



Short communication

C-C chemokine receptor type 4 antagonist Compound 22 ameliorates experimental autoimmune encephalomyelitis

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ABSTRACT

Chemokines and chemokine receptors play important roles in the immune response. We previously reported the pathogenic role of C-C chemokine receptor type 4 (CCR4) in experimental autoimmune encephalomyelitis (EAE). Here, we examined whether CCR4 antagonism modulates the disease course of EAE. Wild-type and CCR4-knockout mice were induced EAE and were administered Compound 22, an antagonist of CCR4. Compound 22 significantly ameliorated the severity of EAE in wild-type mice, but not in the CCR4-knockout mice. Compound 22 inhibited Th1 and Th17 polarization of antigen-induced T-cell responses. Therefore, CCR4 antagonists might be potential therapeutic agents for multiple sclerosis.

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1. Introduction

C-C chemokines and chemokine receptors (CCRs) play important roles in the immune response by regulating leukocyte migration, lymphocyte differentiation, and cytokine production. They are crucial for the development of autoimmune diseases such as multiple sclerosis (MS). Chemokine receptors have become therapeutic targets for various human diseases, such as malignant lymphoma and HIV infection, which are treated using anti-CCR4 antibodies and CCR5 antagonists, respectively. They are also expected to be useful for autoimmune diseases and many clinical trials are being carried out.

However, in MS, there are no agents available that target CCRs, such as CCR4 and CCR6, which regulate the functions of T helper 17 (Th17) cells. C-X-C chemokine receptor type 3 (CXCR3) and CCR5 are also important in MS, because they are necessary for the migration of pathogenic Th1 cells into the central nervous system (Comerford et al., 2013). Unfortunately, a previous clinical trial using CCR2 antagonists completed at 2008, however, that provided no positive data (NCT00239655, ClinicalTrials.gov, 2005). CCR5 deletion does not have

an essential effect on the development or disease course of MS (Ghorban et al., 2013).

We previously reported the pathogenic role of CCR4 in experimental autoimmune encephalomyelitis (EAE) using the CCR4 and CCR6 knockout mouse models (Moriguchi et al., 2013). Elimination of CCR4 ameliorated EAE; however, the downregulation of T cell response in the induction phase and the reinforcement of Th1 polarization were not observed. Recently, a novel small-molecule CCR4 antagonist, 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 reported to efficiently inhibit the CCR4-mediated Ca²⁺ mobilization and chemotaxis in vitro, and Compound 22 is a >500-fold less effective inhibitor of other chemokine receptors (CCR2, CCR3, and CXCR3) (Purandare et al., 2007). Compound 22 is reported to ameliorate allergic airway inflammation by suppressing Th2-mediated cytokine production (Kaminuma et al., 2012).

In the present study, we examined whether CCR4 antagonism ameliorates the disease course of EAE using Compound 22.

2. Materials and methods

2.1. Active immunization for inducing EAE

C57BL/6 female mice were injected subcutaneously with 100 µg myelin oligodendrocyte glycoprotein (MOG)_{35–55} in complete Freund's adjuvant (CFA) in a total volume of 0.2 ml, and 200 ng pertussis toxin

Abbreviations: CCR, C-C chemokine receptor; DMSO, dimethyl sulfoxide; EAE, experimental autoimmune encephalomyelitis; MS, multiple sclerosis; MOG, myelin oligodendrocyte glycoprotein.

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was administered intraperitoneally on day 0 and day 2 after immunization. Compound 22 (10 mg/kg; $n = 9$) or dimethyl sulfoxide (DMSO) as control ($n = 10$) was injected intraperitoneally on days 0, 7, 14, 21, and 28 after immunization to test whether Compound 22 prevent EAE. To confirm whether Compound 22 was effective after onset of the disease, Compound 22 ($n = 5$) or DMSO ($n = 6$) was injected on day 14, 21, and 28 after immunization. Compound 22 was dissolved in DMSO. Immunized mice were examined and scored daily as follows:

0, no clinical signs; 1, limp tail; 2, partial hind leg paralysis; 3, total hind leg or partial hind and front leg paralysis; 4, total hind leg and partial front leg paralysis; 5, moribund or dead. Mice were examined daily for the signs of EAE in a blind fashion (Miyamoto et al., 2001).

To confirm that the effects of Compound 22 were mediated by blocking CCR4, EAE was induced in the CCR4 knockout (CCR4-KO) mice (Compound 22, $n = 5$; DMSO, $n = 8$). Spinal cords 35 days after immunization were harvested and embedded in paraffin. Tissues were

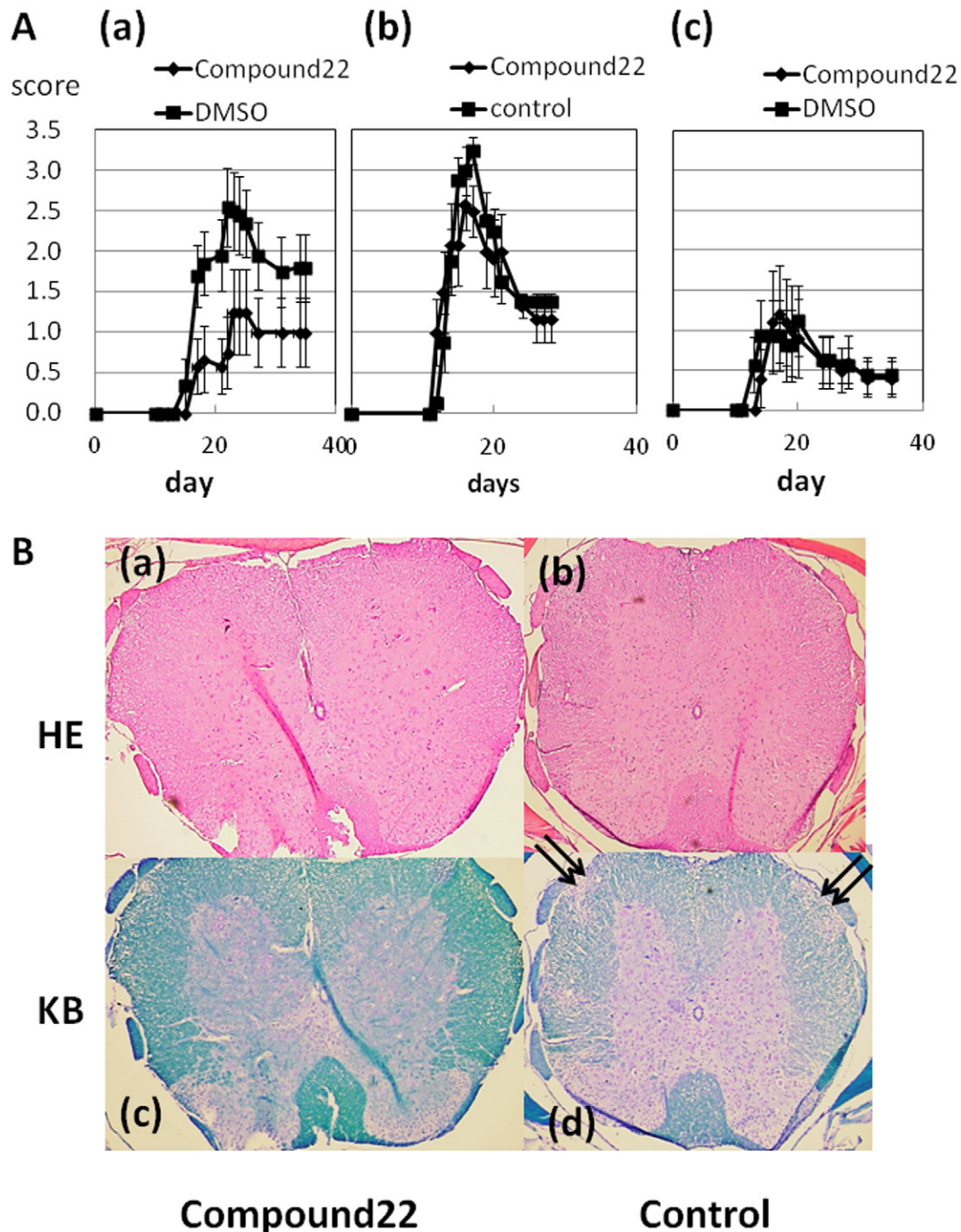


Fig. 1. Experimental autoimmune encephalomyelitis (EAE) was induced in wild-type and C-C chemokine receptor type 4 (CCR4)-KO C57BL/6 mice. Mice were treated with either Compound 22 or dimethyl sulfoxide (DMSO) as control (wild type mice: groups treatment was started before onset of the disease; Compound 22, $n = 9$; DMSO, $n = 10$; groups after onset of the disease; Compound 22, $n = 6$; DMSO, $n = 5$; CCR4-KO: Compound 22, $n = 5$; DMSO, $n = 8$). (a), In groups treated before onset of the disease, disease severity was significantly ameliorated in the Compound 22-treated wild type mice based on the cumulative score (Compound 22 vs. DMSO, 10.3 ± 10.6 vs. 23.0 ± 12.3 , mean \pm standard deviation, $P < 0.05$). (b), In groups treated after onset, cumulative scores in mice administered Compound 22 and DMSO were 22.5 ± 8.06 and 25.5 ± 4.29 ($P > 0.05$), respectively. (c), In CCR4-KO mice, Compound 22 had no beneficial effects on disease severity (Compound 22 vs. DMSO, 8.8 ± 10.7 vs. 9.4 ± 11.8 ; $P > 0.05$). **Fig. 1B.** Pathological findings of spinal cords 35 days after EAE induction were evaluated. (a) and (b) were stained with hematoxylin-eosin (HE). (c) and (d) were Klüver-Barrera stain (KB). Compound 22-treated mice showed mild demyelination (arrow head).

stained with hematoxylin-eosin (HE) Klüver-Barrera stain (KB) to assess pathological findings. CCR4-KO mice were obtained from Dr. Yoshie (Moriguchi et al., 2013). The mice were backcrossed for at least 8 generations onto the C57BL/6 background. All experiments were performed according to the regulations of the institutional ethics committee. All mice used in these experiments were 8–16 weeks old.

2.2. Peptides

MOG_{35–55} (single-letter amino acid code: MEVGVYRSPFSRVVHL YRNGK) was purchased from Medical and Biological Laboratories Co., Ltd. (Nagano, Japan). HPLC showed that the peptides were >90% pure.

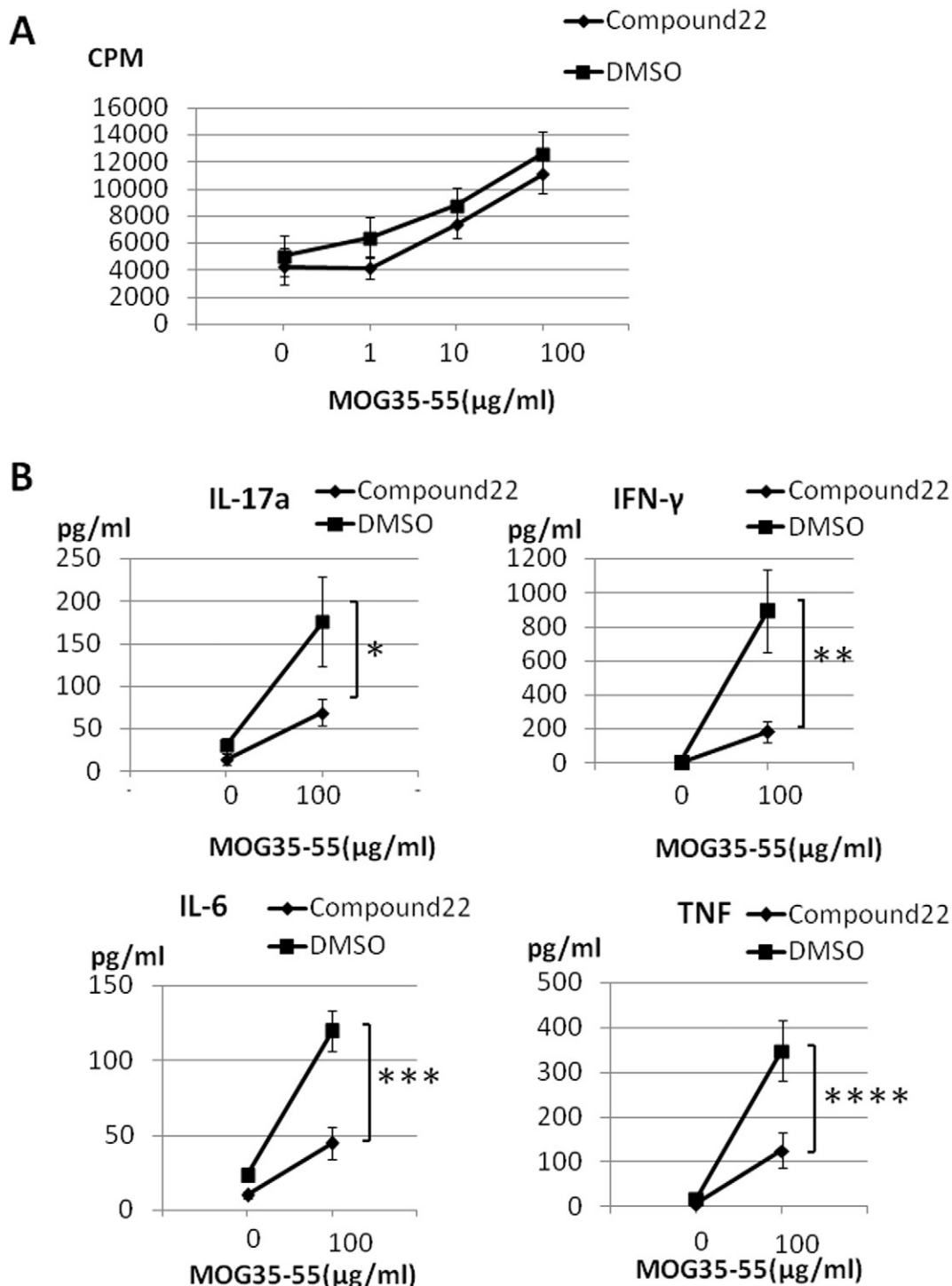


Fig. 2. Comparison of myelin oligodendrocyte glycoprotein (MOG)_{35–55}-induced T cell responses in Compound 22-treated or control mice (Compound 22, n = 5; dimethyl sulfoxide [DMSO], n = 5). A, Proliferative response was determined based on the uptake of [³H]-thymidine. Proliferation was downregulated in the Compound 22-treated mice, although this effect was not significant (Compound 22 vs. DMSO; 11150 ± 3888 vs. 12648 ± 4337). B, Cytokine concentrations in the culture medium were measured using cytometric bead array. Interleukin (IL)-17a (Compound 22 vs. DMSO; 70.0 ± 33.7 vs. 177 ± 118 pg/ml; *P < 0.05), interferon (IFN)-γ (Compound 22 vs. DMSO; 183 ± 138 vs. 893 ± 490 pg/ml; **P < 0.05), and IL-6 (Compound 22 vs. DMSO; 44.9 ± 23.7 vs. 119 ± 30.6 pg/ml; ***P < 0.05), and TNF (Compound 22 vs. DMSO; 127 ± 85.9 vs. 349 ± 151 pg/ml; ****P < 0.05) production were significantly suppressed.

2.3. T cell proliferation assay

For the proliferation assay, mice were immunized with the peptides and CFA as described above, but they were not treated with pertussis toxin. Mice were administered 10 mg/kg Compound 22 ($n = 7$) or DMSO ($n = 7$) intraperitoneally on days 0 and 7. A single-cell suspension was prepared from both inguinal lymph nodes (LNs) on day 12 after immunization. Cells were cultured in RPMI GlutaMAX (Gibco, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum (FBS), and were seeded into 96-well flat-bottomed plates (5×10^5 cells/well). Cells were restimulated with the MOG peptide at 0, 1, 10, and 100 $\mu\text{g/ml}$ for 48 h at 37 °C in a humidified atmosphere with 5% CO_2 . To measure cell proliferation, [^3H]-thymidine was added (1 $\mu\text{Ci/well}$), and radioisotope uptake during the final 18 h of culturing was measured with a beta-1205 counter (Pharmacia, Uppsala, Sweden).

2.4. Cytokine production assay

In parallel, LN cells obtained from the immunized mice were cultured in the presence of the MOG peptide. Culture medium was harvested 48 h after activation, and the levels of various cytokines were measured. Concentrations of interleukin (IL)-2, IL-4, IL-6, IL-10, IL-17a, interferon (IFN)- γ , and tumor necrosis factor (TNF) were measured using a cytometric bead array (BD Bioscience, San Jose, CA). The assay was performed according to the manufacturer's guidelines.

3. Results

3.1. Compound 22 ameliorated the severity of EAE

To examine whether CCR4 antagonism ameliorates the disease course of EAE, C57BL/6 wild type and CCR4-KO mice were immunized to induce EAE. In wild-type mice, Compound 22 significantly ameliorated the severity of EAE, when treatment was started before onset of EAE. When treatment was started after onset, EAE score was slightly lower in Compound 22-treated group, although not significant. In groups treated before onset, maximal scores in mice treated with Compound 22 and DMSO were 0.8 ± 1.1 and 2.6 ± 1.5 , respectively (mean \pm standard deviation; $P < 0.05$ by Mann–Whitney U test). Cumulative scores (summation of the clinical scores from days 1 to day 35) in mice administered Compound 22 and DMSO were 10.3 ± 10.6 and 23.0 ± 12.3 , respectively (mean \pm standard deviation, $P < 0.05$ by Mann–Whitney U test). In groups treated after onset, maximal scores in mice treated with Compound 22 and DMSO were 3.0 ± 0.91 and 3.5 ± 0.55 ($P > 0.05$), respectively. Cumulative scores in mice administered Compound 22 and DMSO were 22.5 ± 8.06 and 25.5 ± 4.29 ($P > 0.05$), respectively.

In CCR4-KO mice, the EAE score was significantly lower than that in the wild-type mice, as previously reported. There was no significant difference between the Compound 22-treated and control groups (8.8 ± 10.7 vs. 9.4 ± 11.8 , $P > 0.05$) (Fig. 1A). Pathological findings revealed that Compound 22-treated mice showed mild demyelination (Fig. 1B).

3.2. Compound 22 did not suppress the proliferative response of MOG-specific T cells

We evaluated the proliferation of MOG_{35–55} responsive T cells obtained from the LNs. Uptake of radioisotopes was downregulated in the cells from the Compound 22-treated mice, although this effect was not significant (Compound 22 vs. DMSO; 11150 ± 3888 vs. 12648 ± 4337 cpm) (Fig. 2A).

3.3. Th1- and Th17-associated cytokine production was suppressed by Compound 22

Cytokine production was measured in the culture medium using the cytometric bead array. IFN- γ (Compound 22 vs. DMSO; 183 ± 138 vs. 893 ± 490 pg/ml; $P < 0.05$), TNF (Compound 22 vs. DMSO; 127 ± 85.9 vs. 349 ± 151 pg/ml; $P < 0.05$), IL-6 (Compound 22 vs. DMSO; 44.9 ± 23.7 vs. 119 ± 30.6 pg/ml; $P < 0.05$), and IL-17a (Compound 22 vs. DMSO; 70.0 ± 33.7 vs. 177 ± 118 pg/ml; $P < 0.05$ by Mann–Whitney U test) production were significantly suppressed (Fig. 2B). These results suggest that CCR4 antagonism might inhibit Th1 and Th17 polarization. IL-2, IL-4 and IL-10 were not detected in both groups.

4. Discussion

EAE is an animal model of human MS mediated by pathogenic T cells expressing various chemokine receptors, of whom Th17 expressing CCR4 is considered to be a major player. In this study, Compound 22 significantly ameliorated EAE severity, and our results suggest that CCR4 antagonism might act mainly during the induction phase. Compound 22 had no impact on the EAE course in CCR4-KO mice, indicating that Compound 22 acts on the CCR4 pathway. Similar to the knockout model, CCR4 antagonism did not augment Th1 polarization. Th2-associated cytokine production was not observed in both the Compound 22-treated and control mice. In Th2 mediated disease model, Compound 22 suppressed chemotaxis and cytokine production of Th2 polarized cells but had little effect on Th1 or CCR4-expressing Th17 polarized cells (Kaminuma et al., 2012).

CCR4 might be a therapeutic target in multiple sclerosis. A CCR4-specific antibody (mogamulizumab) is already available for clinical use to treat adult T cell lymphoma. However, mogamulizumab is not suitable for animal experiments, because it is specific for human CCR4. CCR4 antagonists might be used to understand if CCR4 can serve as a therapeutic target.

Our results suggest that CCR4 antagonism might be used to treat MS. CCR2 antagonists were not successful in human MS, even though CCR2 knockout mice are resistant to murine EAE (Gaupp et al., 2003), and it is possible to treat EAE with CCR2 antagonists (Wang et al., 2009). Therefore, the translation of CCR4 antagonists into clinical research must be planned carefully.

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