation-regulated chemokine: TARC) and CCL22 (macrophage-derived chemokine:

MDC) are a pair of CC chemokines known to selectively bind to CC chemokine receptor 4 (CCR4) (Proudfoot, 2002). CCR4 is mainly, even if not selectively, expressed on T helper 2 (Th2) cells that produce interleukin (IL)-4, IL-5 and IL-10 (Bonecchi et al., 1998; Imai et al., 1999). These reports suggest that CCL17, CCL22 and CCR4 are involved in Th2-predominant allergic inflammation, whereas CCR4⁺ cells are also reported to produce interferon-γ (Andrew et al., 2001).

Atopic dermatitis is an inflammatory skin disease characterized by pruritic and eczematous lesions, and many reports support the hypothesis that a Th1/Th2 imbalance, at least in part, contributes to the occurrence. The imbalance prefers Th2 cells in the initial stage, followed by additional acquisition of Th1 cell phenotypes (Pastar et al., 2005). Clinical data demonstrate that CCL17, CCL22 and CCR4 are much involved in the pathogenesis of atopic dermatitis. CCL17 (Vestergaard et al., 2000) and CCL22 (Galli et al., 2000) are mainly produced by keratinocytes and dendritic cells, respectively, in atopic dermatitis patients, and serum levels of CCL17 and CCL22 reflect the disease severity (Horikawa et al., 2002). In the same way as that of ligands, CCR4 is expressed on keratinocytes (Fujimoto et al., 2008)

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and its expression on peripheral blood CD4⁺ cells is also strongly correlated with the severity of atopic dermatitis (Vestergaard et al., 2003; Wakunaga et al., 2001). Furthermore, skin-homing cutaneous lymphocyte-associated antigen (CLA⁺) lymphocytes overexpress CCR4 (Campbell et al., 1999; Vestergaard et al., 2004). A plausible function of CLA and CCR4 is that they are utilized to synergistically recruit Th2 cells, subsequently exaggerating inflammation at the skin. Tsunemi et al. (2006) generated transgenic mice which overexpress CCL17 in keratinocytes and Th2-type responses to allergic stimuli were exaggerated in the transgenic mice. These series of data strongly indicate that CCR4 antagonists are a promising medicine to cure atopic dermatitis (Hwang, 2001; Wakunaga et al., 2002). Therefore, much effort has been made to develop as a CCR4 antagonist as a therapeutic agent; however, no medicines have succeeded in clinical trials so far.

In this study, we screened an in-house library utilizing a binding assay to search for compounds having a profile as a CCR4 antagonist. From among the screening hits, we focused on 3-{2-[(2*R*)-2-phenyl-4-(4-pyridin-4-ylbenzyl)morpholin-2-yl]ethyl}quinazoline-2,4 (1*H*,3*H*)-dione (named RS-1154, Fig. 1A) and further investigated its pharmacological profile using the following two experimental assays: a chemotaxis assay of Th2 cells *in vitro* and an ovalbumin-induced ear swelling assay *in vivo*. The latter is an easy assay for evaluating atopic dermatitis-like allergic response by measuring the thickness of the ears (Gonda et al., 2005; Togawa et al., 2001).

2. Materials and Methods

2.1. Animals

Five or six-week-old female BALB/c mice and DO11.10 mice were purchased from Charles River Japan, Inc. (Yokohama, Japan) and CLEA Japan, Inc. (Tokyo, Japan), respectively, and housed under specific pathogen-free conditions. The mice were acclimatized for at least 7 days before the start of experiments in a breeding room. In the room, the environmental conditions were maintained with a temperature range from 21°C to 23°C, and artificial illumination from 7:00 to 19:00 throughout the experimental period. The mice were fed an FR-2 solid diet (Funabashi Farm Co., Funabashi, Japan) and allowed access to tap water *ad libitum*. All experiments were performed according to the Guidelines for the Care and Use of Laboratory Animals of Daiichi Sankyo Co., Ltd.

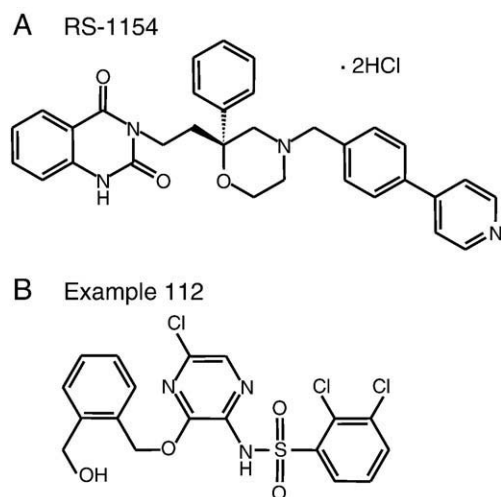


Fig. 1. Chemical structures of RS-1154 and Example 112. RS-1154: C₃₂H₃₀N₄O₃ · 2HCl, molecular weight 591.53; Example 112: C₁₈H₁₄Cl₃N₃O₄S, molecular weight 474.75.

2.2. Reagents

RS-1154 and 2,3-dichloro-*N*-[5-chloro-3-(2-hydroxymethylphenylmethoxy)-2-pyrazinyl]benzenesulfonamide (named Example 112, Fig. 1B) were synthesized in our laboratory, and each diluted in a solution consisting of 70% dimethyl sulfoxide and 30% methanol at a concentration of 5 mM in *in vitro* experiments. In *in vivo* experiments, RS-1154 was suspended in saline containing 0.5% methyl cellulose #400 (Nacalai Tesque, Inc., Kyoto, Japan). Gqi5α-coupled CHO cells were obtained from Molecular Devices Corp. (Sunnyvale, CA, USA) and [¹²⁵I]human CCL17 was from Amersham Place Ltd. (Buckinghamshire, UK). CD4 microbeads (clone L3T4) were purchased from Miltenyi Biotec GmbH (Bergisch Gladbach, Germany). Anti-mouse CD28 antibody (clone 37.51), anti-mouse CD3 antibody (clone 145-2C11), Cytofix/Cytoperm solution, Perm/Wash solution and mouse CCL1 (T cell activation gene 3: TCA-3) were from BD Biosciences (San Jose, CA, USA). Mouse IL-4, mouse IL-12, mouse CCL17, mouse CCL22, anti-mouse IL-4 antibody, anti-mouse IL-12 antibody, anti-mouse CCL17 antibody (clone 110946), anti-mouse CCL22 antibody, IgG2A isotype control (clone 54447) and an IL-4 ELISA kit were from R&D Systems Inc. (Minneapolis, MN, USA). Anti-mouse CCR4 antibody was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Phycoerythrin-conjugated anti-goat IgG, prostaglandin D₂ (PGD₂) and ovalbumin were from Sigma Chemical Co. (St. Louis, MO, USA). Alum was from LSL Co., Ltd. (Tokyo, Japan).

2.3. Production of CCR4-expressing CHO cells

Human CCR4 was cloned from a thymus cDNA library (Marathon-Ready cDNA Human Thymus, Clontech Laboratories, Inc., Mountain View, CA, USA) by polymerase chain reaction and the primers for human CCR4 cDNA were designed based on information from GeneBank (accession number NM_005508). The human CCR4 cDNA was cloned into a pCneo vector (Promega Corp., Madison, WI, USA). The CHO cells constitutively expressing the chimeric Gqi5α subunit protein were maintained in Ham's F12 medium containing 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin, 100 µg/ml streptomycin and 200 µg/ml hygromycin B in a humidified atmosphere (5% CO₂-95% room air) at 37 °C. The cells were transfected with the plasmid pCneo carrying human CCR4 cDNA using FuGENE 6 transfection reagent (F. Hoffmann-La Roche Ltd, Basel, Switzerland). Stably transfected CHO cells were selected with 400 µg/ml Geneticin (G418, Invitrogen Corp., Carlsbad, CA, USA) and evaluated for receptor expression by a ligand binding assay.

2.4. Binding assay

CHO cells stably expressing CCR4 were plated at a density of 40,000 cells per well on 96-well plates and cultured in a humidified atmosphere (5% CO₂-95% room air) at 37 °C. One day after plating, the culture was washed using Ham's F12 medium three times. The medium was changed to binding buffer (final concentrations: 0.15 nM [¹²⁵I]CCL17, various concentrations of reagents, 20 mM HEPES [pH 7.6], 5 mM MgCl₂, 0.1% NaN₃ and 0.5% bovine serum albumin), and then the culture was incubated at room temperature for 90 min. The reaction was terminated by washing (washing buffer: 25 mM HEPES [pH 7.6], 5 mM MgCl₂, 1 mM CaCl₂, 0.1% NaN₃, 0.5% bovine serum albumin and 0.5 M NaCl) and homogenates were collected into vials by adding 150 µl of 1 N NaOH. The radioactivity counts in the vials were measured using a γ counter (COBRA II, Hewlett-Packard, Palo Alto, CA, USA), and non-specific binding was determined using Gqi5α-coupled CHO cells and was subtracted from the total binding.

2.5. Preparation of Th2 and Th1 cells

Th2 cells were generated according the methods described in Dzialo-Hatton et al. (2001) and Wenner et al. (1997) with some

modifications. Single-cell suspensions of DO11.10 spleen cells were prepared in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum. CD4⁺ T cells were isolated from the suspensions by positive sorting (autoMACS, Miltenyi Biotec GmbH) with anti-CD4 micro beads. The purity of the sorted cells was constantly more than 95%. The cells were plated with 2.5 µg/ml anti-CD3 antibody, 1 µg/ml anti-CD28 antibody, 50 ng/ml IL-4 and 0.5 µg/ml anti-IL-12 antibody in RPMI 1640 medium containing antibiotics at a density of 4×10^6 cells per well on 24-well plates, and cultured in a humidified atmosphere (5% CO₂-95% room air) at 37 °C. When generating Th1 cells, 2.5 µg/ml anti-CD3 antibody, 1 µg/ml anti-CD28 antibody, 5 µg/ml anti-IL-4 antibody and 40 ng/ml IL-12 were added. Three days after plating, the culture medium was changed to the same medium with stimulants, and then the cells were cultured for 4 more days and used for experiments.

2.6. Flow cytometry

Th1 and Th2 cells were suspended in staining buffer containing 1% fetal bovine serum and 0.05% NaN₃ in phosphate-buffered saline. Cells were washed with the staining buffer two more times, fixed with Cytofix/Cytoperm solution for 20 min, washed with Perm/Wash solution and incubated with 5 µg/ml phycoerythrin-labeled anti-mouse CCR4 antibody for 20 min. After washing with Perm/Wash solution, cells were stained by phycoerythrin-conjugated anti-goat IgG antibody (1:100 dilution) for 30 min, washed by Perm/Wash solution two times and suspended in 2% paraformaldehyde in phosphate-buffered saline. The above procedures were conducted on ice and flow cytometry analyses were performed by FACSCalibur (BD Biosciences).

2.7. Chemotaxis assay

A chemotaxis assay using 24-well transwell plates (polycarbonate membrane transwell inserts, Corning Inc., Corning, NY, USA) was performed. Th2 cells were collected from the plates, washed with RPMI 1640 medium containing 10% fetal bovine serum, and then loaded into the upper chamber at a density of 500,000 cells per well. Beneath a 5 µm polycarbonate filter, CCL17 diluted at the concentration of 300 ng/ml in the same medium was loaded into the lower chamber with or without various concentrations of compounds. Ninety minutes after incubation at 37 °C, the migrated cells in the lower chamber were collected and counted.

2.8. Ovalbumin-induced ear swelling assay

To generate ovalbumin-induced ear swelling, mice were intraperitoneally sensitized with 500 µl immunizing solution on Days 0 and 14. The immunizing solution consisted of 10 µg of ovalbumin and 4 mg of alum in saline. After being housed for another 7 days, the right ears of the sensitized mice were subcutaneously challenged with 10 µl ovalbumin solution using a 31 gauge needle after light anesthesia with ether on Day 21, and the left ears were left untreated. The solution contained 1 µg of ovalbumin in 10 µl of saline. To investigate the effect of reagents, RS-1154 was orally administered a total of two times, once each on Days 14 and 21, 30 min before immunization and challenge, respectively. RS-1154 was suspended in 0.5% methyl cellulose solution and administered at the dose of 10, 30 or 100 mg/kg. At the same timing, anti-CCL17 antibody or anti-CCL22 antibody in saline was intravenously administered at the dose of 20 µg per animal. In some groups, only saline, control antibody or 0.5% methyl cellulose solution was administered to mice instead of administration of reagents and/or challenge with ovalbumin. Right ear thickness was measured using a dial thickness gauge (cat. no. G-1A, Ozaki MFG. Co., Tokyo, Japan) in mm to two decimal places.

2.9. IL-4 ELISA

In an ovalbumin-induced ear swelling assay, blood was collected 24 h after the subcutaneous challenge with ovalbumin and centrifuged to obtain serum at approximately 2,000×g. The concentration of IL-4 in serum was measured according to the manufacturer's instructions.

2.10. Statistical analysis

All data in this study are expressed as means ± S.E.M. Significant differences of data were calculated by Tukey's test after analysis of variance. Probability values of $P < 0.05$ were considered to represent significant differences. Values of IC₅₀ were calculated by the program Prism (GraphPad Software, Inc., San Diego, CA, USA).

3. Results

3.1. Inhibition of [¹²⁵I]CCL17 binding to human CCR4

When screening for CCR4 antagonists, there were no commercially available CCR4 antagonists. Therefore, we searched patents and selected Example 112 as a reference CCR4 antagonist, patented by AstraZeneca UK Ltd. Example 112 was reported to inhibit fluorescence-labeled CCL22 binding to human CCR4-expressing CHO cells with an IC₅₀ value of 0.32 nM (Baxter et al., 2003). In our binding assay system, Example 112 also showed competitive displacement activity during the binding of [¹²⁵I]CCL17, although the IC₅₀ value was determined as 138.3 nM (Fig. 2). Compared to Example 112, we found that RS-1154 more strongly displaced the binding of [¹²⁵I]CCL17 to human CCR4-expressing CHO cells with an IC₅₀ value of 27.7 nM. To check the selectivity, we also investigated competitive displacement of RS-1154 in binding of [¹²⁵I]CCL3 (macrophage inflammatory protein-1α: MIP-1α) to CCR1, [¹²⁵I]CCL2 (monocyte chemoattractant protein-1: MCP-1) to CCR2b and so on; however, RS-1154 did not displace the binding of these ligands even at the concentration of 40 µM (data not shown).

3.2. Inhibition of CCL17-induced migration of Th2 cells

Before conducting an ovalbumin-induced ear swelling assay in mice, we checked the validity using DO11.10 spleen cell-derived Th2 cells. A chemotaxis assay was performed to investigate the functional inhibitory effect of RS-1154 against mouse CCR4. Expression of CCR4 on the Th2 cells was confirmed by flow cytometry (Fig. 3A). Under the condition of the presence of 300 ng/ml CCL17, the ratio of migrated Th2 cells significantly increased, suggesting that the Th2 cells functionally express CCR4 (Fig. 3B). The effect of RS-1154 was investigated in the range of 0.23–300 nM and RS-1154 dose-dependently inhibited CCL17-induced migration of Th2 cells with an IC₅₀ value of 1.5 nM. To check the

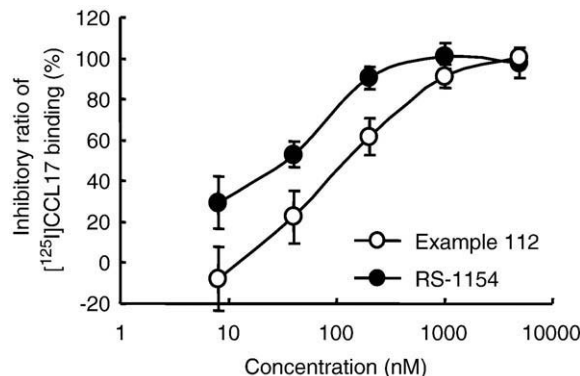


Fig. 2. Inhibitory effect of RS-1154 on the binding of [¹²⁵I]CCL17. Example 112 (open circles, IC₅₀ = 138.3 nM), RS-1154 (closed circles, IC₅₀ = 27.7 nM), n = 4.

selectivity for CCR4, we also investigated CCL1- and PGD₂-induced migration of Th2 cells, because their receptors, CCR8 and chemoattractant receptor-homologous molecule expressed on Th2 cells (CRTH2), were also reported to be expressed on Th2 cells (Romagnani, 2002). In the presence of 600 ng/ml CCL1, significant increase in migrated cells was observed, whereas the increase was smaller compared to CCL17 (Fig. 3C). Although 1000 nM RS-1154 showed a tendency to inhibit the chemotaxis, it did not reach statistical significance. In the case of PGD₂, significant increase was not observed even at the concentration of 5000 nM. We finally investigated the inhibitory effect of RS-1154 against 300 ng/ml CCL3 and 300 ng/ml CXCL10-induced chemotaxis using DO11.10 spleen cell-derived Th1 cells; however, no inhibitory effect was observed in the presence of RS-1154 even at the concentration of 10 μ M (Fig. 3D).

3.3. Inhibition of ovalbumin-induced ear swelling

The administration of 1 μ g of ovalbumin to ovalbumin-sensitized mice elicited bi-phasic reactions (Fig. 4A, closed circles), and the ear thickness was similar to that observed in our previous report (Sato et al., 2003). Ear thickness in the early phase peaked at 30 min after challenge with ovalbumin and in the late phase peaked at 3 h after the challenge. To clarify whether the CCR4 system contributed to the swelling, we examined the effect of anti-CCL17 and anti-CCL22 antibodies. Administration of 20 μ g per animal of a control antibody did not show any effect on the ear swelling; however, a 20 μ g per animal of an anti-CCL17 antibody inhibited the ear swelling in both phases (Fig. 4A, closed triangles; Fig. 4B). An anti-CCL22 antibody also decreased ear swelling, although the inhibitory effect was inferior to that of an anti-CCL17 antibody. These results verify that this ovalbumin-induced ear swelling is partially dependent on the CCR4 system and suitable for screening for CCR4 antagonists. We next investigated the effect of RS-1154 on ovalbumin-induced ear swelling in the range of 10–100 mg/kg. The ear thickness in the RS-1154-orally

administered groups also showed a similar pattern to that observed in the anti-CCL17 and anti-CCL22 antibody-administered groups (Fig. 5A). The values at 3 h after the challenge in the RS-1154 30 and 100 mg/kg-administered groups were significantly lower than that of the ovalbumin-challenged group (Fig. 5B).

3.4. Inhibition of serum level of IL-4

To further elucidate the roles of Th2 cells and their cytokines, we investigated the serum level of IL-4. Sensitization and challenge with ovalbumin significantly increased IL-4 (Fig. 6). Administration of an anti-CCL17 antibody and RS-1154 decreased the increase in IL-4 with statistical significance. We also investigated the serum levels of IL-5, IL-13 and CCL17, but these levels did not significantly change after sensitization and challenge with ovalbumin (data not shown).

4. Discussion

In this study, we succeeded in obtaining RS-1154, a newly-synthesized CCR4 antagonist, and demonstrated that RS-1154 inhibits binding of [¹²⁵I]CCL17 on human CCR4-expressing CHO cells, suppresses chemotaxis of Th2 cells and orally ameliorates ovalbumin-induced ear swelling in mice. Although the potency *in vivo* was not superior to an anti-CCL17 antibody, the observed effect of RS-1154 raise the possibility that RS-1154 or one of its derivatives have a potential as a therapeutic agent for allergic diseases such as atopic dermatitis. Loss of species cross-reactivity becomes the main problem in developing chemokine antagonists (Proudfoot, 2002), but RS-1154 has a potential to antagonize mouse CCR4 as human CCR4. This point would give an advantage to RS-1154 to prove its efficacy in animals and to be developed in the future.

Intravenous administration of anti-CCL17 and anti-CCL22 antibodies ameliorated ear swelling, suggesting that the CCR4 system is involved in the ear swelling. Oral administration of RS-1154 also

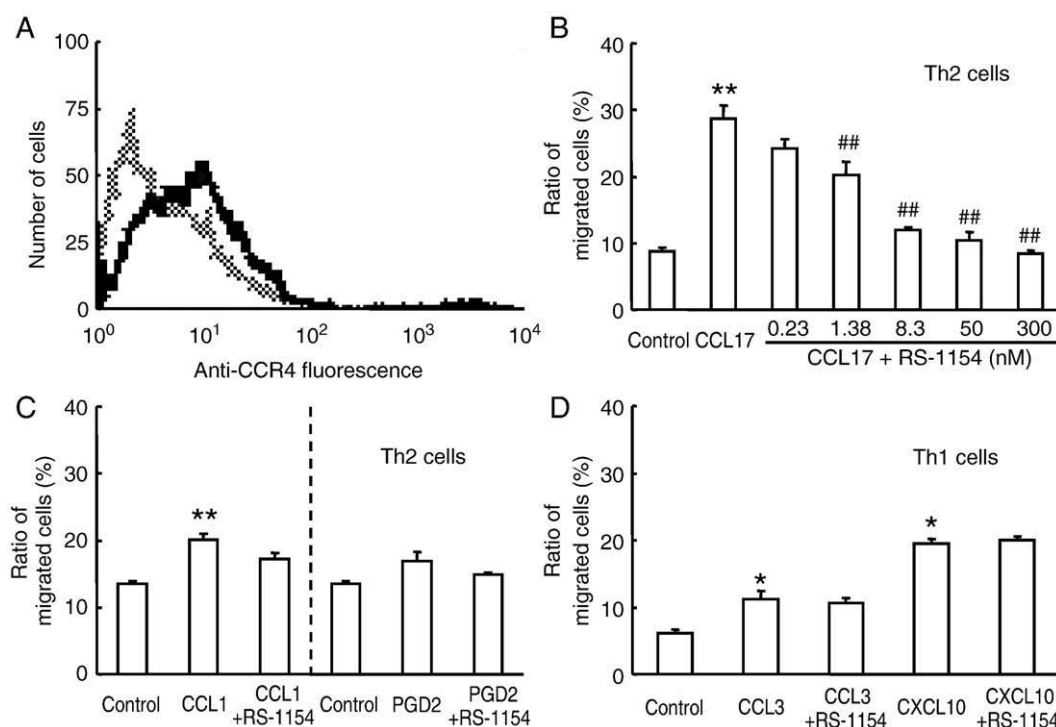


Fig. 3. Selective inhibitory effect of RS-1154 on CCL17-induced chemotaxis of Th2 cells. (A) Expression of CCR4 on Th2 cells. Dotted and solid lines indicate Th1 and Th2 cells, respectively. (B) Inhibitory effect of RS-1154 on CCL17-induced chemotaxis of Th2 cells. The IC₅₀ value was 1.5 nM. CCL17 was added at the concentration of 300 ng/ml. (C) No effect of RS-1154 on CCL1-induced chemotaxis of Th2 cells. CCL1 and PGD₂ were added at the concentrations of 600 ng/ml and 5000 nM, respectively. (D) No effect of RS-1154 on CCL3- or CXCL10-induced chemotaxis of Th1 cells. CCL3 and CXCL10 were added at the concentrations of 300 ng/ml. The concentration of RS-1154 was 10 μ M. **P* < 0.05 vs. control, ***P* < 0.01 vs. control, ###*P* < 0.01 vs. CCL17, *n* = 3.

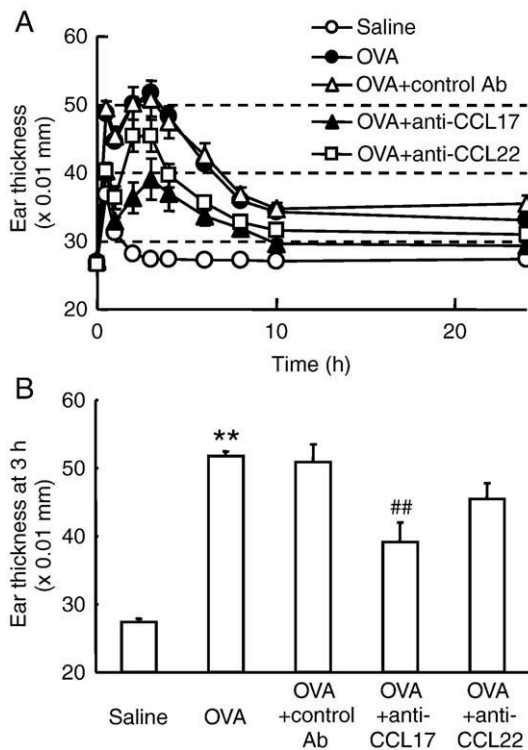


Fig. 4. Inhibitory effect of anti-CCL17 and anti-CCL22 antibodies on ovalbumin-induced ear swelling. (A) Time course of ear thickness after ovalbumin injection. Saline-challenged group (open circles), ovalbumin-challenged group (closed circles), control antibody-administered ovalbumin-challenged group (open triangles, 20 μ g per animal), anti-CCL17 antibody-administered ovalbumin-challenged group (closed triangles, 20 μ g per animal) and anti-CCL22 antibody-administered ovalbumin-challenged group (open squares, 20 μ g per animal). Saline was intravenously administered to mice in the former two groups. The mice in the saline group were sensitized with ovalbumin but challenged with saline. (B) Ear thickness 3 h after ovalbumin injection. OVA: ovalbumin, Ab: antibody, $P < 0.01$ vs. saline, $^{##}P < 0.01$ vs. ovalbumin, $n = 5-17$ from 3 independent experiments.

significantly inhibited the ear swelling and decreased IL-4 production. This result is consistent with that of a previous study in which it was observed that an IL-4 antibody significantly decreased IL-4 production and the ear thickness in an ovalbumin-induced ear swelling assay (Togawa et al., 2001). Considering the function of CCR4, administration of the antibodies and RS-1154 would hamper CCL17- and CCL22-dependent recruitment of CCR4⁺ Th2 cells into the ovalbumin-challenged ears. Clusters of CCR4⁺ Th2 cells and dendritic cells might be also downregulated in the skin and secondary lymphoid tissues (Katou et al., 2001). In addition, significant increase in CCL17 in serum was not observed after injection challenge with ovalbumin in our study; however, it is likely that focal small increase would occur in the ear. This point should be further investigated in detail by other approaches such as histochemical and gene expression analysis.

We focused on atopic dermatitis and utilized an ovalbumin-induced ear swelling assay to investigate the effect of RS-1154. In the assay, the ear swelling shows typical bi-phasic reactions, namely, the early phase reaction and the late phase reaction. It is considered that the early phase reaction is mainly triggered by chemical mediators released from mast cells and basophils after allergen-specific IgE cross-linking, and the late phase reaction is induced by the local infiltration of inflammatory cells such as T cells, eosinophils and monocytes (Gaga et al., 1991). Although the precise mechanism of the late phase reaction is not clear yet, the ear swelling in the late phase reaction would have a close connection with the condition in atopic dermatitis patients. Plausible atopic dermatitis-inducing factors such as CD4⁺ T cells, IgE antibodies, eosinophils, (Sawada et al., 1997), IL-4, mast cells (Togawa et al., 2001) and CD44 (Gonda et al., 2005) have been shown to be involved in the ear swelling in ovalbumin-challenged mice. As in

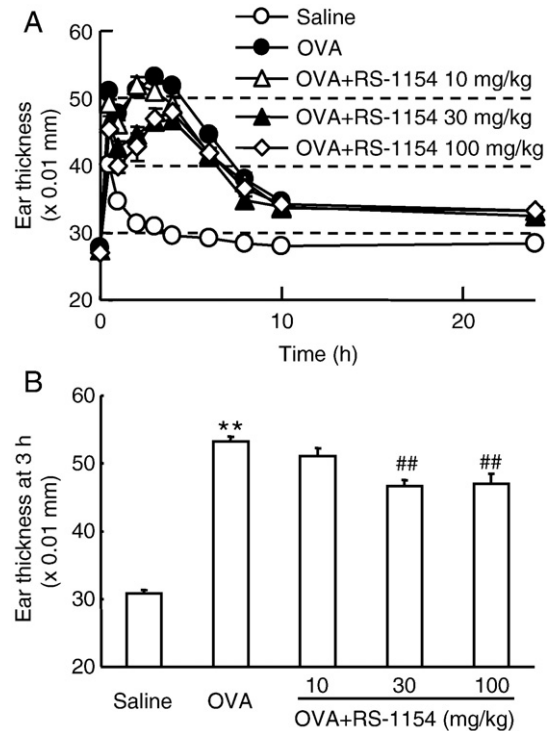


Fig. 5. Inhibitory effect of RS-1154 on ovalbumin-induced ear swelling. (A) Time course of ear thickness after ovalbumin injection. Saline-challenged group (open circles), ovalbumin-challenged group (closed circles), 10 mg/kg RS-1154-administered ovalbumin-challenged group (open triangles), 30 mg/kg RS-1154-administered ovalbumin-challenged group (closed triangles) and 100 mg/kg RS-1154-administered ovalbumin-challenged group (open diamonds). (B) Ear thickness 3 h after ovalbumin injection. OVA: ovalbumin, $^{**}P < 0.01$ vs. saline, $^{##}P < 0.01$ vs. ovalbumin, $n = 5-16$ from 3 independent experiments.

the case of injection of ovalbumin into the ear in the above reports, patch sensitization of ovalbumin also showed atopic dermatitis-like dermatitis characterized by infiltration of T cells, eosinophils and neutrophils (Spergel et al., 1998). On the other hand, Togawa et al. (2001) proved that intravenous administration of an anti-IL-5 antibody suppressed eosinophil accumulation, but did not inhibit the ear swelling. Furthermore, the ear swelling lasts for only several hours, contrary to chronic inflammation in atopic dermatitis. Taken together, measuring the ear thickness in an ovalbumin-induced ear swelling assay seems to be both easy and suitable for screening for medicines against atopic dermatitis; however, it would not be a sufficient level as an atopic dermatitis model. Although many other systems such as

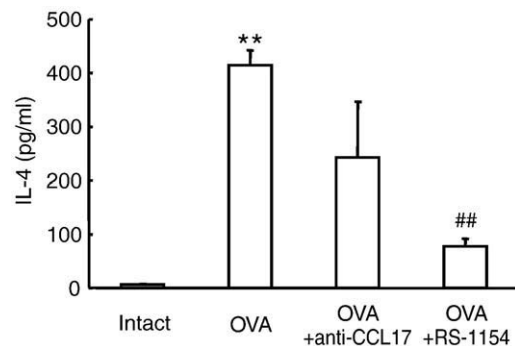


Fig. 6. Inhibitory effect of RS-1154 on ovalbumin-induced IL-4 production. Mice in the intact group were neither sensitized nor challenged with ovalbumin. An anti-CCL17 antibody was intravenously administered at the dose of 20 μ g per animal and RS-1154 was orally administered at the dose of 30 mg/kg. OVA: ovalbumin, $^{**}P < 0.01$ vs. intact, $^{##}P < 0.01$ vs. ovalbumin, $n = 4-5$.

NC/Nga mice (Matusda et al., 1997), NOA mice (Natori et al., 1999), IL-4 transgenic mice (Chan et al., 2001), caspase-1 transgenic mice (Yamanaka et al., 2000) and human skin-transplanted model (Carballido et al., 2003) have been reported, further easy-handling advanced animal models are indispensable for the development of new anti-atopic dermatitis medicines efficiently.

The balance of the chemokine system is orchestrally regulated by approximately 50 chemokine ligands and 20 chemokine receptors (Proudfoot, 2002). CCR4 is mainly considered responsible for excessive recruitment of Th2 cells, but this concept has become more complicated by the finding that CD4⁺CD25⁺ regulatory T cells also strongly express CCR4 (Iellem et al., 2001). One of our authors also showed the pure CD4⁺CD25^{high}Foxp3⁺ regulatory T cells circulating in peripheral blood express uniformly high levels of CCR4 (Hirahara et al., 2006). The plausible functions of these cells are attenuating activated T cells and suppressing antigen-presenting cells at inflammatory sites, consequently terminating inflammatory responses. In some cases, the functions might lead to inhibiting recruitment of CCR4⁺ Th2 cells. Further work is necessary to determine whether such an offsetting mechanism occurs and whether other chemokine systems are involved in the migration of CCR4⁺ Th2 cells (Lee et al., 2005; Aheren et al., 2009).

Asthma is also one of the target diseases for a CCR4 antagonist (Lloyd and Rankin, 2003). Both anti-CCL17 (Kawasaki et al., 2001) and anti-CCL22 (Gonzalo et al., 1999) antibodies, and CCR4-deficient antigen-specific Th2 cells (Luster, 2001) ameliorated ovalbumin-induced pulmonary inflammation and airway hyperreactivity in mice. A chemical CCR4 antagonist, compound 8c, was reported to be effective against ovalbumin-induced airway inflammation in mice, although the administration is a subcutaneous route (Purandare et al., 2006). Contrary to the findings in these reports, CCR4-deficient mice did not show the phenotypes observed when using antibodies or a compound (Chvatchko et al., 2000). Because of the diversity of chemokines and their receptors, long-term depletion of CCR4 might be compensated for by other systems. The same group also reported that airway hyperresponsiveness was attenuated when using *Aspergillus* antigens instead of ovalbumin (Schuh et al., 2002). Whether this discrepancy is caused by the system studied and/or antigens to validate proof-of-concept of CCR4 antagonists should be further elucidated. Clinical data also strongly suggest the involvement of the CCR4 system in asthma (Panina-Bordignon et al., 2001). Currently, much effort has been made by the pharmaceutical industry to develop small molecule CCR4 antagonists, therefore, the approach could be successful soon.

In conclusion, we found a newly-synthesized compound, RS-1154, which inhibits the CCL17-induced chemotaxis of Th2 cells *in vitro*, and also has a pharmacological profile to ameliorate ovalbumin-induced ear swelling *in vivo*. The potency *in vivo* was partial probably because of insufficient pharmacokinetic profiles of RS-1154. The plasma concentration 8 h after single oral administration of 100 mg/kg RS-1154 was > 1000 nM, but its protein-free fraction rate was below 1% (our unpublished data). Further optimization of the chemical structure is necessary to improve pharmacokinetic profiles, and then to be developed to a clinical stage. However, at present, our results demonstrate that RS-1154 or its derivatives have a potential to be developed as a novel anti-atopic dermatitis medicine in the future.

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