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Suppressive effects of a novel CC chemokine receptor 4 antagonist on Th2 cell trafficking in ligand- and antigen-induced mouse models

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ABSTRACT

CC chemokine receptor 4 (CCR4) has been implicated as a preferential marker for T helper type 2 (Th2) cells, and is believed to be involved in the pathology of allergic diseases by controlling Th2 cell trafficking into inflamed tissues. The objective of the study was to characterize the pharmacological properties of E0001-163, a novel CCR4 antagonist. E0001-163 was tested in both *in vitro* chemotaxis assays as well as *in vivo* mouse models of CCR4 ligand-induced air pouch and antigen-induced airway inflammation by utilizing *in vitro*-polarized Th2 cells. *In vitro*, E0001-163 inhibited migratory response of human Th2-polarized cells to CCL22, a CCR4 ligand, with an IC_{50} value of 11.9 nM. E0001-163 significantly suppressed CCL22-induced Th2 cell trafficking into mouse air pouch in a dose-dependent manner at doses of 3 and 10 mg/kg, suggesting that E0001-163 has an inhibitory effect on CCR4-mediated T cell trafficking *in vivo*. In addition, E0001-163 partially decreased Th2 cell trafficking and the level of IL-4 in the lungs in Th2-tansferred and ovalbumin (OVA)-challenged mice. T cell trafficking involves multiple chemokine receptors both in acute and chronic phases, and our findings suggest that CCR4, together with other chemokine receptors, may be involved in Th2 cell trafficking under disease conditions.

1. Introduction

$CD4^+$ helper T cells have a pivotal role in mediating adaptive immunity to diverse pathogens and in promoting autoimmune diseases and allergic inflammation (Zhu et al., 2010). During inflammation and after T cell receptor activation, specific cytokines and distinct master transcription factors program naive $CD4^+$ T cells to differentiate into at least 4 distinct subsets, namely, Th1, Th2, Th17, and Treg cells (Islam and Luster, 2012; Jiang and Dong, 2013). Polarizing cytokines and master transcription factors that promote $CD4^+$ T cell subset differentiation also program the expression of unique set of chemokine receptors on $CD4^+$ T cell subsets during priming. Th1 cells are programmed during lineage differentiation to selectively express CCR5 and CXCR3, while Th2 cells CCR3, CCR4, CCR8, and the prostaglandin D2 chemoattractant receptor DP2 or CRTH2 (Andrew et al., 2001; Bonecchi et al., 1998; Kim et al., 2003; Sallusto et al., 1998; Wang et al., 2009).

Th2 cells express a variety of chemokine receptors, even though the majority of research has centered on the role of CCR4 as a key regulator of Th2 cell trafficking. CCR4 has been implicated in the recruitment of antigen-specific Th2 cells to inflamed tissues (Campbell et al., 1999; Nouri-Aria et al., 2002; Zheng et al., 2003). In asthmatic patients, $CCR4^+$ T cells are preferentially accumulated in airway and produce Th2-type cytokines (Panina-Bordignon et al., 2001; Vijayanand et al., 2010). Furthermore, concentrations of CCR4 ligands, CCL17 and CCL22, are elevated in the lung of asthmatic patients (Pilette et al., 2004; Sekiya et al., 2000; Ying et al., 2008). However, deletion of CCR4 gene and/or CCR4 pharmacological intervention has yielded some conflicting results in mouse models, and the contribution of CCR4 in allergic inflammation still remains controversial (Chvatchko et al., 2000; Conroy et al., 2003; Kaminuma et al., 2012; Othy et al., 2012; Purandare et al., 2007; Sato et al., 2009). Recent studies on *in vivo* role of trafficking studies suggest that the kinetics of chemokine and chemokine receptor expression on antigen-specific T cells might be changed during acute or chronic allergen challenge (Mikhak et al., 2009; Oyoshi et al., 2011). T cell trafficking during those phases involves multiple chemokines and chemokine receptors, and the roles of each receptor will vary depending on the inflamed organ and temporal phase of the disease.

Here, we present data detailing the characteristics of E0001-163, a novel small molecule antagonist of CCR4. To evaluate the potency

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of the CCR4 antagonist *in vivo*, we developed a mouse air pouch model in which infiltration of lymphocytes after injection of a stimulus can be observed. We performed the experiments in mice transferred with *in vitro*-polarized Th2 cells and injected with CCL22-expressing cells, displayed a sustained production of CCL22 in air pouch. We also used OVA-induced airway inflammation mouse model by adoptively transferring DO11.10 Th2 cells into mice which were then exposed to OVA aerosol to monitor the accumulation of transferred-Th2 cells and eosinophils in the bronchoalveolar lavage fluid. Effects of E0001-163 not only on Th2 cell trafficking in mice, but on migratory function of human Th2 cells *in vitro* have been characterized.

2. Materials and methods

2.1. Chemicals

E0001-163, N-[5-bromo-3-({3-[2-(dimethylamino)ethoxy]-4-methoxybenzyl}oxy)-2-pyrazinyl]-4-methylbenzenesulfonamide (Fig. 1) was synthesized and purified by Ono Pharmaceutical Co., Ltd. (Osaka, Japan).

2.2. Experimental animals

Male BALB/cAnNCrj and male DBA/2NCrj mice were obtained from Charles River Laboratories Japan (Yokohama, Japan). Male BALB/c-TgN (DO11.10) mice were obtained from The Jackson Laboratory (Maine, USA). Animal studies were conducted in compliance with the Guidelines for Animal Studies established by Research Headquarters, Ono Pharmaceutical Co., Ltd.

2.3. Binding assay

CCRF-CEM cells (ATCC, Virginia, USA) were seeded into 96-well plates at 5×10^5 cells per well in binding buffer (RPMI-1640 (Life Technologies, Carlsbad, CA, USA) medium containing 20 mM Hepes (Life Technologies) and 1% of bovine serum albumin (BSA; Sigma-Aldrich, St Louis, MO, USA)). Binding reactions were performed at 4 °C for 1 h in the presence of [^{125}I] CCL17 (Perkin Elmer Life and Analytical Sciences, Waltham, MA, USA) and various concentrations of E0001-163. After incubation, the reaction mixtures were harvested using 0.5% of polyethyleneimine-pretreated Unifilter-96 GF/B plates, and the filters were dried and counted on TopCount with MicroScint-20 (all reagents and equipment purchased from Perkin Elmer Life and Analytical Sciences).

2.4. Preparation of Th1 and Th2 Cells from human peripheral blood

Human peripheral blood was obtained from two healthy volunteers who provided a signed informed consent. The studies using human blood were approved by the institutional ethics committee. Peripheral blood mononuclear cells (PBMC) from blood were separated by Lymphoprep (Accu-Prep, Oslo, Norway) gradient density centrifugation. CD4$^+$ T cells were isolated from PBMC fraction using human T-cell enrichment column (R&D Systems, Minneapolis, MN, USA) according to the manufacture's protocol. 24-well plates were coated overnight with 2 μg/mL anti-CD3 Ab (OKT3; Janssen Pharmaceuticals, Titusville, NJ, USA) and then washed with phosphate-buffered saline (PBS). CD4$^+$ T cells were cultured at 2×10^5 cells per well in culture medium (RPMI1640 supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich), 2-mercaptoethanol, Penicillin–Streptomycin, 10 mM Hepes, 0.1 mM MEM non-essential amino acid solution, 1 mM MEM sodium pyruvate (all reagents but FBS purchased from Life Technologies)), 4 ng/mL IL-2, and 10 μg/mL anti-CD28 (Day 0). 5 ng/mL IL-12 and 1 μg/mL anti-IL-4 were used to differentiate Th1, while 5 ng/mL IL-4 and 1 μg/mL anti-IFN-γ were used to differentiate Th2 (all proteins and antibodies purchased from BD Biosciences, San Jose, CA, USA). After 5 days, the activated Th1 and Th2 cells were washed in RPMI1640 and expanded for 4 days in culture medium containing 1 ng/mL IL-2. Following this, the resting Th1 and Th2 cells were restimulated for 5 days with OKT3, anti-CD28, and cytokines described above, but with the addition of 1 mg/ml anti-CD95L (BD Biosciences) to prevent apoptosis. After 5 days, the activated Th1 and Th2 cells were washed and then expanded again with IL-2 for 7 days. Activated and resting Th1/Th2 cells were maintained in this way for three cycles.

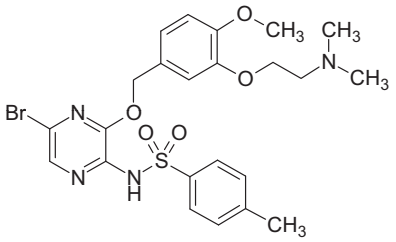

Fig. 1. Chemical structure of E0001-163. N-[5-bromo-3-({3-[2-(dimethylamino) ethoxy]-4-methoxybenzyl}oxy)-2-pyrazinyl]-4-methylbenzenesulfonamide.

2.5. Analysis of chemokine receptor expression

CD4$^+$ T cells at Day 0, Th1- and Th2-polarized cells on Day 5, 9, 14, 17, 24, 29 were stained with fluorochrome-conjugated Abs against CD4, CCR4 or CXCR3 (all purchased from BD Biosciences) in PBS containing 2% FBS and 0.1% NaN_3 for 0.5 h on ice and then washed. The fluorescence intensity of antibodies bound to receptors was measured using flow cytometry (FACSCalibur, BD Biosciences). For each sample, at least 15,000 events were collected. To calculate the relative expression level of CCR4 or CXCR3, the median fluorescence intensity (MFI) values of receptor expression was obtained by subtracting the MFI of the isotype control antibody. Data were analyzed using FACSCalibur software (BD Biosciences).

2.6. Preparation of Th1 and Th2 Cells from mouse spleen

For chemotaxis assays and CCL22-induced T cell trafficking model, CD4$^+$ T cells were isolated from spleens of BALB/c mice by using mouse CD4 subset enrichment column (R&D Systems). Purified CD4$^+$ T cells were activated in a 5 μg/mL anti-mouse TCRβ (BD Biosciences)-coated 24 well plate for 3 days. Th2 cells were generated by activating the cells in the presence of 1 μg/mL anti-CD28 (Southern Biotechnology Associates, Birmingham, Alabama, USA), 20 ng/mL IL-4 (BD Biosciences), and 2.5 μg/mL anti-IL-12 (BD Biosciences), while Th1 cells by 1 μg/mL anti-CD28, 20 ng/mL IL-12 (BD Biosciences), and 2.5 μg/mL anti-IL-4 (BD Biosciences). Th2-polarized cells were then cultured with 2 ng/mL IL-2 (BD Biosciences) for 3 days and used for experiments. To assess the degree of Th1 and Th2 polarization, cells were activated in an anti-mouse TCRβ-coated plate at 37 °C for 6 h in the presence of monensin (Santa Cruz Biotechnology, CA, USA). The cells were fixed with 4% paraformaldehyde and permeabilized with 0.5% Triton X-100 and then stained with anti-IL-4 (BD Biosciences) and anti-IFN-γ (BD Biosciences).

For antigen-induced inflammation model, OVA-specific naïve CD4$^+$ T cells were isolated from the spleens of DO11.10/RAG2$^{-/-}$ mice by using mouse CD4 subset enrichment column (R&D Systems). 5.0×10^5 CD4$^+$ T cells were then cultured with 5.0×10^5 mitomycin C (Sigma-Aldrich)-treated BALB/c splenocytes at 37 °C in culture