

Selective down-regulation of Th2 cell-mediated airway inflammation in mice by pharmacological intervention of CCR4

O. Kaminuma^{1,2}, T. Ohtomo², A. Mori², D. Nagakubo³, K. Hieshima³, Y. Ohmachi⁴, Y. Noda⁴, K. Katayama¹, K. Suzuki¹, Y. Motoi¹, N. Kitamura¹, M. Saeki¹, T. Nishimura¹, O. Yoshie³ and T. Hiroi¹

¹Department of Allergy and Immunology, The Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan, ²Clinical Research Center for Allergy and Rheumatology, National Hospital Organization, Sagami National Hospital, Kanagawa, Japan, ³Department of Microbiology, Kinki University Faculty of Medicine, Osaka, Japan and ⁴Department of Advanced Technologies for Radiation Protection Research, National Institute of Radiological Science, Chiba, Japan

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Summary

Background The chemokine receptor CCR4 has been implicated in Th2 cell-mediated immune responses. However, other T cell subsets are also known to participate in allergic inflammation.

Objective The role of CCR4 in Th1, Th2, and Th17 cell-mediated allergic airway inflammation was investigated.

Method We generated an allergic airway inflammation model by adoptive transfer of *in vitro*-polarized ovalbumin (OVA)-specific Th1, Th2, and Th17 cells. The effect of a low-molecular weight CCR4 antagonist, Compound 22, on this model was examined.

Results Upon *in vitro* polarization of DO11.10 naïve T cells, Th1- and Th2-polarized cells dominantly expressed CXCR3 and CCR4, respectively, while Th17-polarized cells expressed CCR6 and CCR4. Intranasal OVA-challenge of mice transferred with each T cell subset induced accumulation of T cells in the lungs. Eosinophils were also massively accumulated in Th2-transferred mice, whereas neutrophils were preferentially recruited in Th1- and Th17-transferred mice. Compound 22, as well as anti-CCL17 or anti-CCL22 antibody selectively suppressed accumulation of Th2 cells and eosinophils in the lungs of Th2-transferred and OVA-challenged mice. Compound 22 also inhibited bronchial hyper-responsiveness but had little effect on goblet cell hyperplasia in Th2-transferred and OVA-challenged mice.

Conclusions and Clinical Relevance There were notable differences in allergic lung inflammation mediated by different T cell subsets. CCR4 blockage was selectively effective for suppression of Th2-mediated allergic inflammation by blocking infiltration of Th2 cells.

Keywords airway inflammation, bronchial hyperresponsiveness, chemokine, eosinophil, lung, T cell

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Correspondence:

Osamu Kaminuma, DVM, PhD,
Department of Allergy and
Immunology, The Tokyo Metropolitan
Institute of Medical Science, 2-1-6
Kamikitazawa, Setagaya-ku, Tokyo
113-8613, Japan.
E-mail: kaminuma-os@igakuken.or.jp

Introduction

At least four distinct CD4⁺ T cell subsets (Th1, Th2, Th17, and Treg) that are characterized by the expression of specific transcription factors and cytokines are currently recognized [1]. Among them, Th2 cells are considered to play the major role in development of allergic inflammation [1, 2]. A number of investigations have demonstrated that different T cell subsets tend to

express a unique set of chemokine receptors. Thus, Th1 cells selectively express CXCR3, while Th2 cells CCR4 [3–6]. Th17 cells are reported to characteristically express CCR6 [7–9]. Accordingly, CCR4 has been implicated in the recruitment of antigen-specific Th2 cells to sites of allergic inflammation [2–6]. In asthmatic patients, antigen exposure was shown to induce the accumulation of CCR4-expressing T cells in the lungs [10, 11]. Furthermore, up-regulation of CCR4 ligands

was demonstrated in asthmatic lungs during the late-phase reaction [11–13]. In murine models, interventions of CCR4 as well as the CCR4 ligands, CCL17 and CCL22, were shown to attenuate development of airway inflammation and bronchial hyperresponsiveness (BHR) [14–16].

However, the importance of CCR4 in allergic diseases still remains controversial, given some contradictory results obtained from CCR4-gene disruption and/or blockade of CCR4 in mouse models of allergic airway inflammation or remodelling [17–19]. As a possible reason for such discrepancy, involvement of other T cell subsets than Th2 cells in the development of allergic inflammation could be considered. Antigen provocation in mice transferred with either Th1 or Th17 cells indeed induced airway inflammation with massive accumulation of neutrophils in the airways followed by the development of BHR [20–22]. Furthermore, Wakashin *et al.* demonstrated that Th17 cells enhanced Th2-mediated airway eosinophilic inflammation [23]. As Th1 and Th17 cells mainly express CXCR3 and CCR6, respectively [2, 3], the CCR4/CCR4-ligand interactions may not play the major role in allergic inflammation mediated by these T cell subsets. Therefore, the efficacy of chemokine receptor blockers may need to be separately evaluated in allergic airway inflammation induced by each T cell subset.

Recently, several CCR4 antagonists applicable for *in vivo* administration have been reported [24, 25]. Among them, Compound 22 ((R)-{4-{4-[(2,4-dichlorobenzyl)amino]pyrido[2,3-d]pyrimidin-2-yl}piperazin-1-yl}-piperidin-2-yl-methanone), has been shown to efficiently inhibit CCR4-mediated Ca^{2+} mobilization and chemotaxis *in vitro*, to be >500-fold less effective against other chemokine receptors (CCR2, CCR3 and CXCR3) and GPCRs (5-HT1A, 5-HT6 and 5-HT7), and to suppress eosinophil accumulation in the lungs of antigen-immunized mice *in vivo* [24].

We have developed a mouse model of airway inflammation in which mice are transferred with *in vitro*-differentiated T cells and challenged with the corresponding antigen. The model has provided relatively consistent and reproducible results and thereby enables us to easily evaluate the efficacy of antibodies and chemical compounds [26–28]. Herein, we employed this model to evaluate the role of CCR4 in the development of allergic inflammation by using Compound 22. Mice transferred with ovalbumin (OVA)-specific Th1, Th2, and Th17 cells were challenged with OVA and monitored for the accumulation of inflammatory cells in the lungs. Compound 22 efficiently suppressed Th2 cell-mediated airway inflammation and BHR, but not those mediated by Th1 or Th17 cells. Thus, our present study provides a proof of principle for the effectiveness of CCR4 blockade on allergic airway inflammation mediated by Th2 cells.

Materials and methods

Reagents

Compound 22 (99.5% purity) was synthesized based on published information [24] with the help of the NARD Institute (Hyogo, Japan).

In vitro polarization of T cells

All experiments and animal care were performed in accordance with the guidelines approved by the animal use committee of the Tokyo Metropolitan Institute of Medical Science. OVA-specific naïve CD4^+ T cells were isolated from the spleens of DO11.10/RAG-2^{-/-} mice by positive selection using CD4 microbeads and a magnetic cell sorting system (Miltenyi, Bergisch Gladbach, Germany). Cells were cultured with X-ray-irradiated BALB/c splenocytes in RPMI1640 medium supplemented with 10% fetal bovine serum, 0.3 μM OVA323–339 peptide and 10 U/mL recombinant IL-2 (BD Bioscience, San Jose, CA, USA). Appropriate cytokines and anti-cytokine antibodies (Abs) were added for Th1 and Th2 cell development as described previously [27]. For Th17 cell development, IL-1 β (10 ng/mL), IL-6 (20 ng/mL), TGF- β (1 ng/mL) (all purchased from Peprotech, Rocky Hill, NJ, USA), and IL-23 (10 ng/mL) (R & D Systems, Minneapolis, MN, USA) were added at the start of culture together with anti-IL-4 (40 $\mu\text{g/mL}$), anti-IL-12 (40 $\mu\text{g/mL}$), and anti-IFN- γ (40 $\mu\text{g/mL}$) (all purchased from eBioscience, San Diego, CA, USA). After 7 days of stimulation, cells were harvested, purified by centrifugation on Ficoll-Paque (GE Healthcare Bio-Science, Uppsala, Sweden) and then used as each T cell subset.

To determine the levels of polarization, cells were stimulated with 5 nM PMA plus 1 μM ionomycin for 6 h at 37°C in the presence of 2 μM monensin. The cells were fixed with 4% paraformaldehyde and permeabilized with 0.5% Triton X-100. After blocking with PBS containing 3% BSA, cells were stained with anti-IL-4-PE-Cy7, anti-IL-10-FITC, anti-IL-17A-Alexa Fluor 647, and/or anti-IFN- γ -Pacific Blue (eBioscience). CCR4 and CCR8 were stained with hamster monoclonal anti-mouse CCR4 (2G12) [29] and anti-mouse CCR8 (1018), respectively, followed by anti-hamster IgG-FITC (eBioscience). 1018 was synthesized by a hybridoma clone established from splenic B cells of Armenian hamsters immunized with the N-terminal 35 amino acid residues of mouse CCR8. We confirmed that 1018 specifically binds to mouse CCR8 by using a panel of L1.2 cell lines, stably expressing mouse CCRs (CCR1–10), CXCRs (CXCR1–CXCR6), CX₃CR1, and XCR1 (data not shown). Anti-CCR3-FITC, anti-CCR6-FITC, anti-CXCR3-PE (all R & D Systems), and anti-CCR5-PE (BD Bioscience) were also used to stain respective chemokine receptors. Flow

cytometric analysis was performed on a FACSCanto flow cytometer (BD Bioscience) with Flowjo software (Tree Star Inc., Ashland, OR, USA). As basal fluorescence levels were different among T cell subsets (Fig. S1), data are expressed as the ratio of mean fluorescence intensity stained with a specific Ab and that with an isotype control (relative expression).

Chemotaxis assay

Chemotactic responses were evaluated as described previously [27]. Briefly, T cells (1×10^6) and appropriately diluted mouse CXCL10, CCL17, CCL20, or CXCL12 (all purchased from R & D Systems) were added to the upper and lower chemotaxis chambers, respectively. After incubation at 37°C for 2 h, cells that migrated to the bottom wells were collected and counted by flow cytometry. Percentage of chemotaxis was calculated from the following formula: (number of cells recovered from lower chamber with chemokine – number of cells recovered from lower chamber without chemokine) / (number of total input cells – number of cells recovered from lower chamber without chemokine) $\times 100$. The test reagents were added to the cells at the start of assay.

Cell transfer and antigen challenge

Polarized T cells were stained with the fluorescein-based dye, CFSE (Invitrogen, Carlsbad, CA, USA), and transferred to recipient mice as described previously [26, 27]. To obtain comparable results, each mouse received the same number (3×10^7) of Th1-, Th2-, or Th17-polarized cells. Staining with CFSE did not affect the functions of T cells *in vitro* and *in vivo* (data not shown), and was more convincing for tracking antigen-specific T cells than staining with an anti-clonotype KJ1-26 Ab, since this Ab also reacts to unknown non-T cells [26]. Twenty-four hours after cell transfer, mice were challenged with intranasal administration of OVA (100 µg in 50 µL PBS) or BSA as control. For blocking experiments, Compound 22 was suspended in PBS containing 0.1% Tween 80 and administered subcutaneously twice, 30 min before and 24 h after antigen challenge. Hamster anti-mouse CCL17 (5H5) [14], rabbit polyclonal anti-mouse CCL22 (kindly provided by ICOS, Bothell, WA, USA) or control IgG (Beckman Coulter, Fullerton, CA, USA) was administered intravenously 30 min before OVA challenge. The vehicle alone did not affect any parameter examined (data not shown).

Assessment of airway inflammation

Forty-eight hours after the last antigen challenge, inflammatory cells in bronchoalveolar lavage fluid

(BALF), BHR and histopathological changes in the lungs were evaluated as described previously [14, 27] with minor modifications.

Bronchial hyperresponsiveness was assessed by restrained whole-body plethysmography (Buxco Electronics, Wilmington, NC, USA) according to the procedures recommended by the manufacturer. Briefly, mice were anaesthetized by intraperitoneal injection of 5 mg/kg pentobarbital, 0.2 mg/kg xylazine, and 0.02 mg/kg pancuronium bromide, tracheally cannulated and ventilated (175 breaths/min, tidal volume: 6 mL/kg). Then, lung resistance (R_L) was measured after exposing the animals to aerosolized PBS containing increasing concentrations of MCh (1.6–6.3 mg/mL). PC3.0, the concentration of exposed MCh by which R_L was reached to 3.0 cmH₂O/mL/sec, was extrapolatory calculated and used for comparison.

Lung sections (5 µm thick) were stained with haematoxylin and eosin or Periodic acid-Schiff. In some experiments, the severity of cellular infiltration and goblet cell hyperplasia were evaluated by scoring each specimen from grade 1 (no change) to 5 (severe).

Measurement of cytokines and chemokines

The concentrations of IL-4, IL-5, IL-17, IFN- γ and CXCL10 in BALF were determined with a Milliplex MAP mouse cytokine/chemokine immunoassay kit (Millipore Corp, St Charles, MI, USA), according to the manufacturer's protocol. Detection limits (DL) of these assays were 0.1, 2, 0.3, 0.1 and 0.3 pg/mL, respectively. TGF- β 1, CCL17, CCL20 and CCL22 were measured using Qantikine mouse Immunoassay kits (R & D Systems) (DL; 0.2, 2, 0.5, and 2 pg/mL, respectively). Neutrophil myeloperoxidase (MPO) was measured by Mouse MPO ELISA kit (Hycult Biotech Inc., Plymouth Meeting, PA, USA) (DL; 0.5 pg/mL).

Statistical analysis

Results are expressed as arithmetic mean $\pm$ SEM. Statistical analysis was performed by one-way ANOVA and Bonferroni's method. $P < 0.05$ was considered to indicate statistical significance.

Results

*Characterization of *in vitro*-differentiated T cells*

Splenic naïve T cells derived from DO11.10 mice were subjected to the respective culture conditions for Th1, Th2, and Th17 differentiation, and the polarization status of the resulting cells was examined (Fig. 1a). Upon stimulation, the majority of Th1-polarized cells produced IFN- γ but hardly produced IL-4, IL-10, or IL-17.

On the other hand, a large fraction of Th2-polarized cells produced IL-4 but not IFN- γ or IL-17. A small fraction of Th2-polarized cells also produced IL-10. Similarly, cells producing IL-17 were seen only in Th17-polarized cells. A small fraction of Th17-polarized cells also produced IFN- γ . Even though the polarization of Th17 cells seemed to be less robust than that of Th1 and Th2 cells, the obtained phenotype was not much different from those reported by several other groups [30–33]. Thus, we considered that naïve OVA-specific T cells were reasonably well polarized into Th1, Th2, and Th17 cells by the single-round stimulatory culture conditions.

Given that T cell subsets are known to express a relatively specific set of chemokine receptors [2, 3], the

expression profile of chemokine receptors on polarized T cells was next determined. As shown in Figs. 1b and S1, highly selective expression of CXCR3 was seen in Th1-polarized cells, while CCR4 was predominantly expressed by Th2-polarized cells. Although the dominant types of chemokine receptor were less clear in Th17-polarized cells, at least they were confirmed to express CCR6 and CCR4. These results were mostly consistent with the reported chemokine receptor expression patterns of these T cell subsets [2, 3].

Effect of Compound 22 on chemotaxis of Th1, Th2 and Th17 cells

To evaluate the functionality of the chemokine receptors expressed by each T cell subset, we next performed chemotaxis assays. CCL17 (a CCR4 ligand) induced vigorous migratory responses in Th2 cells, whereas CXCL10 (a CXCR3 ligand) in Th1 cells (Fig. 2a). On the other hand, CCL20 (the CCR6 ligand) induced poor chemotactic responses only in Th2 cells. Although Th17 cells expressed CCR6 and CCR4 at substantial levels, they failed to migrate to CCL20 or CCL17 (Fig. 2a). To test the basic chemotactic ability of Th17 cells, we also examined the effect of CXCL12 (the CXCR4 ligand). However, CXCL12 failed to induce efficient cell migration in any of these T cell subsets (Fig. 2a).

Compound 22 was reported to inhibit human CCR4-mediated chemotaxis with an IC_{50} of 7 nM [24]. We corroborated the CCR4 selective antagonism of Compound 22 by using a panel of murine L1.2 cell lines stably expressing the whole set of human chemokine receptors: CCR (CCR1–10), CXCR (CXCR1–4), CX3CR (CX3CR1), and XCR (XCR1) (data not shown). It was, however, not clear whether this compound was also effective on mouse CCR4. We therefore examined the effect of Compound 22 on chemotaxis of Th1- and Th2-polarized DO11.10 cells. Even though with an IC_{50} of about 100 nM, Compound 22 dose-dependently suppressed chemotaxis of Th2 cells induced by mouse CCL17 (Fig. 2b). On the other hand, Compound 22 failed to affect chemotaxis of Th1 cells induced by mouse CXCL10. We also confirmed that anti-mouse CCL17 mAb specifically inhibited CCL17-induced chemotaxis of Th2 cells but not CXCL10-induced chemotaxis of Th1 cells (Fig. 2b). On the other hand, Compound 22 did not affect proliferation (Fig. 2c) or production of IL-4 and IL-5 (Fig. 2d) by stimulated Th2 cells even at the concentration of 10 μ M. The CCR4 specificity of Compound 22 was further demonstrated by the findings that it did not affect mouse CCL5-induced chemotaxis of L1.2 cells expressing mouse CCR5 (data not shown). Taken together, Compound 22 is a highly selective antagonist not only for human CCR4 but also for mouse CCR4.

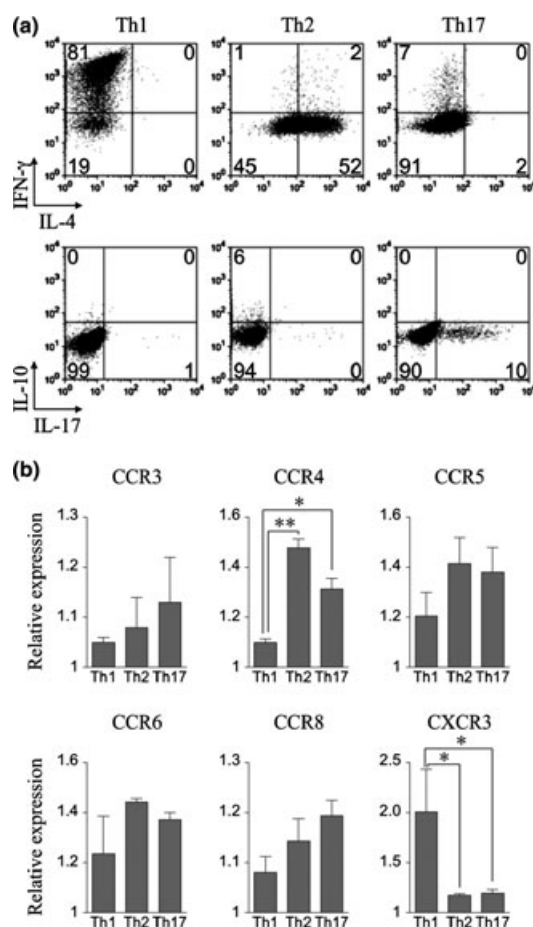


Fig. 1. Expression of cytokines and chemokine receptors in Th1, Th2 and Th17 cells. After 7 days of culture under Th1, Th2, and Th17-skewing conditions, polarized T cells were stimulated with 5 nM PMA + 1 μ M ionomycin for 6 h and stained for intracellular IFN- γ , IL-4, IL-10, and IL-17 (a). The results shown are representatives from three separate experiments. The surface expression of chemokine receptors on polarized cells was detected by staining with respective antibodies (b). Data are expressed as mean $\pm$ SEM of relative mean fluorescence intensity compared with that obtained with isotype control ($n = 3$). * P < 0.05, ** P < 0.01 (Bonferroni's method).

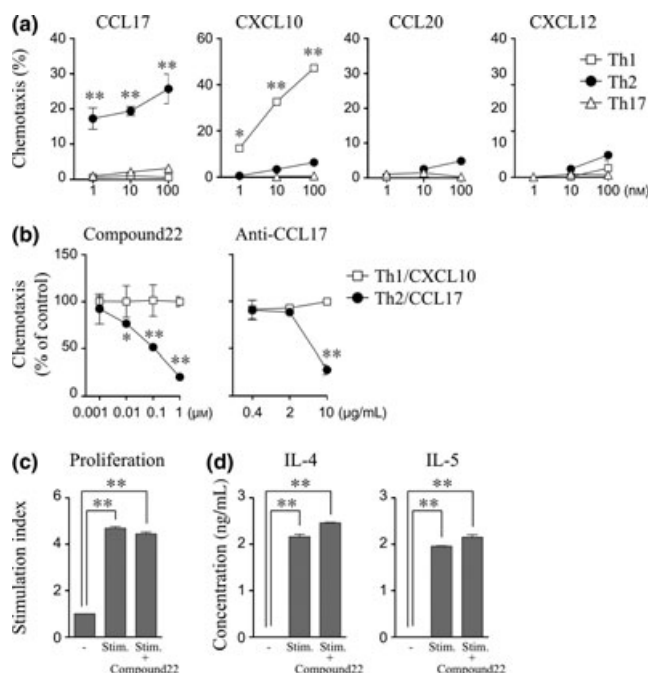


Fig. 2. Chemotactic activity of induced T cell subsets. Standard transwell chemotaxis assays were performed for 2 h using Th1-, Th2-, and Th17-polarized cells and increasing concentrations of CCL17, CXCL10, CCL20, and CXCL12 (a). Chemotaxis of Th1- and Th2-polarized cells to 1 nM CXCL10 and 10 nM CCL17, respectively, was examined in the presence of Compound 22 or anti-CCL17 mAb (b). Polarized Th2 cells were stimulated with anti-CD3 + anti-CD28 Ab (Stim.) in the presence or absence of 10 μ M Compound 22 for 72 h. Then, proliferative response (c) and the concentration of IL-4 and IL-5 (d) in the culture supernatant were assayed. Data are expressed as mean $\pm$ SEM of quadruplicate cultures. * P < 0.05, ** P < 0.01, compared with non-treated (a, c, d) or chemokine-treated (b) control (Bonferroni's method).

Ovalbumin-induced airway inflammation in cell-transferred mice

We next examined OVA-induced airway inflammation in mice transferred with *in vitro*-polarized T cell subsets. In transferred mice, the polarized T cell subsets appeared to accumulate in the lungs upon antigen challenge. Thus, upon challenge with OVA but not BSA, the concentrations of IFN- γ , IL-4/IL-5, and IL-17 were selectively increased in BALF of Th1-, Th2-, and Th17-transferred mice, respectively (Fig. 3). Upon challenge with OVA but not BSA, various chemokines (CXCL10, CCL17/CCL20, and CCL22) were also highly elevated in BALF. However, the repertoire of chemokines elevated in BALF did not show any obvious correlation with the type of infused T cell subsets (Fig. 3). Thus, the main producers of chemokines may be the residential tissue cells in these mice.

To trace the mobilization of infused T cells, we next used CFSE-labelled T cells. Upon challenge with OVA but not BSA, CFSE-labelled OVA-specific T cells were

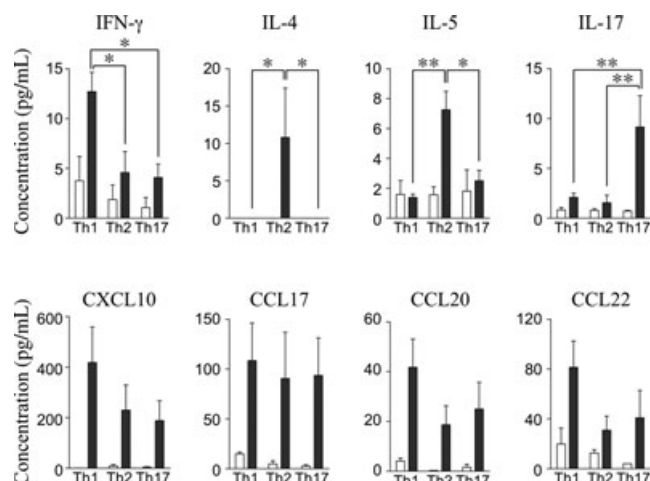


Fig. 3. Antigen-induced production of cytokines and chemokines in lungs of T cell-transferred mice. Recipient mice were transferred with Th1-, Th2-, or Th17-polarized cells by intravenous injection (3×10^7 cells/animal). After 24 h, mice were challenged with intranasal administration of ovalbumin (OVA) (filled) or BSA (open). Bronchoalveolar lavage (BAL) was performed 48 h after challenge, and the concentrations of indicated cytokines and chemokines in BALF were determined. Data are expressed as mean $\pm$ SEM of four to eight animals from three separate experiments. * P < 0.05, ** P < 0.01, compared with Th2 cell-transferred and OVA-challenged animals (Bonferroni's method).

indeed recovered in the BALFs of Th1 or Th2 cell-transferred mice but not those transferred with Th17 cells (Fig. 4a). The latter observation might be partly due to the relatively poor chemotactic responses of Th17-polarized cells *in vitro* (Fig. 2). Notably, however, the accumulation of bystander lymphocytes was efficiently induced not only in Th1- and Th2-transferred mice but also in Th17-transferred mice (Fig. 4b). This might be due to the elevated production of various chemokines in these mice (Fig. 3). In Th2-transferred mice, eosinophils dominantly and neutrophils to a lesser extent also accumulated in the lungs (Figs 4c and d). In contrast, neutrophils dominantly and eosinophils to a lesser extent accumulated in the lungs of Th1- and Th17-transferred mice (Figs 4c and d). These results were mostly consistent with previous reports [20–22].

Effect of Compound 22 on lung cell infiltration

The effect of Compound 22 on OVA-induced cell accumulation in the lungs was also investigated. Compound 22 significantly suppressed the increase in OVA-specific T cells and eosinophils in Th2-transferred mice (Figs 4a and c). It should be mentioned that CFSE-labelled cells were clearly distinguishable from resident cells, as only a small portion of OVA-activated transferred cells would undergo cell division and thus weaken their

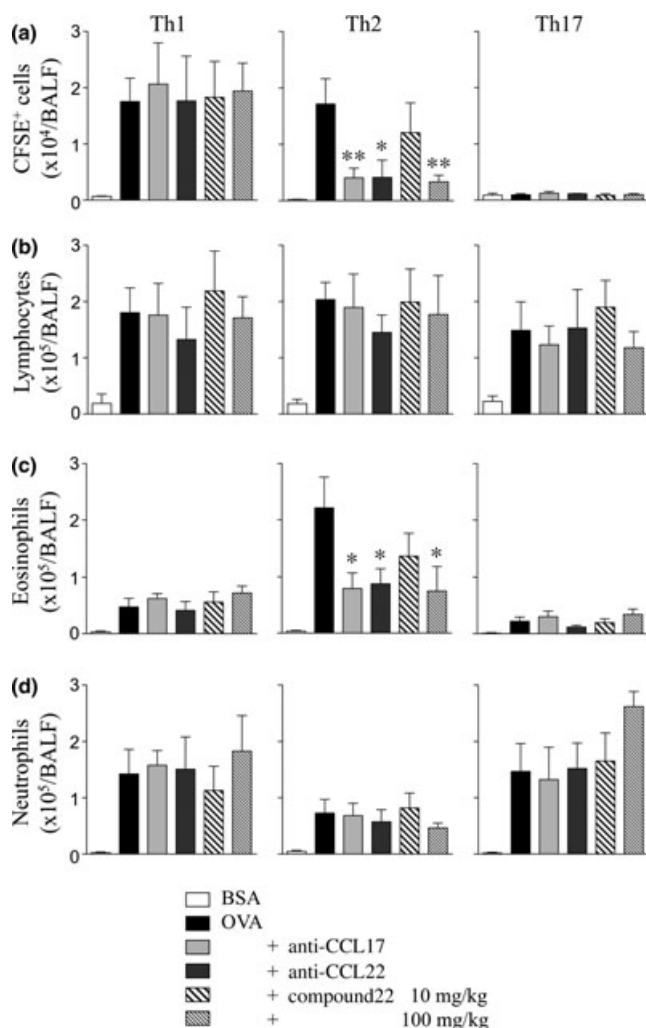


Fig. 4. Effect of the CCR4 antagonist on antigen-induced cellular infiltration in lungs of T cell-transferred mice. *In vitro*-polarized Th1, Th2, and Th17 cells were stained with CFSE and transferred to recipient mice by intravenous injection (3×10^7 cells/animal). After antigen challenge and BAL as shown in Fig. 3, the number of CFSE-positive cells in BALF was determined by flow cytometry (a). Lymphocytes (b), eosinophils (c), and neutrophils (d) were also differentially counted in BALF. Compound 22 was administered subcutaneously twice, 30 min before and 24 h after ovalbumin (OVA) challenge. Anti-CCL17 and anti-CCL22 (5 mg/kg) were intravenously injected 30 min before OVA challenge. Data are expressed as mean $\pm$ SEM of 3–13 animals from three separate experiments. * $P < 0.05$, ** $P < 0.01$, compared with OVA-challenged control animals (Bonferroni's method).

fluorescence intensity at this time point (48 h after antigen challenge) [26]. Given that Compound 22 did not affect proliferation of Th2 cells (Fig. 2c), the reduction of antigen-specific T cells in the lungs of Compound 22-treated mice was most likely by the inhibition of cell migration. Notably, however, the bystander lymphocyte accumulation was not significantly reduced by Compound 22. This might be due to the

Table 1. Effect of Compound 22 on antigen-induced cytokine production in T cell-transferred mice

Cytokine	Subset	Concentration (pg/mL)		
		BSA	OVA	OVA + Compound 22
IFN- γ	Th1	$0.5 \pm 0.1^{**}$	9.8 ± 1.4	11 ± 6.2
IL-4	Th2	$1.3 \pm 0.8^{**}$	17 ± 7.6	11 ± 4.5
IL-5	Th2	$2.0 \pm 0.1^{**}$	9.7 ± 2.6	5.0 ± 3.0
IL-17	Th17	$0.3 \pm 0.1^{**}$	7.2 ± 2.5	6.9 ± 3.2
TGF- β 1	Th1	$1.2 \pm 0.8^{**}$	4.3 ± 1.1	4.5 ± 0.6
	Th2	$1.0 \pm 0.7^{**}$	6.5 ± 1.2	$2.9 \pm 0.6^*$
	Th17	$0.6 \pm 0.3^*$	2.2 ± 0.1	2.2 ± 0.1

Twenty-four hours after transfer of Th1-, Th2-, or Th17-polarized cells, mice were challenged with ovalbumin (OVA) or BSA as shown in Fig. 3. Compound 22 (100 mg/kg) was administered as indicated in Fig. 4. Data are expressed as mean $\pm$ SEM of 3–13 animals from three separate experiments.

* $P < 0.05$,

** $P < 0.01$, compared with OVA-challenged mice (Bonferroni's method).

chemokines produced by resident tissue cells. Essentially the same results were obtained with the administration of anti-CCL17 or anti-CCL22 (Figs 4a and c). In the presence of Compound 22, there was also a tendency towards a reduction of Th2 cytokines in BALF of mice transferred with Th2 cells and challenged with OVA, but this was not statistically significant (Table 1). In Th1-, Th2-, and Th17-transferred mice, TGF- β 1, a Treg-related cytokine, was also significantly elevated upon OVA challenge (Table 1). Notably, Compound 22 also suppressed TGF- β 1 production in Th2- but not in Th1- or Th17-transferred mice. Collectively, these results support that the CCL17/CCL22-CCR4 axis plays the major role in Th2-mediated airway inflammation primarily by recruiting antigen-specific Th2 cells.

Effect of Compound 22 on bronchial hyperresponsiveness

It has been reported that BHR can be induced not only by Th2 cells but also by Th1 and Th17 cells, at least in mouse models of airway inflammation [20–22]. Therefore, our next investigation was performed to evaluate the contribution of CCR4 to OVA-induced BHR in T cell-transferred mice. Upon OVA challenge, bronchial responsiveness to inhaled methacholine (MCh) was significantly up-regulated in Th1- or Th2-transferred mice (Fig. 5) However, Th17-transferred mice failed to show significant BHR in the present study. Consistent with its selective effect on Th2 infiltration in the lungs (Fig. 4), Compound 22 suppressed BHR in Th2- but not Th1-transferred mice to the control levels (Fig. 5). The results were also confirmed by comparing the two experimental groups with the use of PC3.0 as a threshold parameter (Table 2).

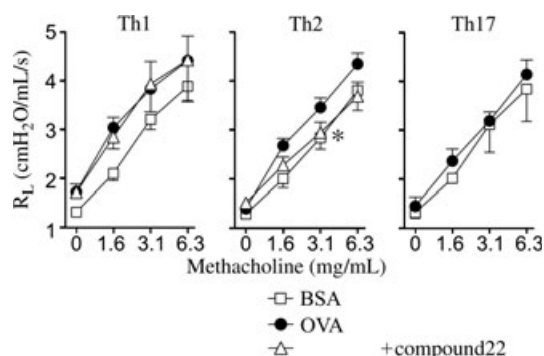


Fig. 5. Effect of Compound 22 on antigen-induced bronchial hyperresponsiveness (BHR) in T cell-transferred mice. Twenty-four hours after transfer of Th1-, Th2-, or Th17-polarized cells, mice were challenged with ovalbumin (OVA) as shown in Fig. 3. Compound 22 (100 mg/kg) was administered as indicated in Fig. 4. Data are expressed as mean $\pm$ SEM of four to eight animals from three separate experiments. * $P < 0.05$, compared with OVA-challenged control animals (Bonferroni's method).

Table 2. Effect of CCR4 antagonist on antigen-induced BHR in T cell-transferred mice

	PC3.0 (mg/mL)		
	BSA	OVA	OVA + Compound 22
Th1	3.65 $\pm$ 0.87	2.06 $\pm$ 0.45*	2.10 $\pm$ 0.40*
Th2	3.78 $\pm$ 0.37	2.34 $\pm$ 0.30**	3.74 $\pm$ 0.94 [†]
Th17	3.92 $\pm$ 0.72	3.38 $\pm$ 0.58	N.D.

From the dose-response curves in Fig. 5, PC3.0, the concentration of exposed MCh by which R_L was reached to 3.0 cmH₂O/mL/sec, was extrapolatory calculated. Data are expressed as mean $\pm$ SEM of four to eight animals from three separate experiments.

* $P < 0.05$,

** $P < 0.01$, compared with BSA-challenged mice and

[†] $P < 0.05$, compared with OVA-challenged mice (Bonferroni's method).

N.D., not determined; BHR, bronchial hyperresponsiveness.

Effect of Compound 22 on goblet cell hyperplasia

To evaluate tissue reactions in the lungs of T cell-transferred and OVA-challenged mice, tissue sections were examined (Fig. 6). Massive infiltration of inflammatory cells, including antigen-specific T cells, was observed in Th1- or Th2-transferred mice 48 h after OVA challenge (Figs 6a and b). In contrast, infiltration of inflammatory cells in the lung interstitium was rather weak in Th17-transferred mice (Figs 6a and b), although a large number of neutrophils were recovered in BALF (Fig. 4). Hyperplasia of goblet cells was specifically seen in Th2-transferred mice (Fig. 6c). Such inflammatory responses were not observed in the lungs of non-transferred mice (Figs 6a–c) or BSA-challenged mice (data not shown). The effect of Compound 22 on OVA-induced infiltration

of total inflammatory cells (Fig. 6d) and antigen-specific T cells (Fig. 6e) as well as goblet cell hyperplasia (Fig. 6f) in the lungs of Th2-transferred mice was evaluated by scoring the severity of these responses or counting CFSE-positive cells. Consistent with the results from BAL examination (Fig. 4), Compound 22 clearly suppressed cellular infiltrates including infused Th2 cells in the lungs (Figs 6a, b, d, and e). However, antigen-induced goblet cell hyperplasia was not significantly suppressed by Compound 22 (Figs 6c and f). We also examined the effect of Compound 22 on the concentration of MPO in BALF of Th2-transferred mice. Consistent with the lack of suppression of Compound 22 on BALF neutrophils, increases in MPO levels upon antigen provocation was not affected by Compound 22 (Fig. 6g).

Discussion

A number of studies have addressed the contribution of CCR4 to the development of allergic inflammation *in vivo* by using neutralizing Abs against CCR4 or CCR4 ligands or using the CCR4 gene-disrupted mice [6, 14–19]. However, the results are still controversial and the potential efficacy of CCR4 blockers in allergic disorders associated with eosinophilic inflammation remains unclear. Compound 22 is a recently reported CCR4 antagonist shown to inhibit eosinophil accumulation in the lungs of antigen-immunized mice *in vivo* [24]. We have generated an allergic airway inflammation model in which mice are transferred with *in vitro*-polarized OVA-specific Th1, Th2, or Th17 cells and challenged with OVA [26–28]. By using this simplified and reproducible model, we demonstrated that Compound 22 selectively suppressed Th2-mediated allergic lung inflammation. Namely, the CCR4 antagonist suppressed lung accumulation of antigen-specific Th2 cells and eosinophils as well as BHR. Since antigen provocation does not necessarily lead to exclusive Th2 responses even in allergic diseases, this selective contribution of CCR4 to Th2 cell-mediated responses is probably the major reason for the previous contradictory findings concerning the role of CCR4 in such disease models [6, 14–19].

Not only Th2-polarized cells but also Th17-polarized cells expressed CCR4 at significant levels. This is consistent with the report by Acosta-Rodriguez et al. [7], demonstrating that human Th17 cells were enriched in the CCR6⁺CCR4⁺ fraction of CD4⁺ T cells. However, a potential functional difference in CCR4 expressed on Th2 and Th17 cells was observed in our present study. CCL17, the CCR4 ligand, induced vigorous chemotaxis in Th2 cells but not in Th17 cells *in vitro*. Furthermore, CCR6 was similarly expressed on Th1, Th2, and Th17 cells, but did not mediate efficient chemotaxis in these

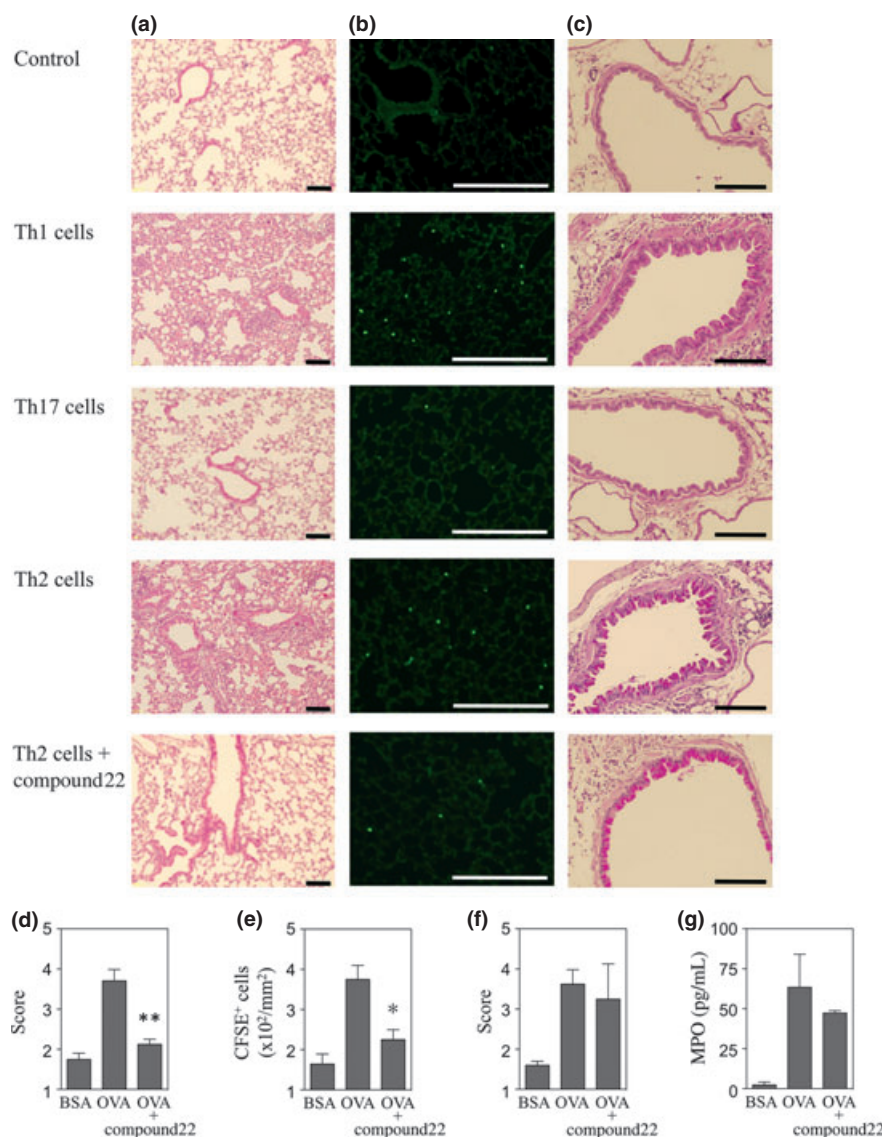


Fig. 6. Effect of Compound 22 on airway inflammation in the lungs of T cell-transferred mice. Twenty-four hours after transfer of CFSE-labelled Th1-, Th2-, or Th17-polarized cells, mice were challenged with ovalbumin (OVA) as shown in Fig. 3. Lung specimens were taken 48 h after challenge from these mice as well as from non-transferred and OVA-challenged control mice. Tissue sections were stained with haematoxylin and eosin (a) or Periodic acid-Schiff (c). Unstained specimens were observed under confocal microscopy (b). Bar = 100 μ m. Compound 22 (100 mg/kg) was administered as indicated in Fig. 4. The effect of Compound 22 on antigen-induced infiltration of inflammatory cells (d), antigen-specific T cells (e), goblet cell hyperplasia (f) in Th2 cell-transferred mice was semi-quantitatively analysed by counting the number of cells appeared in the sections (e) or scoring the severity of these responses (d and f). The concentration of MPO in bronchoalveolar lavage fluid (BALF) recovered as described in Fig. 3 was also determined (g). Data are expressed as mean $\pm$ SEM of four to seven animals from four separate experiments. * P < 0.05, ** P < 0.01, compared with OVA-challenged control animals (Bonferroni's method).

subsets to its ligand, CCL20 in this study. Th17 cells also did not respond to CXCL12 either, suggesting that at least Th17-polarized cells employed in this study might be generally poor chemotactic responders. The reason for such poor responses of Th17 cells was not clear, but it has been reported that the chemokine receptors can be uncoupled from downstream signalling pathways in some cases. Thus, D'Amico *et al.* demonstrated that concomitant exposure to LPS and IL-10

caused unresponsiveness of dendritic cells against stimulation through CCR1, CCR2, CCR5, and CCR7 [34]. Similarly, Collington *et al.* reported that bone marrow-derived mast cells cultured without stem cell factor failed to migrate to CCL2, even though they expressed substantial levels of CCR2 [35]. Hirota *et al.* [9] also demonstrated CCR6 expression on *in vitro*-polarized Th17 cells and their Th17 cells seemed to express more CCR6 as well as IL-17 in comparison with those devel-

oped in our present study. Thus, more rigorous polarization of Th17 cells may be necessary for their acquisition of chemotactic and other functions. Therefore, the pathological role of Th17 cells in our model and the effect of CCR4 blockage still remain to be fully explored.

Mucus hypersecretion by hyperplastic goblet cells in the airways has been implicated in the pathogenesis of bronchial asthma [36]. In a murine model of asthma, Nakanishi et al. demonstrated that intratracheal administration of antisense RNA against gob-5, a member of the calcium-activated chloride channel family, efficiently suppressed both BHR and mucus hypersecretion [37]. However, a direct contribution of the goblet cell hyperplasia to BHR still remains to be confirmed. Even though a series of Th2 cytokines have been implicated in mucus hypersecretion [38], Compound 22 did not show clear reduction of goblet cell hyperplasia in mice transferred with Th2 cells. This might be consistent with the findings that Compound 22 was less effective in reduction of Th2 cytokine production in the lungs, even though it clearly suppressed Th2 cell migration to the lungs. Previously, we showed that a fraction of infused Th2 cells spontaneously distributed in the lungs even before antigen provocation and they could be the main source of Th2 cytokines responsible for goblet cell hyperplasia [27]. Thus, the suppression of antigen-evoked accumulation of Th2 cells by Compound 22 might not be sufficient to reduce the local Th2 cytokine production by such cells.

Spontaneous distribution of a fraction of infused T cells in the lungs might also explain the efficient accumulation of neutrophils and bystander lymphocytes in Th17-transferred mice even though Th17-polarized cells migrated to the lungs less efficiently than Th1- or Th2-polarized cells upon antigen challenge. On the other hand, recruitment of Treg cells in the lungs might be induced along with the migration of antigen-specific T cells. Thus, TGF- β 1, a Treg-related cytokine, was elevated in Th1-, Th2-, and Th17-transferred mice after antigen challenge. In Th17-transferred mice in which only weak migration of infused Th17 cells was observed, the increase in TGF- β 1 levels was also less potent than in Th1- and Th2-transferred mice. In addition, Compound 22 specifically suppressed the elevation of TGF- β 1 only in Th2-transferred mice. Thus, although Treg cells are considered to express CCR4 [39, 40], the direct contribution of CCR4 to the recruitment of Treg cells was not confirmed in the present model. Further studies may be necessary to clarify the role of CCR4 in recruitment of Treg cells in allergic airway inflammation.

Our present findings may also suggest that goblet cell hyperplasia is not directly related to BHR and thus CCR4 antagonists may be less effective on the mucus

secretion. Indeed, Schuh et al. reported that BHR but not airway remodelling was attenuated in *Aspergillus fumigatus*-sensitized mice by the gene disruption of CCR4 [18]. The decline of BHR has also been achieved by treatment with anti-CCL17 and anti-CCL22 Abs in actively sensitized mice [14, 15] and by treatment with anti-human CCR4 Ab in severe combined immunodeficiency mice reconstituted with peripheral blood mononuclear cells of allergic patients [16], even though the effect on airway remodelling was not fully evaluated in those studies. Notably, Chvatchoko et al. demonstrated that the extent of antigen-induced BHR in CCR4-deficient mice was equivalent to that in wild-type mice [19]. They also did not see any significant differences in airway infiltration of inflammatory cells including eosinophils and lymphocytes between wild-type and CCR4-knockout mice [19]. Although the exact reason for such discrepancies is not clear at present, the blockade of CCR4 or its ligands using antibodies or small compounds successfully attenuated not only BHR but also cellular infiltration as shown in our present study and previous reports [14–16]. Thus, some compensatory mechanisms involving other chemokine receptors might have operated in the case of CCR4-deficient mice. At least in our present model, it has been clearly demonstrated that CCR4 plays the major role in Th2-mediated airway inflammation.

Significant BHR was also induced, despite the lack of goblet cell hyperplasia, in Th1 cell-transferred mice. We have recently demonstrated that, unlike mouse T cells, human peripheral T cells are programmed to produce IFN- γ even under Th2-skewing conditions [41]. Furthermore, IFN- γ -secreting Th1 cells were also increased in the lungs of asthmatic patients [42, 43]. Thus, although the relative contribution of Th1 cells to the pathogenesis of bronchial asthma remains to be seen, asthmatic patients may still suffer from Th1 cell-mediated BHR even after treatment with CCR4 antagonists for Th2 cell-mediated inflammatory responses and BHR. In this context, Takaoka et al. reported that BHR as well as airway neutrophilia in OVA-specific Th1 cell-transferred mice was inhibited by anti-human CXCR2 [20]. By employing CXCR2-deficient mice, however, Cui et al. demonstrated that although Th1 cell-mediated neutrophilia was greatly reduced, BHR was still as strong as that seen in wild-type mice [21]. We also observed neutrophil accumulation at similar levels in the lungs of Th1- and Th17-transferred mice. However, no significant BHR was observed in mice transferred with Th17 cells. In Th2-transferred mice, antigen-induced BHR but no increase in MPO was suppressed by Compound 22. These findings suggest that neutrophils play a minor role in the development of BHR. Previously, McKinley et al. [22] demonstrated development of BHR in antigen-specific Th17 cell-transferred mice. Although the

reason for their discrepancy with the present study is unclear at present, the experimental conditions were different. In the study of McKinley *et al.*, mice were challenged with antigen for three consecutive days and BHR was assessed 24 h after the last antigen challenge, while we examined BHR 48 h after a single OVA challenge. Even though neutrophil-rich airway inflammation was induced in both studies, much severe inflammation might have occurred in the conditions employed by McKinley *et al.*, possibly leading to the development of BHR. This explanation is supported by the fact that BHR that developed in Th2-transferred mice in their study was also much more severe than that in our study. It should also be mentioned that Th17 cell-dependent BHR was still less severe than that induced by Th2 cells in their report [22]. Further investigations are required to elucidate the mechanisms underlying Th1- and Th17-mediated BHR, including the role of neutrophils.

In conclusion, we have demonstrated that intervention against CCR4 is effective for selective suppression of antigen-induced lung infiltration of Th2 cells together with accompanying eosinophilic inflammation and BHR development. Even though airway mucus hypersecretion may be less responsive, specific CCR4 antagonists are promising for the treatment of Th2 cell-mediated allergic diseases such as bronchial asthma.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Representative expression pattern of CXCR3 on Th1, Th2, and Th17 cells

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Supporting Information Fig. S1.

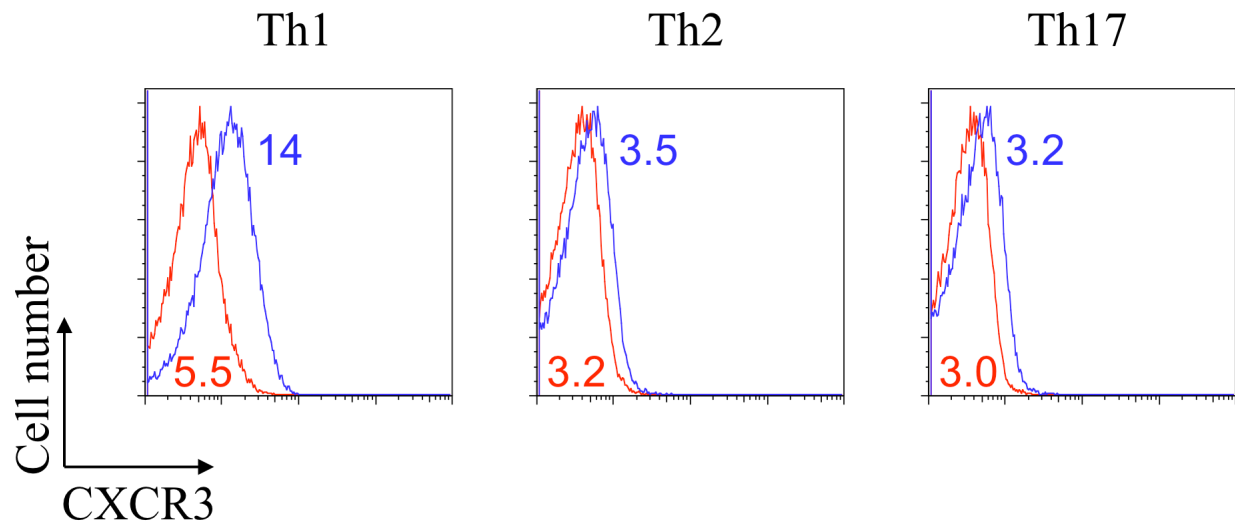


Fig. S1. Representative expression pattern of CXCR3 on Th1, Th2, and Th17 cells.

After 7 days of culture under Th1-, Th2-, and Th17-skewing conditions, the surface expression of CXCR3 was examined. The mean fluorescence intensity of the cells stained with anti-CXCR3 Ab and its isotype control Ab was described in blue and red, respectively. The results shown were the representatives of at least three independent experiments.