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TITLE PAGE

E6130, a novel CX3CR1 modulator, attenuates mucosal inflammation and reduces CX3CR1^{+} leukocyte trafficking in mice with colitis

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RUNNING TITLE PAGE

Running title:

Characterization of a modulator of chemokine receptor CX3CR1

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Nonstandard Abbreviations:

CX3CR1, CX3C chemokine receptor 1; MFI, mean fluorescence intensity; NK cells, natural killer cells

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ABSTRACT

The chemokine fractalkine (CX3C chemokine ligand 1; CX3CL1) and its receptor, CX3CR1, are involved in the pathogenesis of several diseases, including inflammatory bowel diseases such as Crohn's disease and ulcerative colitis, rheumatoid arthritis, hepatitis, myositis, multiple sclerosis, renal ischemia, and atherosclerosis. There are no orally available agents that modulate the fractalkine/CX3CR1 axis. E6130 is an orally available highly selective modulator of CX3CR1 that may be effective for treatment of inflammatory bowel disease. We found here that E6130 inhibited the fractalkine-induced chemotaxis of human peripheral blood natural killer cells (IC_{50}, 4.9 nM), most likely via E6130-induced down-regulation of CX3CR1 on the cell surface. E6130 had agonistic activity via CX3CR1 with respect to GTPγS binding in CX3CR1-expressing CHO-K1 membrane but did not have any antagonistic activity. Orally administered E6130 ameliorated several inflammatory bowel disease–related parameters in a murine $CD4^{+}CD45RB^{high}$ T-cell-transfer colitis model and a murine oxazolone-induced colitis model. In the $CD4^{+}CD45RB^{high}$ T-cell transfer model, E6130 inhibited the migration of $CX3CR1^{+}$ immune cells and decreased the number of these cells in the gut mucosal membrane. These results suggest that E6130 is a promising therapeutic agent for treatment of inflammatory bowel disease.

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INTRODUCTION

Inflammatory bowel disease is a group of idiopathic, chronic intestinal inflammatory diseases that primarily includes two distinct conditions: Crohn's disease and ulcerative colitis, which are both the result of inappropriate immune responses to antigens produced by commensal microorganisms. Although the signs of Crohn's disease and ulcerative colitis manifest primarily in the gastrointestinal tract, these diseases affect the entire human body (Baumgart and Sandborn, 2007). Therapeutic treatments that inhibit cytokines are currently in widespread clinical use; however, the serious adverse events and lack of long-term efficacy of these treatments remain serious concerns.

Infiltration of leukocytes from the peripheral circulation into tissues such as the gut mucosa is an important aspect of immune surveillance. To enter the mucosal tissue from the blood, leukocytes must cross the endothelial barrier. They do this by interacting with cytokine- or other pro-inflammatory stimuli-activated endothelial cells via either leukocyte cell-surface chemokine receptors (and their respective endothelial and mucosal ligands) or via integrins and immunoglobulin superfamily cellular adhesion molecules (Charo and Ransohoff, 2006; Thomas and Baumgart, 2012).

Chemokines and their receptors are also important means of orchestrating tissue-specific and cell type–selective leukocyte trafficking (Nishimura et al., 2009). For

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example, fractalkine (also known as CX3C motif ligand 1; CX3CL1) is a chemokine prominently expressed by epithelial and endothelial cells that functions as both an adhesion molecule and a chemotactic factor (Bazan et al., 1997; Nishimura et al., 2002). Fractalkine binds to its sole receptor, CX3C chemokine receptor 1 (CX3CR1), which is expressed on the surfaces of monocytes, macrophages, dendritic cells, microglia, natural killer (NK) cells, and cytotoxic effector T-cells that contribute to the development of chronic inflammation (Imai et al., 1997; Yoshie et al., 2001). In patients with inflammatory bowel disease, the expression of fractalkine by intestinal epithelial cells and microvascular cells and of CX3CR1 by peripheral blood T-cells increases with the level of disease activity. Thus, the fractalkine/CX3CR1 axis appears to be directly involved in the pathogenesis of inflammatory bowel disease (Brand et al., 2006; Kobayashi et al., 2007; Sans et al., 2007).

Here, we characterized E6130, a previously identified novel modulator of CX3CR1. We found that E6130 inhibited fractalkine-induced chemotaxis and induced down regulation of CX3CR1 on the cell surface in NK cells from human peripheral blood. E6130 also prevented the trafficking of CX3CR1-expressing leukocytes into colonic tissue in a murine $CD4^{+}CD45RB^{high}$ T cell–transfer colitis model, and it ameliorated other inflammatory bowel disease–related parameters in both the murine

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$CD4^{+}CD45RB^{high}$ T-cell-transfer colitis model and a murine oxazolone-induced colitis model. Together, these results suggest that E6130 is a promising agent for treating the inflammatory bowel disease.

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MATERIALS AND METHODS

Animals

BALB/c mice (female, 7–10 weeks old) were obtained from Charles River Laboratories Japan, Inc. (Tokyo, Japan). SCID mice (female, 6 weeks old) were obtained from CLEA Japan, Inc. (Tokyo, Japan). The mice were group-housed under controlled conditions (temperature, 22 ± 3 °C; humidity, 55% ± 5%; 12-h light/dark cycle) with *ad libitum* access to water and standard pelleted food. All animal experiments were approved by the Committee for the Welfare of Laboratory Animals, Eisai Co. Ltd.

Test compound and reagents

E6130 was synthesized by Eisai Co., Ltd. at its plant located in Kashima, Japan (Yoshida et al., 2013). The chemical structure of E6130 is shown in Fig. 1. Oxazolone was purchased from Sigma-Aldrich (St. Louis, MO). Recombinant human fractalkine was purchased from R&D Systems (Minneapolis, MN).

CX3CR1 down-regulation assay

Assay medium (RPMI 1640 medium containing 10% fetal bovine serum) (80 μL/well)

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and E6130 (10 μL/well) were added to each well of a 96-well culture plate. Written informed consent was obtained from each subject before blood draw. Human peripheral blood was drawn from the forearm vein of three healthy male volunteers and an aliquot (10 μL) was added to each well. After incubation for 30 min at 37 °C under an atmosphere of 5% CO_2, the human peripheral blood cells were washed twice with Flow cytometry (FCM) buffer (phosphate-buffered saline [PBS] containing 1% fetal bovine serum, 1 mM EDTA, 0.1% bovine serum albumin). The cells were incubated with FcR Blocking Reagent (Miltenyi Biotec, Germany) for 10 min on ice to block Fc receptor and then stained for CD56 (NK cell surface marker) and CX3CR1 by incubation with allophycocyanin-conjugated anti-CD56 monoclonal antibody (Miltenyi Biotec) and phycoerythrin-conjugated anti-CX3CR1 monoclonal antibody (BioLegend, CA), respectively, for 30 min on ice. The cells were then washed once and resuspended in FCM buffer. After incubation for 10 min on ice with 7-amino actinomycin D (Sigma-Aldrich) to discriminate dead cells, the fluorescence intensity of the stained cells was determined by using a FACSCanto cell analyzer (Becton Dickinson, NJ). Loss of CX3CR1 staining on the cell surface was assumed to represent the induction of down-regulation of CX3CR1 on the cell surface. The mean fluorescence intensity (MFI) of the $CX3CR1^+$ cell population among the total NK cell population was also

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determined by using a FACSCanto cell analyzer. The percentage MFI of the control was determined by using the following formula:

$$\% \text{ MFI of control} = (MFI_{\text{test well}} - MFI_{\text{background well}}) / (MFI_{\text{control well}} - MFI_{\text{background well}}) \times 100,$$

where "test well" refers to wells containing E6130 and staining antibodies, "control well" refers to wells containing staining antibodies only, and "background well" refers to wells containing neither the staining antibodies nor E6130.

Isolation of NK cells from human peripheral blood

An aliquot (25 mL) of human peripheral blood was added to a plastic centrifugation tube containing 100 units of heparin sodium (Ajinomoto, Japan). An aliquot (8 mL) of physiological saline containing 6% dextran (Nacalai Tesque, Japan) was added to the tube and the mixture was allowed to stand at room temperature for 30 min to allow sedimentation of the erythrocytes. The supernatant was transferred to another plastic centrifugation tube, mixed with an equivalent volume of PBS, and then centrifuged at 1800 rpm for 7 min at room temperature. The resultant hemocyte fraction was suspended in 4 mL PBS and the suspension was superposed on 4 mL Ficoll-Paque Plus (GE Healthcare Life Sciences, Japan). After centrifugation of the suspension at 2200

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rpm for 30 min at room temperature, the cells in the intermediate layer (peripheral blood mononuclear cells) were collected, suspended in PBS, and centrifuged at 1800 rpm for 7 min. The supernatant was removed and the precipitate was suspended in PBS containing 5 mM EDTA and 0.5% bovine serum albumin. NK cells were purified from the peripheral blood mononuclear cell population by means of negative selection using an NK cell isolation kit (Miltenyi Biotec) and a MACS LS column (Miltenyi Biotec). Cells passing through the column were considered NK cells and were collected, washed, and suspended in chemotaxis buffer (RPMI 1640 medium containing 10% fetal bovine serum, 50 μM 2-mercaptoethanol, and 10 mM HEPES).

Chemotaxis assay

Human fractalkine solution (solved in chemotaxis buffer, 60 μL/well) and chemotaxis buffer (480 μL/well) were added to the lower wells of a 24-well chemotaxis assay chamber (Boyden chamber, pore size: 5.0 μm) with final concentration of 0.2 nM. E6130 solution (60 μL/well) was also added to the lower wells. A mixture of NK cell suspension and E6130 was added to the upper wells and the chambers were incubated for 2 h at 37 °C under an atmosphere of 5% CO_2. After incubation, the content of the upper wells was discarded and the assay chambers were centrifuged at 1800 rpm for 5

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min. The supernatant was removed and a CellTiter-Glo reagent (Promega Corporation, Japan) was added to the precipitate. Chemiluminescence intensity was measured by using a Wallac 1420 ARVOsx multi-label counter (PerkinElmer Japan). The number of cells in each well was calculated by using a standard curve fitting the regression line for cell number. The percentage of cells exhibiting chemotaxis was determined by using the following formula:

% of control = (cell number$_{\text{test well}}$ − cell number$_{\text{blank well}}$) / (cell number$_{\text{control well}}$ − cell number$_{\text{blank well}}$) × 100,

where “test well” refers to wells containing fractalkine and E6130, “blank well” refers to wells containing neither fractalkine nor E6130, and “control well” refers to wells containing fractalkine only.

GTPγS binding assay

Scintillation Proximity Assay (SPA) [^{35}S]GTPγS experiments were conducted using CHO-K1 membranes stably expressing human chemokine receptors incubated in assay buffer (20 mM HEPES [pH 7.4], 100 mM NaCl, 10 μg/mL saponin, 1 mM $MgCl_2$). In the agonistic activity assay, E6130 or reference ligand, assay buffer, CHO-K1 membrane - GDP mix, [^{35}S]GTPγS and WGA-PVT beads (Perkin Elmer, MA) mix were

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successively added to the wells of an OptiPlate (Perkin Elmer, MA). In the antagonistic activity assay, E6130 or reference ligand and CHO-K1 membrane-GDP mix were successively added to the wells of an OptiPlate and the plates were incubated for 15 min at room temperature. A reference agonist at its historical EC_{80} was then added, followed by the addition of [^{35}S]GTPγS and WGA-PVT beads.

In both assays, the plates were covered with a top seal, shaken on an orbital shaker for 2 min, and then incubated for 1 h at room temperature. The plates were then centrifuged for 10 min at 2000 rpm and incubated at room temperature for 1 h. Each well was counted for 1 min by using a PerkinElmer TopCount reader.

PathHunter β-arrestin recruitment assay

PathHunter is a trademark of DiscoveRx (Bassoni et al., 2012; Zhao et al., 2008). PathHunter cell lines were expanded from freezer stocks in T25 flasks according to standard procedures and maintained in selective growth media before assay. Once it was established that the cells were healthy and growing normally, the cells were passaged from the flasks by using cell dissociation reagent and seeded into white-walled, clear-bottomed, 384-well microplates. Cells were seeded at a density of 5000/well in a total volume of 20 μL and were allowed to adhere and recover overnight before the

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addition of E6130.

In the agonistic activity assay, intermediate dilutions of E6130 stock solutions were generated such that 5 μL of E6130 could be added to each well to produce a final concentration of DMSO of 1% of the total volume. The cells were incubated in the presence of E6130 at 37 °C for 90 min.

In the antagonistic activity assay, agonist dose curves were constructed and used to determine EC_{80} values. Cells were pre-incubated with antagonist; this was followed by agonist challenge, as follows: 5 μL of E6130 was added to the cells and the plates were incubated at 37°C for 30 min. Then, 5 μL of agonist at its EC_{80} was added to the cells and the plates were incubated at 37 °C for 90 min.

The assay signal was generated through the addition of 12.5 or 15 μL (50% v/v) of PathHunter Detection reagent cocktail for the agonistic and antagonistic activity assays, respectively, followed by incubation for 1 h at room temperature. The microplates were then read with a ParkinElmer EnVision multilabel plate reader.

Dose curves in the presence of E6130 were plotted by using GraphPad Prism or Activity Base software. In the agonistic activity assay, percentage activity was calculated by using relative light units (RLU) and the following formula: % Activity = 100% × (mean $RLU_{test\ sample}$ − mean $RLU_{vehicle\ control}$) / (mean MAX $RLU_{control\ ligand}$ −

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mean $RLU_{vehicle\ control}$). In the antagonistic activity assay, percentage inhibition was calculated by using the following formula: % Inhibition = 100% × (1 − [mean $RLU_{test\ sample}$ − mean $RLU_{vehicle\ control}$] / mean $RLU_{EC80\ control}$ − mean $RLU_{vehicle\ control}$).

$CD4^+CD45RB^{high}$ T-cell preparation and T-cell-transfer colitis model

$CD4^+CD45RB^{high}$ T cells were isolated from BALB/c splenocytes. BALB/c mice were euthanized by means of cervical dislocation and their spleens were removed. The spleens were passed through a cell strainer (BD Biosciences) and a single-cell suspension of splenocytes was prepared. After hemolysis, the $CD4^+$ T-cell population was purified from the splenocytes by means of negative selection by using a mouse $CD4^+$ T-cell Isolation Kit II (Miltenyi Biotec) and a MACS LS column. For the preparation of $CD4^+CD45RB^{high}$ T cells, the isolated total $CD4^+$ T cells were labeled with phycoerythrin-conjugated mouse anti-CD4 monoclonal antibody (eBioscience, CA) and fluorescein isothiocyanate-conjugated mouse anti-CD45RB monoclonal antibody (eBioscience), and two-color cell sorting was performed by using a FACSAria cell sorter (Becton Dickinson). The sorted $CD4^+CD45RB^{high}$ T cells were suspended in PBS and intravenously transferred into SCID mice. After 2 weeks, E6130 or vehicle (0.5% methyl cellulose (0.5% MC)) was orally administered once daily to the SCID

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mice from Day 14 to Day 27. Stool consistency and body weight of all mice were recorded on Days 14, 16, 18, 20, 22, 24, 26, and 28. On Day 28, the mice were euthanized, their colons were removed, and the weight and length of each colon was recorded. Stool consistency was scored as follows: (0) Normal, (1) Soft stool (well-formed pellets), (2) Soft stool (very soft but formed pellets), (3) Loose stool (pasty stool), and (4) Diarrhea (liquid stool that sticks to the anus). Relative body weight was calculated by using the following formula: Relative body weight (%) = body weight (g) on day of measurement / body weight (g) on Day 14 × 100.

Next, the colons were washed with PBS and then incubated for 30 min at 37 °C in PBS containing 1 mM dithiothreitol, 1 mM EDTA, and 1% fetal calf serum to remove epithelial cells. The remaining tissue was washed with PBS, cut into 1- to 2-mm sections, and incubated for 2.5 h at 37 °C in RPMI 1640 containing 0.5 mg/mL collagenase A, 1 μg/mL DNase, and 5% fetal calf serum. After incubation, the suspension was passed through a cell strainer and the resultant cell suspension was centrifuged at 700 × g for 7 min at room temperature. The resultant enterocyte precipitate was suspended in 4 mL of PBS and the suspension was superposed on Percoll (GE Healthcare Life Sciences). After centrifugation at 700 × g for 20 min at room temperature, the cells in the intermediate layer were collected, suspended in PBS,

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and centrifuged at 700 × g for 7 min at 4 °C. The supernatant was removed and the precipitate was suspended in FACS buffer (PBS containing 1 mM EDTA, 0.1% bovine serum albumin, and 1% fetal calf serum). $CX3CR1^{+}$ cells were labeled with phycoerythrin-conjugated goat anti-CX3CR1 polyclonal antibody (R&D Systems) and the proportion of $CX3CR1^{+}$ cells was determined with a FACSCanto cell analyzer (Becton Dickinson).

Pharmacokinetics and histological analysis in T-cell-transfer colitis model

For pharmacokinetic study, the sorted $CD4^{+}CD45RB^{high}$ T cells, as described above, were suspended in PBS and intravenously transferred into SCID mice. After 2 weeks, E6130 or vehicle was orally administered once daily to the SCID mice from Day 15 to Day 30. Blood samples were withdrawn at 30 min, 2 and 6 h after administration of E6130 on Day 29. After centrifugation of mouse blood, concentrations were quantitatively determined by a mass spectrometer equipped with a high performance liquid chromatography system. Plasma protein binding was evaluated in plasma at 2 h by equibrium method.

For histological analysis, the colon was collected from mice on Day 30, fixed in 10% neutral buffered formalin, embedded in paraffin, and processed for microscopic

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examination of hematoxylin and eosin (H&E) stained sections.

Oxazolone-induced colitis model

BALB/c mice were sensitized with oxazolone on Day 0 by painting 3% oxazolone ethanol solution onto the skin of the abdomen. Five days after sensitization, oxazolone solution was intrarectally injected into the mice under isoflurane anesthesia to induce colitis. In vehicle group, ethanol/water (1:1) vehicle solution was intrarectally injected into the mice instead of oxazolone solution. E6130 was orally administered 1 h before intrarectal injection of oxazolone. The daily administration of E6130 continued from Day 5 to Day 7. Two hours after the administration of E6130 on Day 7, the mice were euthanized by cervical dislocation, their colons were removed, and the length (mm) of the colons was recorded. Body weight ratio was calculated by using the following formula: Body weight ratio (%) = (Body weight at Day 7) / (Body weight at Day 5) × 100.

Statistical analysis

Data are presented as means ± SEM. In stool score and relative body weight of a murine $CD4^+$ $CD45RB^{high}$ T-cell transfer colitis model, differences between

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$CD4^+CD45RB^{high}$ T-cell-transferred group and total-$CD4^+$ T-cells- or E6130-treated group were analyzed by repeated measures analysis of variance (RM-ANOVA) followed by Fisher's LSD test adjusted. In other parameters, differences between $CD4^+CD45RB^{high}$ T-cell-transferred group and total-$CD4^+$ T-cells- or E6130-treated group were analyzed by 1-way analysis of variance (ANOVA) followed by Fisher's LSD test. We used Bonferroni correction for a final multiple comparison to correct for inflation of type 1 error, and significance level was set to 0.001 (0.05 ÷ 48). In a murine oxazolone-induced-colitis model, differences between oxazolone-injected group and vehicle (ethanol/water)-injected or E6130-treated group were analyzed by 1-way analysis of variance (ANOVA) followed by Fisher's LSD test. We used Bonferroni correction for a final multiple comparison to correct for inflation of type 1 error, and significance level was set to 0.00625 (0.05 ÷ 8). Statistical analyses were performed by using GraphPad Prism software (GraphPad Software).

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RESULTS

Effect of E6130 on fractalkine-induced chemotaxis by NK cells isolated from human peripheral blood

Previously, E6130 (Structure: Fig. 1) was identified from a G-protein coupled receptor (GPCR)-focused library by using a fluorometric microvolume assay technology scanner designed to perform high-throughput screening assays using multiwell plates (Yoshida et al., 2013). The library was screened by using an in vitro chemotaxis assay and an in vivo murine inflammatory bowel disease model (unpublished results).

We used a cell-based assay to assess the effects of E6130 on fractalkine-induced chemotaxis. Human NK cells were used, because most NK cells in human peripheral blood express CX3CR1 on their surfaces and NK cells can be easily isolated in large numbers. NK cells were preincubated with E6130 and chemotaxis was induced by incubation with fractalkine for 2 h by using a multiwell chemotaxis chamber. Marked migration of NK cells was observed against 0.2 nM fractalkine (number of cells in blank well: 887 ± 324, number in fractalkine-containing well: 8615 ± 2936). However, E6130 inhibited the chemotaxis of NK cells, with an IC_{50} value of 4.9 nM (Fig. 2a).

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Effect of E6130 on CX3CR1 down-regulation on the cell surface of human peripheral blood NK cells

E6130 exhibited no antagonistic activity on [^{35}S]GTPγS binding via CX3CR1 (Table 1). Therefore, we hypothesized that E6130 prevents fractalkine from binding to the receptor by inducing down-regulation of CX3CR1 on the cell surface, leading to inhibition of the fractalkine-induced chemotaxis of human leukocytes. To test this hypothesis, human peripheral blood was incubated with E6130 for 30 min at 37 °C and then stained with monoclonal antibodies to CD56 and CX3CR1. The fluorescence intensity of CX3CR1 on CD56$^+$ NK cells was evaluated by means of flow cytometry. E6130 induced the down-regulation of CX3CR1 on the cell surface of CD56$^+$ NK cells with an EC_{50} value of 5.2 nM (Fig. 2b).

Effect of E6130 on GTPγS binding via CX3CR1

When activated, CX3CR1 changes conformation to expose a G-protein complex binding site. Once this G-protein complex is bound, the G_α subunit releases GDP and binds GTP. Agonist-activated GPCR induces receptor phosphorylation mediated by GPCR kinases, leading to the recruitment of β-arrestin. Upon receptor phosphorylation and β-arrestin binding, most GPCRs internalize into clathrin-coated vesicles. Therefore,

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next we examined the effects of E6130 on [^{35}S]GTPγS binding and β-arrestin recruitment by CX3CR1 in CX3CR1-expressing CHO-K1 membrane. E6130 had agonistic activity with respect to [^{35}S]GTPγS binding (EC_{50} = 133 nM) and β-arrestin recruitment (EC_{50} = 2.4 μM) through CX3CR1 (Fig. 3). No antagonistic activity of E6130 was observed in the presence of fractalkine (IC_{50} value: >10 μM) (data not shown).

The agonistic and antagonistic activities of E6130 with respect to [^{35}S]GTPγS binding and β-arrestin recruitment were also examined by using CHO-K1 membrane expressing human chemokine receptors. E6130 had agonistic activity through chemokine orphan receptor 1 for β-arrestin recruitment at 10 μM. However, no significant agonistic or antagonistic activity (EC_{50} and IC_{50} values: >10 μM) of E6130 was observed with respect to [^{35}S]GTPγS binding or β-arrestin recruitment through any other chemokine receptor (Table 1).

Effects of E6130 in a murine CD4^{+}CD45RB^{high} T-cell-transfer colitis model

The in vivo efficacy of E6130 after oral administration was assessed by using a murine CD4^{+}CD45RB^{high} T-cell-transfer colitis model. E6130 (10 or 30 mg/kg) was orally administered to the SCID mice once a day. Oral availability of E6130 was

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confirmed with pharmacokinetic study in the mice (Table 2). The total $CD4^+$ T-cell population was used as the negative control. Stool consistency and body weight were recorded, and colons were excised and their weight and length were determined because colon length-to-weight ratio correlates well with histopathological scores (Ostanin et al., 2009). The number of $CX3CR1^+$ lymphocytes in the lamina propria was determined by means of flow cytometry. Oral administration of E6130 significantly ($p < 0.001$) improved stool consistency from Day 18 at doses of 10 and 30 mg/kg compared with $CD4^+CD45RB^{high}$ T-cell-transferred group, and significantly ($p < 0.001$) ameliorated body weight loss from Day 22 at doses of 10 and 30 mg/kg (Fig. 4a, b). Furthermore, oral administration of E6130 significantly ($p < 0.001$) ameliorated the increased colon weight to length ratio as well as the increase in the number of $CX3CR1^+$ lymphocytes in the lamina propria at doses of 10 and 30 mg/kg (Fig. 4c, d). These effects of E6130 were histopathologically confirmed by a marked reduction in mucosal thickness due to much less inflammatory cell infiltration and crypt hyperplasia in treated animals (Fig. 4e, f).

Effects of E6130 in a murine oxazolone-induced-colitis model

The in vivo efficacy of E6130 after oral administration was assessed in a murine

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oxazolone-induced-colitis model. Body weight loss and shrinkage of the colon are well recognized as features of hapten-induced colitis model (Kojima et al., 2004; Ishiguro et al., 2010). At a dose of 30 mg/kg, E6130 significantly ($p < 0.00625$) prevented the colon-length shortening, and significantly ($p < 0.00625$) suppressed the body weight loss on day 7 (Fig. 5).

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DISCUSSION

Inflammatory bowel diseases such as Crohn's disease and ulcerative colitis are characterized by a chronic clinical course of relapse and remission. In both Crohn's disease and ulcerative colitis, leukocyte infiltration into the intestine is a fundamental event in the development and progression of the disease. Indeed, $CX3CR1^{+}CD4^{+}$ T cells are increased in the peripheral blood and inflamed tissues of patients with inflammatory bowel disease. Fractalkine is expressed by epithelial cells in the colonic tissue and the expression is greater in the inflamed tissues of patients with Crohn's disease or ulcerative colitis (Kobayashi et al., 2007), suggesting that the fractalkine/CX3CR1 axis plays an important role in the pathogenesis of inflammatory bowel disease. Therefore, inhibition of the trafficking of $CX3CR1^{+}$ leukocytes represents a potential means of treating inflammatory bowel disease.

We found here that E6130 had an inhibitory activity against fractalkine-induced chemotaxis in NK cells isolated from human peripheral blood. The down-regulation of CX3CR1 on the cell surface induced by E6130 mainly contribute to the inhibition of fractalkine-induced chemotaxis, because the down-regulation of CX3CR1-inducing activity of E6130 (EC_{50} = 5.2 nM) was more potent than its agonistic activity with respect to GTPγS binding (EC_{50} = 133 nM) and β-arrestin recruitment (EC_{50} = 2.4 μM).

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E6130 exhibited no antagonistic activity on [^{35}S]GTPγS binding via CX3CR1. We cannot completely ruled out the possibility that E6130 acts through a mechanism other than CX3CR1, because E6130 was shown to affect ACKR3/CMKOR1 in β-arrestin recruitment at the high concentration of E6130 (10 μM) (Table 1). In vitro data suggested that E6130 induced down-regulation of CX3CR1 on the cell surface, thereby inhibiting the interaction between fractalkine and CX3CR1, leading to inhibition of fractalkine-induced chemotaxis.

Numerous efforts have been made to clarify the in vivo role of the fractalkine/CX3CR1 axis in intestinal inflammation; however, it remains unclear whether the fractalkine/CX3CR1 axis contributes to the progression of colitis. Kostadinova et al. demonstrated that knockout of *CX3CR1* in mice suppressed the development of dextran sulfate sodium–induced colitis and inhibited the trafficking of inducible nitric oxide synthase–expressing macrophages into the mucosa (Kostadinova et al., 2010). Likewise, Niess and Adler (2010) demonstrated in a CD4$^+$ T-cell adoptive-transfer colitis model that CX3CR1$^{gfp/gfp}$/RAG2$^{-/-}$ mice had less colitis signs than RAG2$^{-/-}$ mice, fewer dendritic cells in the mesenteric lymph nodes, and reduced serum interferon gamma and interleukin 17 concentrations. In contrast, Medina-Contreras et al. (2011) demonstrated that deletion of *CX3CR1* aggravates the signs of colitis in mice, and Kayama et al. (2012) demonstrated the

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importance of intestinal CX3CR1^{high} myeloid cells for the prevention of intestinal inflammation). These inconsistent results may be due to a lack of expression of CX3CR1 in a specific stage during fetal development or an excessive compensation by other chemokine pathway or, a possibility that both too much and too little receptor might predispose to pathology. Therefore, to fully understand the mechanistic role of the fractalkine/CX3CR1 axis in intestinal inflammation, the fractalkine/CX3CR1 axis needs to be interrupted at the exact time at which inflammation begins. Here, in a murine CD4^{+}CD45RB^{high} T-cell-transfer colitis model, we used E6130 to interrupt the fractalkine/CX3CR1 axis during intestinal inflammation and found that E6130 suppressed the signs of colitis by inhibiting the infiltration of CX3CR1^{+} leukocytes into inflamed colon tissue.

Massive infiltration of leukocytes—including lymphocytes, macrophages, and neutrophils—into inflamed colonic mucosa is a hallmark of human inflammatory bowel disease (Nishimura et al., 2009; Thomas and Baumgart, 2012). In this regard, chemokine/receptor axes play central roles in the trafficking of leukocytes from the blood into inflamed intestinal mucosa (Nishimura et al., 2009). Previous studies have also shown the importance of the chemokine/receptor axes in the onset of T-cell-dependent, hapten-induced experimental colitis (Khan et al., 2006; Bento et al.,

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2008). Khan et al. reported the critical role of monocyte chemoattractant protein-1 (MCP-1) in a dinitrobenzenesulfonic acid-induced colitis model. MCP-1 is a ligand for CCR2, CCR4, and CCR11, and these receptors are expressed by monocytes, macrophages, and certain subsets of T lymphocytes. Khan et al. (2006) revealed that knockout of the gene encoding MCP-1 in mice attenuates the onset and severity of the signs of colitis by reducing the infiltration of $F4/80^+$ macrophages and $CD3^+$ lymphocytes into inflamed mucosa. Most chemokines, including MCP-1, can bind to several receptors, and most receptors bind many chemokines; however, fractalkine binds only to CX3CR1. Because the expression of MCP-1 mRNA is significantly decreased in CX3CR1-deficient mice compared with in wild-type mice, it is possible that E6130 suppresses the expression of MCP-1 in inflamed colonic tissues (Morimura et al., 2016). This suggests that the fractalkine/CX3CR1 axis exists upstream of other chemokine/receptor axes that together form a specialized system that regulates chemokine-induced leukocyte infiltration into the colonic mucosa.

In conclusion, here we characterized E6130, a novel CX3CR1 modulator. We found that E6130 induced down-regulation of CX3CR1 on the cell surface, leading to suppression of fractalkine-induced chemotaxis of $CX3CR1^+$ human NK cells. Oral administration of E6130 ameliorated inflammatory bowel disease-related parameters in

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both a murine $CD4^{+}CD45RB^{high}$ T-cell-transfer colitis model and a murine oxazolone-induced colitis model. In the murine $CD4^{+}CD45RB^{high}$ T-cell-transfer colitis model, E6130 inhibited the trafficking of $CX3CR1^{+}$ immune cells into the mucosa and decreased the number of these cells in the gut mucosal membrane. Thus, E6130 is a potentially useful therapeutic agent for treatment of inflammatory bowel diseases such as ulcerative colitis and Crohn's disease.

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AUTHORSHIP CONTRIBUTIONS

Participated in research design: HW, TY, YK

Conducted experiments: HW, TY, YK

Performed data analysis: HW, TY, YK

Wrote or contributed to the writing of the manuscript: HW, TI

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FOOTNOTES

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LEGENDS FOR FIGURES

Figure 1. Chemical structure of E6130.

Figure 2. Effects of E6130 on fractalkine-induced chemotaxis and down-regulation of CX3CR1 on the cell surface in human peripheral blood natural killer cells. (a) Effects of E6130 on fractalkine-induced chemotaxis in human peripheral blood natural killer cells. Data are presented as means ± SEM from three independent experiments performed in duplicate for the blank and control groups, and performed in a single well for the test compound group. (b) Effects of E6130 on the induction of down-regulation of CX3CR1 on the cell surface in human peripheral blood natural killer cells. Data are presented as means ± SEM (n = 3). MFI, mean fluorescence intensity.

Figure 3. Agonistic activity of E6130 against CX3CR1 with respect to GTPγS binding and β-arrestin recruitment. Agonistic activity of E6130 against CX3CR1 in (a) an [^{35}S]GTPγS binding assay and (b) an β-arrestin recruitment assay. The agonistic activity of E6130 is expressed as a percentage of the activity of the reference agonist (fractalkine) at its EC_{100} concentration.

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Figure 4. Effect of E6130 in a murine $CD4^+CD45RB^{high}$ T-cell-transfer colitis model. (a) Stool score was assessed on the indicated days in $CD4^+CD45RB^{high}$ T-cell-transfer mice given vehicle (solid squares), 10 mg/kg E6130 (solid circles), 30 mg/kg E6130 (solid triangles), or total $CD4^+$ T cells (open diamonds). (b) $CD4^+CD45RB^{high}$ T-cell-transfer mice were administered vehicle (solid squares), 10 mg/kg E6130 (solid circles), or 30 mg/kg E6130 (solid triangles). Mice that received total $CD4^+$ T cells were treated with vehicle (open diamonds). Body weight is expressed as the percentage of that at Day 14. $*p < 0.001$ versus vehicle in $CD4^+CD45RB^{high}$ T-cell-transferred group (repeated measures analysis of variance (RM-ANOVA) followed by Fisher's LSD test in each time slice). (c) On Day 28 after T-cell transfer, the mice were euthanized and the colons were excised. Leukocytes were extracted from the colon tissue by using collagenase. $CX3CR1^+$ cells were labeled with phycoerythrin-conjugated anti-CX3CR1 antibody and the size of the $CX3CR1^+$ cell population was determined by using a FACSCanto cell analyzer. (d) The colon weight to length ratio of each mouse was assessed on Day 28 after T-cell transfer. Results are presented as means ± SEM (n = 8/group). $*p < 0.001$ versus vehicle in $CD4^+CD45RB^{high}$ T-cell-transferred group (1-way ANOVA followed by Fisher's LSD test). Histopathology of colon from vehicle (e) or E6130 at 30 mg/kg (f) treated $CD4^+CD45RB^{high}$ T-cell-transfer colitis model. Massive thickening of intestinal mucosa with inflammatory cell infiltration

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and reactive hyperplasia of mucosal epithelial cells with cell debris in the glandular lumen of the vehicle treated animal and almost normal structure of colon in the E6130 treated animal. Bars 100μm. H&E stain.

Figure 5. Effect of E6130 in a murine oxazolone-induced colitis model. (a) Mice were euthanized, colons were excised, and colon length was measured. (b) Mice that intrarectally received oxazolone solution were administered vehicle (0.5% MC solution) (solid squares), 3 mg/kg E6130 (solid inverted triangles), 10 mg/kg E6130 (solid circles), or 30 mg/kg E6130 (solid triangles). Mice that received same vehicle (ethanol/water) were treated with vehicle (0.5% MC solution) (open diamonds). Body weight is expressed as the percentage of that at Day 5. Data are presented as means ± SEM (n = 9/group). *$p < 0.00625$ versus vehicle in oxazolone-injected group (1-way analysis of variance (ANOVA) followed by Fisher's LSD test).

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Table 1. Agonistic and antagonistic activities of E6130 (10 μmol/L) towards chemokine receptors

[^{35}S]GTPγS binding[a], Agonist mode		[^{35}S]GTPγS binding[a], Antagonist mode	
Receptor	% Activation[c]	Receptor	% Inhibition
CCR1	1.6	CCR1	15.1
CCR2	3.1	CCR2	24.9
CCR3	−8.1	CCR3	54.8
CCR4	−6.1	CCR4	44.0
CCR6	−5.2	CCR6	18.7
CCR7	0.9	CCR7	−15.2
CCR8	8.9	CCR8	9.7
CCR10	−5.1	CCR10	9.1
CX3CR1	95.2	CX3CR1	-18.6
CXCR1	−2.2	CXCR1	23.3
CXCR2	−3.4	CXCR2	9.1
CXCR3	2.4	CXCR3	22.7
CXCR4	0.0	CXCR4	13.2
CXCR6	−1.3	CXCR6	10.9
XCR1	−4.3	XCR1	23.8

β-arrestin[b], Agonist mode		β-arrestin[b], Antagonist mode	
Receptor	% Activation[c]	Receptor	% Inhibition
CCR2	−3	CCR2	−13
CCR3	−2	CCR3	5
CCR4	−2	CCR4	−11
CCR5	−2	CCR5	0
CCR6	−2	CCR6	2
CCR7	−5	CCR7	10
CCR8	−1	CCR8	15
CCR9	−3	CCR9	6
CCR10	−1	CCR10	−15
CX3CR1	179	CX3CR1	0
CXCR1	−1	CXCR1	−10
CXCR2	−1	CXCR2	6
CXCR3	−1	CXCR3	−2
CXCR4	3	CXCR4	10
CXCR5	−2	CXCR5	4
CXCR6	1	CXCR6	−10
ACKR3/CMKOR1	157	ACKR3/CMKOR1	0

[a] GTPγS binding assay was performed for chemokine receptors.

[b] PathHunter β-arrestin assay was performed for chemokine/chemokine-like receptors

[c] Agonist activity of E6130 is expressed as a percentage of the activity of the reference agonists at their EC_{100} concentrations.

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Table 2. Plasma concentrations of E6130 after oral administration in mice

Dose (mg/kg)	Plasma concentration (nmol/L)		
	30 minutes	2 hours	6 hours
10	236 ± 57.3	26.4 ± 11.6	10.7 ± 5.24
30	1430 ± 237	65.8 ± 12.6	22.8 ± 15.9

Data are shown as the mean values ± SEM (n = 3 - 6)

Plasma Protein Binding of E6130 in mice : 93.3%

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Figure 1

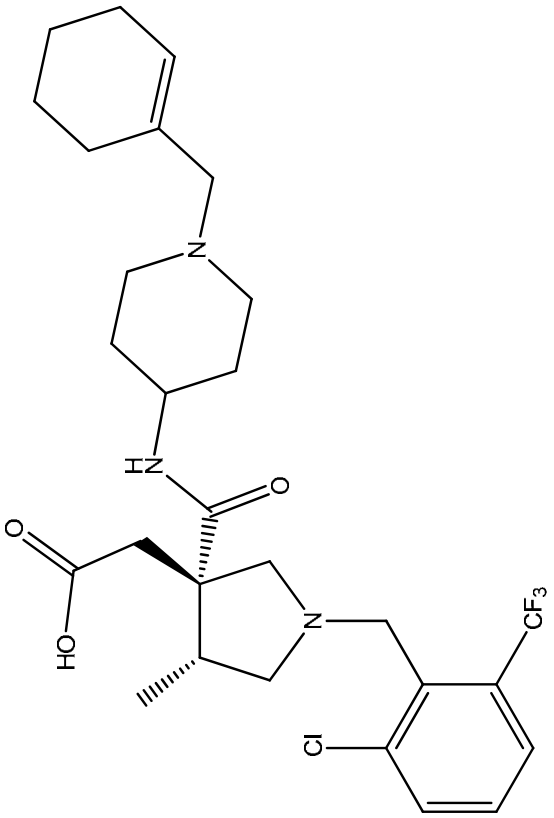

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Figure 2

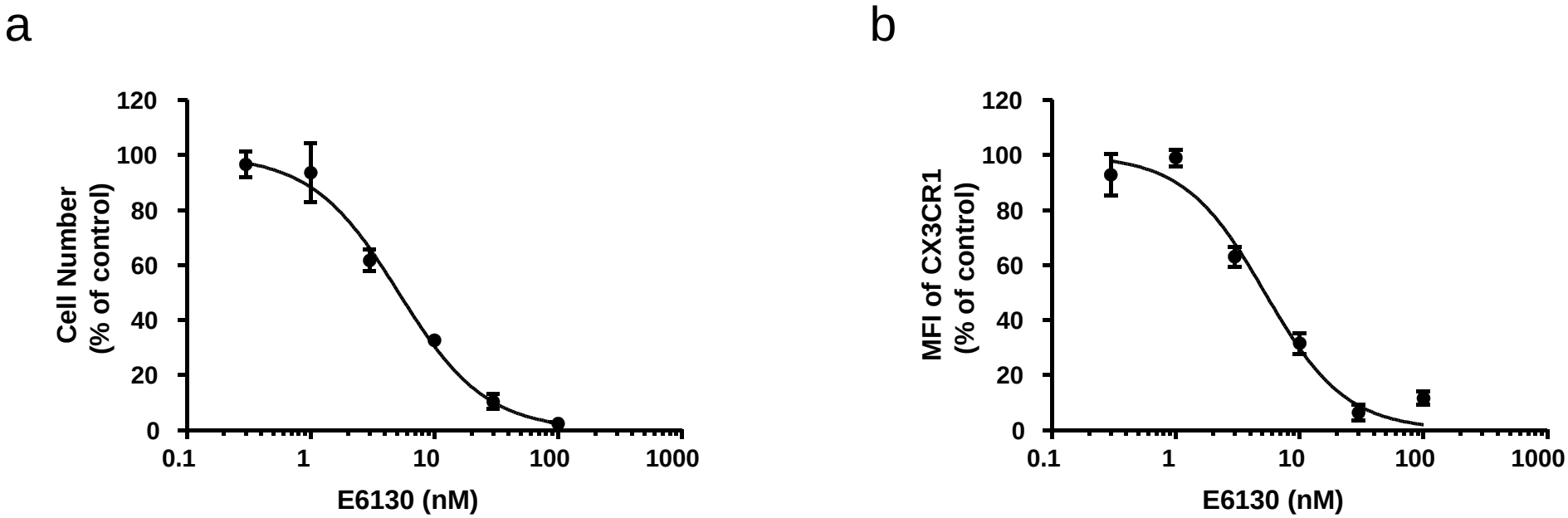

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Figure 3

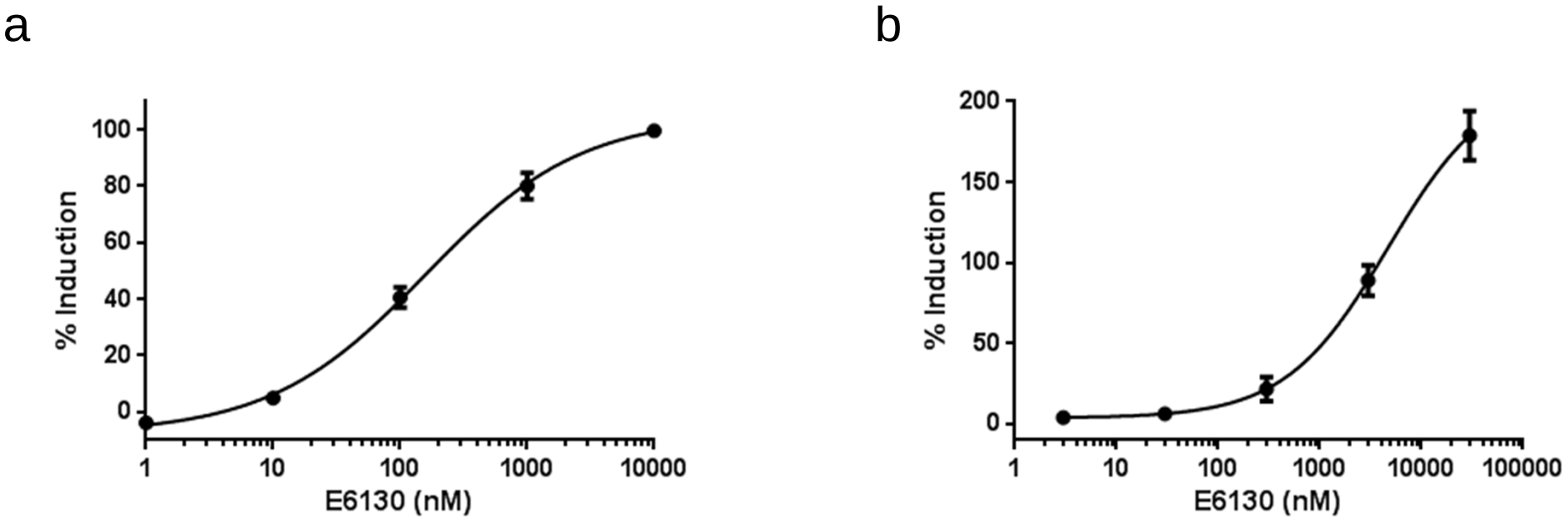

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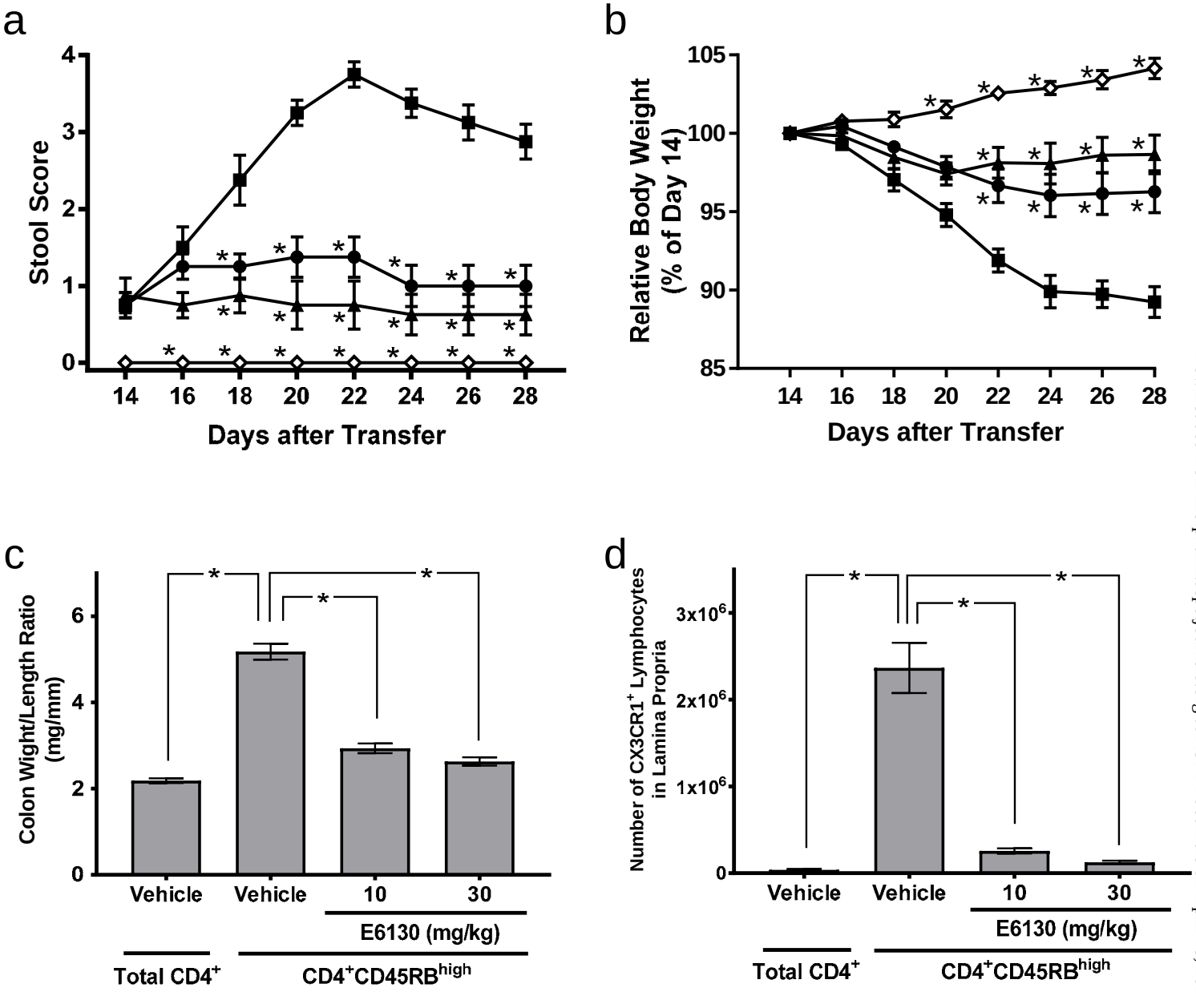
Figure 4



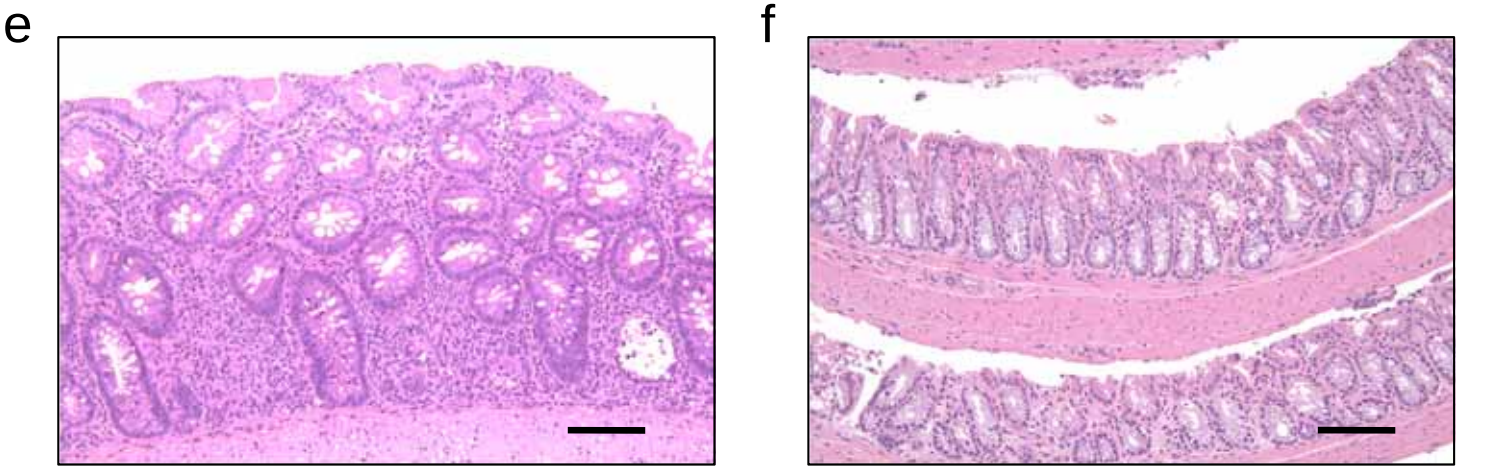


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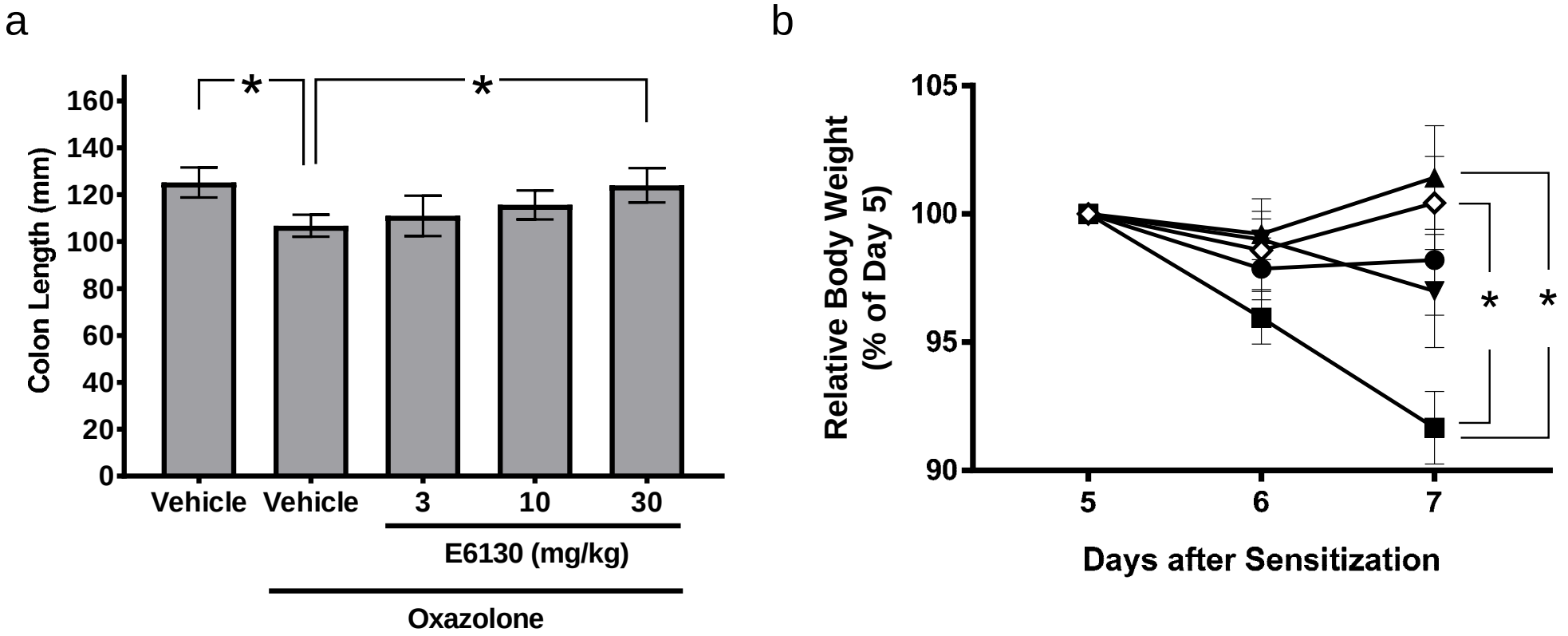
Figure 5



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