Creation of a Claudin-2 Binder and Its Tight Junction–Modulating Activity in a Human Intestinal Model[§]

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ABSTRACT

Disruption of the gastrointestinal epithelial barrier is a hallmark of chronic inflammatory bowel diseases (IBDs). The transmembrane protein claudin 2 (CLDN2) is a component of epithelial tight junctions (TJs). In the intestines of patients with IBDs, the expression of the pore-forming TJ protein CLDN2 is upregulated. Although CLDN2 is involved in these leaky barriers, whether it can be a target to enhance TJ integrity is unknown because a CLDN2-specific inhibitor has not been developed. Here, we used DNA immunization to generate a monoclonal antibody (mAb) that recognized an

extracellular loop of CLDN2. Treatment of epithelial cell monolayers with the mAb increased barrier integrity. In addition, the anti-CLDN2 mAb attenuated the decrease in TJ integrity induced by the proinflammatory cytokine tumor necrosis factor- α (TNF- α), and cotreatment of cells with anti-TNF- α mAb and anti-CLDN2 mAb showed additive attenuating effects. These findings indicate that CLDN2 may be a target for enhancing TJ integrity, and CLDN2 binder may be an enhancer of mucosal barrier integrity and a potential therapeutic option for IBDs.

Introduction

Inflammatory bowel diseases (IBDs), which include Crohn's disease and ulcerative colitis, are characterized by chronic relapsing idiopathic inflammation of the gastrointestinal tract that is associated with long-term and sometimes irreversible impairment of gastrointestinal structure and function (Podolsky, 2002). Patients with IBDs suffer from

inflammation-induced "leak flux" diarrhea caused by a passive loss of ions and water from the circulation into the intestinal lumen as a result of an impaired intestinal barrier (Hering et al., 2012). The chronic intestinal inflammation is induced by multiple exogenous and endogenous signals and mediated by various immune cells (de Souza and Fiocchi, 2016). The exogenous substances include dietary antigens, gut microbiota-derived microbe-associated molecular patterns, pathogens, and xenobiotics, all of which undergo epithelial translocation and thus activate the intestinal immune responses in patients with IBDs (de Souza and Fiocchi, 2016). Therefore, one therapeutic strategy for IBDs is to suppress inflammation, and anti-inflammatory agents including steroids, thiopurines, methotrexate, and monoclonal antibodies to tumor necrosis factor α (TNF- α) have been incorporated into treatment regimens (de Souza and Fiocchi, 2016). However, many patients have to stop these therapies because of adverse drug effects or lack of therapeutic responses (Bravatà et al., 2015).

Despite the role of epithelial translocation of exogenous substances in the activation of intestinal immune responses in IBDs (de Souza and Fiocchi, 2016), therapeutic interventions

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ABBREVIATIONS: CLDN, claudin; FITC, fluorescein isothiocyanate; hCLDN, human claudin; IBD, inflammatory bowel disease; mAb, monoclonal antibody; mCLDN, mouse claudin; MDCKI, Madin-Darby canine kidney I; PBS, phosphate-buffered saline; PI3K, phosphatidylinositol-3-kinase; TEER, transepithelial electrical resistance; TJ, tight junction; TNF- α , tumor necrosis factor α .

targeted at the epithelial translocation have never been fully developed.

Chronic inflammation in IBDs compromises the intestinal epithelial monolayer. Concentrations of the proinflammatory cytokine TNF- α are increased in Crohn's disease and ulcerative colitis (Podolsky, 2002). To maintain the barrier function of the intestinal epithelium, its intercellular spaces are sealed by tight junctions (TJs), which prevent the free movement of solutes (ions, water, and noxious antigens derived from foods or microorganisms) across epithelial sheets (Powell, 1981). The intercellular TJ seal is impaired in colonic samples from IBD patients (Zeissig et al., 2007), and TNF- α plays a pivotal role in the disruption of TJ seals, perhaps through altered regulation of TJ components (Schmitz et al., 1999; Bürgel et al., 2002; Amasheh et al., 2009; Mankertz et al., 2009).

Freeze-fracture analysis showed that TJ seals consist of a set of continuous, anastomosing intramembranous strands (Staehelin, 1974). TJs comprise transmembrane proteins [junctional adhesion molecules, occludin, claudins (CLDNs), and tricellulin] and intracellular scaffolding proteins (zonula occludens) (Van Itallie and Anderson, 2014). CLDNs are key components of the TJ seal. CLDNs are a family containing 27 members that show tissue-specific expression profiles. CLDN1, 3, 4, 5, 7, and 8 are frequently observed in tight epithelia, whereas CLDN2 is primarily expressed in leaky epithelia (Luettig et al., 2015). Colon biopsy samples from patients with IBDs showed decreased expression of CLDN3, 5, and 8 and occludin and increased expression of CLDN2 (Zeissig et al., 2007).

CLDN2 is a 24.5-kDa tetra-transmembrane protein with two extracellular loop domains (Furuse et al., 1998b). Transfection with a plasmid encoding CLDN2 decreases the integrity of TJs, due to the formation of discontinuous TJ strands (Furuse et al., 2001). Treatment with TNF- α upregulates the expression of CLDN2 in part via phosphatidylinositol-3-kinase (PI3K) signaling (Prasad et al., 2005; Heller et al., 2008; Mankertz et al., 2009; Amasheh et al., 2010; Suzuki et al., 2011), whereas anti-TNF- α monoclonal antibody (mAb) prevents the TNF- α -induced loss of TJ integrity and the associated downstream effects on PI3K and CLDN2 (Fischer et al., 2013). Furthermore, the administration of an anti-TNF- α antibody restores intestinal barrier function in patients with Crohn's disease (Suenae et al., 2002; Noth et al., 2012). These findings indicate that the inhibition of CLDN2 may restore the diminished TJ integrity in IBDs. However, whether CLDN2 is a feasible target for IBD therapy has been unknown because a CLDN2-specific inhibitor had not been developed.

In this study, we used a DNA immunization method to generate a mAb that specifically recognizes the first extracellular loop of CLDN2, and we investigated the effects of this mAb on TNF- α -induced loss of TJ integrity in cell culture models.

Materials and Methods

Reagents. Anti-histidine tag mAb, fluorescein isothiocyanate (FITC)-conjugated antibody, rat IgG, anti- β -actin antibody, and TNF- α were obtained from Thermo Fisher Scientific (Waltham, MA), Jackson ImmunoResearch (West Grove, PA), BD Biosciences (Franklin Lakes, NJ), Sigma-Aldrich (St. Louis, MO), and R&D Systems (Minneapolis, MN), respectively. Anti-CLDN1 mAb (7A5),

anti-CLDN4 mAb (5D12), and anti-CLDN3 and -4 mAb (5A5) were prepared as described previously (Fukasawa et al., 2015; Kuwada et al., 2015). The anti-TNF- α monoclonal antibodies infliximab (Remicade; Mitsubishi Tanabe Pharma Corporation, Osaka, Japan) and adalimumab (Humira; Eisai, Tokyo, Japan) were purchased from reagent distributors.

Cell Cultures. Because they are CLDN negative, HT1080 cells (a human fibrosarcoma cell line) are used to characterize CLDN binders (Neesse et al., 2013; Fukasawa et al., 2015; Mosley et al., 2015; Nakajima et al., 2015). HT1080 cells mock transfected or stably transfected with human CLDN1–CLDN7 or CLDN9 (mock/HT1080, hCLDN1/HT1080 to hCLDN7/HT1080, and hCLDN9/HT1080 cells, respectively) were previously established (Li et al., 2014). hCLDN/HT1080 cells were grown in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum at 37°C under 5% CO₂.

L cells that stably expressed mouse CLDN1 or CLDN2 (mCLDN1/L and mCLDN2/L cells, respectively) were provided by Dr. S. Tsukita (Kyoto University, Kyoto, Japan). mCLDN/L cells were cultured in modified Eagle's medium containing 10% fetal bovine serum at 37°C under 5% CO₂.

Human intestinal epithelial cells (Caco-2; American Type Culture Collection, Manassas, VA) were grown in modified Eagle's medium containing 10% fetal bovine serum at 37°C under 5% CO₂.

Madin-Darby canine kidney I (MDCKI) cells (European Collection of Animal Cell Cultures, Porton Down, UK) were grown in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum at 37°C under 5% CO₂.

Animals. Female Wistar rats (6 weeks of age) and female BALB/c Slc-nu/nu mice (8 weeks of age) were purchased from Shimizu Laboratory Supplies (Kyoto, Japan) and SLC, Inc. (Shizuoka, Japan), respectively. All animals were maintained under controlled conditions of a 12-hour light/dark cycle at 23 $\pm$ 1.5°C. The rodents had unrestricted access to food and water. All of the experimental protocols conformed to the ethics guidelines of the Graduate School of Pharmaceutical Sciences, Osaka University.

Preparation of a CLDN-Binding Peptide. We previously created m19, a peptide that binds to CLDNs 1, 2, 4, and 5, by using the CLDN3/4-binding polypeptide C-terminal fragment of *Clostridium perfringens* enterotoxin as a template (Takahashi et al., 2012). We then prepared m19 tagged with a histidine tag at the N terminus. Briefly, pET16b plasmid encoding m19 was transformed into *Escherichia coli* strain BL21 (DE3), and the production of the recombinant protein was induced by adding isopropyl-D-thiogalactopyranoside. Harvested cells were lysed in buffer A [10 mM Tris-HCl (pH 8.0), 400 mM NaCl, 5 mM MgCl₂, 0.1 mM phenylmethanesulfonyl fluoride, 1 mM 2-mercaptoethanol, and 10% glycerol]. Supernatants were applied to a HiTrap Chelating HP column (GE Healthcare, Little Chalfont, UK), and the recombinant protein was eluted with buffer A containing imidazole. The solvent was exchanged with phosphate-buffered saline (PBS) by using a PD-10 column (GE Healthcare), and the purified protein was stored at –80°C until use. Purification of the recombinant protein was confirmed by SDS-PAGE followed by staining with Coomassie Brilliant Blue. Protein was quantified by using a BCA Protein Assay Kit (Pierce Biotechnology, Waltham, MA) and bovine serum albumin as a standard.

Generation of Anti-CLDN2 mAb. Female Wistar rats (6 weeks of age) were immunized with a eukaryotic expression vector encoding human CLDN2 every 2 weeks for 2 months according to proprietary technology (Genovac, Freiburg, Germany). Lymphocytes were removed 7 days after the last immunization and fused with P3UI cells in the presence of polyethylene glycol 1000, thus generating hybridoma cells. Hybridoma cells producing anti-CLDN2 mAbs were initially screened for the ability of their conditioned medium to bind to hCLDN2/HT1080 cells but not to mock/HT1080 cells, resulting in the isolation of a hybridoma that produced mAb against hCLDN2 (clone 1A2). The Ig class and subclass of anti-CLDN2 1A2 were determined by using a rat Ig-isotyping enzyme-linked immunosorbent assay kit (BD Biosciences).

Purification of mAb. According to standard procedures, female BALB/c *Slc-nu/nu* mice (8 weeks of age; Japan SLC, Inc., Hamamatsu, Japan) were each intraperitoneally injected with the adjuvant pristane (Sigma-Aldrich) and 1×10^7 mAb-producing hybridoma cells. Ascites was collected, and the mAb was purified by using a protein G column (GE Healthcare). Eluted antibodies were dialyzed in PBS. The concentration of the purified antibody was determined by measuring the absorbance at 280 nm.

Flow Cytometry. hCLDN/HT1080 or mCLDN/L cells (5×10^5 cells) were incubated with PBS containing 1% bovine serum, m19 (10 μ g/ml), or mAbs (5 μ g/ml) for 1 hour at 4°C. Cells treated with m19 were then treated with an anti-histidine tag mAb (5 μ g/ml; Thermo Fisher Scientific). The cells were then incubated with FITC-conjugated secondary antibody (10 μ g/ml; Jackson ImmunoResearch) and stained with propidium iodide solution (1 μ g/ml; Miltenyi Biotec, Bergisch Gladbach, Germany) to exclude dead cells from flow cytometric analysis. The mAb-bound cells were detected with a FACSCalibur Flow Cytometer (BD Biosciences), and data were analyzed by using CellQuest Software (BD Biosciences).

Chimeric CLDN2/HT1080 Cells. In CLDN2 cDNA, the sequences encoding the first extracellular loop domain (corresponding to amino acids 28 through 78) of hCLDN2, the second extracellular loop domain (corresponding to amino acids 144 through 162) of hCLDN2, or the first and second loop domains of hCLDN2 were genetically replaced by those of hCLDN4, resulting in hCLDN2/EL1-hCLDN4, hCLDN2/EL2-hCLDN4, or hCLDN2/EL1 and EL2-hCLDN4 cDNA. The resultant cDNAs were cloned into pcDNA3.1(–) (Invitrogen, Carlsbad, CA). The chimeric hCLDN2 expression vectors were then transfected into HT1080 cells by using X-tremeGENE HP DNA transfection reagent (Roche Diagnostics, Basel, Switzerland), and G418-resistant clones (stable transfectants for each chimeric hCLDN2) were isolated. Chimeric hCLDN/HT1080 cells were maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum.

Assay of TJ Integrity. Caco-2 or MDCK I cells were recovered by gentle pipetting with trypsin until a single-cell suspension was obtained, and these cells were seeded into 24-well Transwell inserts (Corning Life Sciences, Tewksbury, MA) at a density of 6.0×10^4 or 5.0×10^4 cells per 0.33-cm² well, respectively. The monolayers were then cultured for 10–14 days before basolateral treatment with mAb (0.01–10 μ g/ml). Transepithelial electrical resistance (TEER) during treatment was measured by using a Millicell-ERS Epithelial Volt-Ohm Meter (Millipore, Billerica, MA). The background TEER value of a well containing medium only was subtracted from the recorded TEER; these values were then normalized to the surface area of the well.

Assay of TJ Integrity in TNF- α -Treated Caco-2 Monolayers. Caco-2 cells were seeded into 24-well Transwell inserts (6.0×10^4 cells/0.33-cm² well; Corning Life Sciences). The Caco-2 monolayers were then cultured for 10 days until the TEER value had reached a plateau, and the cells were used for various analyses.

To investigate the effects of anti-CLDN2 mAb on TNF- α -induced reduction of TJ integrity, the cells were cotreated with TNF- α (10 ng/ml; R&D Systems) and either rat IgG (10 μ g/ml; R&D Systems) or anti-CLDN2 mAb (10 μ g/ml). TEER values were measured 24 hours before and 24 hours after treatment. Alternatively, the Caco-2 cells were pretreated with TNF- α (10 ng/ml) for 24 hours, and then rat IgG (10 μ g/ml) or anti-CLDN2 mAb 1A2 (10 μ g/ml) was added; the cells were cultured, and TEER values were measured.

To investigate the effects of anti-TNF- α mAb and anti-CLDN2 mAb, the cells were pretreated with TNF- α (10 ng/ml) for 24 hours, after which anti-TNF- α mAb (infliximab or adalimumab), anti-CLDN2 mAb 1A2, or a mixture of anti-TNF- α mAb and anti-CLDN2 mAb 1A2 was added to the medium (10 μ g/ml/reagent). TEER values were measured after treatment.

Immunoblot Analysis. MDCK I or Caco-2 cells were collected by using a cell scraper and lysed in buffer (50 mM Tris-HCl, 150 mM NaCl, 0.1% SDS, 1% NP40, 0.5% sodium deoxycholate, and 1%

protease inhibitor cocktail; Nacalai Tesque, Kyoto, Japan). The resultant supernatants were recovered by centrifugation. Lysates (10 μ g/sample) underwent SDS-PAGE in a 15% polyacrylamide gel; the separated proteins were blotted onto a polyvinylidene difluoride membrane. Membranes were incubated with Tris-buffered saline containing 0.1% (v/v) Tween-20 and 2% (v/v) bovine serum albumin for 2 hours and then incubated with the primary antibody for 1 hour [anti-CLDN2 at 0.5 μ g/ml (Invitrogen) or anti- β -actin at 0.4 μ g/ml (Sigma-Aldrich)]. After several washes in Tris-buffered saline containing 0.1% Tween-20, the membrane was incubated with a horseradish peroxidase-conjugated secondary antibody (goat anti-mouse IgG at 0.5 μ g/ml; Millipore) for 1 hour. Immunoreactive bands were detected by using a chemiluminescence reagent (Nacalai Tesque) and an image analyzer (LAS 4010; GE Healthcare).

Statistical Analysis. Data were analyzed by using Tukey's test. The statistical significance for all comparisons was set at $P < 0.05$.

Results

Generation of Anti-CLDN2 mAbs. CLDN2 forms homodimers with adjacent CLDN2 or heterodimers with CLDN3, but does not interact with CLDN1 (Furuse et al., 1999), and single-molecule force spectroscopy revealed that these *trans*-interactions are mediated through the first extracellular loop domain of CLDN2 (Lim et al., 2008). We therefore hypothesized that a molecule that binds to an extracellular domain of CLDN2 would prevent the formation of leaky barriers caused by CLDN2, thus attenuating the CLDN2-mediated impairment of the TJ seal in IBDs. To address this hypothesis, we first tried to generate mAbs that recognized one or both of the extracellular loop domains of CLDN2. We previously generated anti-CLDN1 and anti-CLDN4 mAbs by immunizing mice and rats with plasmid DNA encoding hCLDN1 and hCLDN4 cDNA, respectively (Li et al., 2014; Fukasawa et al., 2015). Therefore, we immunized rats with plasmid DNA encoding hCLDN2 and selected hybridoma cells that produced anti-CLDN2 mAbs, which bound to hCLDN2/HT1080 cells but not to mock/HT1080 cells; these efforts resulted in the generation of a hybridoma clone that produced anti-CLDN2 mAb (1A2). The Ig subtype of 1A2 is IgG2b. 1A2 bound to hCLDN2/HT1080 cells but not to HT1080 cells that stably expressed hCLDN1, 3, 4, 6, 7, or 9 (Fig. 1A); 1A2 also bound to mCLDN2/L cells (Fig. 1A). These findings indicate that mAb 1A2 is a specific binder of CLDN2.

To determine the extracellular domains to which 1A2 binds, we replaced either or both of the extracellular loop domains of CLDN2 with those of CLDN4. The broadly reactive CLDN binder m19, which binds to CLDNs 1–5, bound to all of these chimeric CLDN2-expressing HT1080 cells (Fig. 1B). In contrast, mAb 1A2 bound only to fractions containing the first extracellular domain of CLDN2 (Fig. 1B). Therefore, mAb 1A2 specifically recognizes the first extracellular loop domain of hCLDN2.

Effects of Anti-CLDN2 mAb on TJ Integrity. Monolayers of Caco-2 cells are widely used as an *in vitro* model of the human intestinal epithelial barrier (Meunier et al., 1995; Wang et al., 2005). Therefore, to assess whether a CLDN2 binder might attenuate the CLDN2-induced impairment of epithelial TJ integrity, we investigated the effects of anti-CLDN2 mAb on TJ integrity in Caco-2 cell monolayers. mAb 1A2 increased TEER values to 133%, and the removal of 1A2 from the medium attenuated changes in TEER (Fig. 2A). Furthermore, 1A2 dose-dependently increased TEER (Fig. 2B).

Fig. 1. Characterization of anti-CLDN2 mAb. (A) CLDN specificity of anti-CLDN2 mAb. hCLDN/HT1080 or mCLDN/L cells were incubated with rat anti-CLDN2 mAb (1A2) or PBS as a control, and then treated with goat anti-rat IgG (H + L) antibody conjugated with FITC. The antibody-bound cells were detected by using a flow cytometer. Gray and white histograms represent vehicle-treated (with PBS) and antibody-treated cells, respectively. Data are representative of three independent experiments. (B) Epitope identification of anti-CLDN2 mAb. Either the first or second extracellular domain or both the first and second extracellular domains of hCLDN2 were genetically replaced by those of hCLDN4 to yield hCLDN2 EL1-hCLDN4, hCLDN2 EL2-hCLDN4, or hCLDN2 EL1-EL2-hCLDN4, respectively. HT1080 cells transfected with hCLDN2, hCLDN4, or each chimeric CLDN2 were incubated with 5 μ g/ml anti-CLDN2 mAb (white histogram), 10 μ g/ml broad-specific CLDN binder (m19; white histogram), or vehicle (PBS; gray histogram) followed by treatment of cells with FITC-conjugated goat anti-rat IgG (H + L) or anti-6 $\times$ His tag mAb and FITC-conjugated goat anti-mouse IgG. Cells that had bound anti-CLDN2 mAb or m19 were detected by using a flow cytometer. Data are representative of three independent experiments.

To confirm the CLDN2 specificity of the 1A2-induced increase in TJ integrity, we investigated the effects of mAbs specific for CLDN1 (7A5), CLDN4 (5D12), and CLDN3/4 (5A5) on TJ integrity (Li et al., 2014; Fukasawa et al., 2015).

Treatment of Caco-2 monolayers with mAb specific for CLDN1 (7A5) or CLDN4 (5D12) (10 μ g/ml) did not significantly change TEER from that of the vehicle-only control, and treatment for 24 hours with the CLDN3/4 dual-specific mAb (5A5) decreased

Fig. 2. Effects of anti-CLDN2 mAb on TJ seals in Caco-2 monolayers. (A) Effects of anti-CLDN2 mAb on TJ integrity. Caco-2 monolayers were cultured for 10 days and then treated with vehicle (PBS), rat IgG (10 µg/ml; as a negative control), or anti-CLDN2 mAb (1A2; 10 µg/ml) on the basal side of the insert. The cells were exposed to the antibodies for 24 hours, washed with medium to remove antibodies, and cultured for an additional 12 hours. Changes in TEER values were monitored throughout treatment. Data are presented as the mean $\pm$ S.D. ($n = 3$). * $P < 0.05$ (Tukey's test). (B) Dose dependency of barrier-modulating activity of anti-CLDN2 mAb in TEER. Caco-2 monolayers were cultured for 10 days and then treated with vehicle (PBS) or anti-CLDN2 mAb (1A2; 0.01, 0.1, 1, or 10 µg/ml) on the basal side of the insert. The cells were exposed to the antibodies for 24 hours. Changes in TEER values were monitored throughout treatment. Data are presented as the mean $\pm$ S.D. ($n = 3$).

TEER to 78% of that of the vehicle control (Fig. 2C). In addition, we transfected MDCKI cells, which do not express CLDN2, to stably express CLDN2 and examined the effect of mAb 1A2 on TJ integrity. Treatment with mAb 1A2 increased TEER in MDCKI/CLDN2 cells but not MDCKI/vector cells (Supplemental Fig. 1). Similarly, mAb 1A2 induced a greater increase in the TEER of Caco-2 cells exogenously expressing CLDN2 compared with Caco-2/vector cells (Supplemental Fig. 2). Therefore, the ability of 1A2 to promote TJ integrity might be due to specific interaction with CLDN2.

Effects of Anti-CLDN2 mAb on TNF- α -Induced TJ Dysfunction. To investigate the effects of the anti-CLDN2 mAb 1A2 on the disruption of TJ integrity in the context of inflammation, we used a Caco-2 model of TNF- α -induced TJ dysfunction (Ma et al., 2005; Graham et al., 2006). Treatment of cells with TNF- α (10 ng/ml) for 24 hours decreased TEER to 70% of that at 0 hour, accompanied by an increase in cellular CLDN2 protein content (Fig. 3A; Supplemental Fig. 3). In contrast, cotreatment with TNF- α and anti-CLDN2 mAb (1A2) attenuated the reduction in TEER (Fig. 3A). To clarify the effects of anti-CLDN2 mAb in the TNF- α -induced dysfunction model, Caco-2 cells were exposed to TNF- α for 24 hours and then treated with anti-CLDN2 mAb for 48 hours. In cells exposed to TNF- α for 24 hours followed by 48-hour treatment with vehicle only or rat IgG, TEER decreased to 37% or 39%, respectively, of that before TNF- α treatment (Fig. 3B). In contrast, anti-CLDN2 mAb treatment attenuated the TNF- α -induced reduction in TEER to approximately 64% of that before TNF- α treatment.

To investigate whether the effects of mAbs against CLDN2 and TNF- α might be additive, we pretreated Caco-2 cells with TNF- α (10 ng/ml) for 24 hours and then exposed the cells for 48 hours to anti-CLDN2 mAb (1A2), anti-TNF- α mAb (infliximab or adalimumab), or both anti-CLDN2 and anti-TNF- α mAbs concurrently. Pretreatment with TNF- α reduced TEER to approximately 48% of that of untreated cells (Fig. 3C). However, exposure to anti-CLDN2 mAb, infliximab, or adalimumab (10 µg/ml each) for 48 hours recovered TEER to 75%, 60%, or 62% of the levels of cells before TNF- α treatment, respectively, and cotreatment with anti-CLDN2 mAb and either infliximab or adalimumab increased TEER to 93% and 85%, respectively, of that of cells before TNF- α treatment. These findings indicate that CLDN2-targeted therapeutics might represent a new option in the treatment of IBDs.

Discussion

The activation of mucosal immune responses and impairment of the intestinal barrier are key characteristics of IBDs (Podolsky, 2002). Various attenuators of these immune responses, including steroids, thiopurines, methotrexate, and anti-TNF- α mAbs, are used clinically to treat patients with

* $P < 0.05$ (Tukey's test). (C) Effects of anti-CLDN1, anti-CLDN4, and anti-CLDN3/4 mAbs on TJ integrity. Caco-2 monolayers were cultured for 10 days and then treated with vehicle (PBS), anti-CLDN1 mAb (7A5), anti-CLDN2 mAb (1A2), anti-CLDN4 mAb (5D12), or anti-CLDN3/4 mAb (5A5) (10 µg/ml) on the basal side of the insert. The cells were exposed to the antibodies for 24 hours, washed with medium to remove antibodies, and cultured for an additional 12 hours. Changes in TEER values were monitored during antibody treatment. Data are presented as the mean $\pm$ S.D. ($n = 3$). * $P < 0.05$ (Tukey's test).

Fig. 3. Effects of anti-CLDN2 mAb on the disrupted TJ integrity in an in vitro model of IBDs. (A) Effects of anti-CLDN2 mAb on TJ dysfunction induced by TNF- α treatment. Once TEER values had reached a plateau (approximately 10 days after cells were seeded on the Transwell membrane), the cells were treated with vehicle (medium) or TNF- α (10 ng/ml) in the presence of vehicle (PBS), anti-CLDN2 mAb 1A2, or rat IgG (10 μ g/ml) for 24 hours. TEER values were measured at 0 hour (white bars) and 24 hours (gray bars) of treatment. Data are shown as the mean $\pm$ S.D. ($n = 3$). * $P < 0.05$ as determined by Tukey's test. (B) Effects of anti-CLDN2 mAb on the TJ integrity disrupted by TNF- α treatment. When TEER values had reached a plateau (approximately 10 days after seeding on the Transwell membrane), the cells were treated with vehicle (medium) or TNF- α (10 ng/ml) for 24 hours followed by treatment with vehicle (PBS), anti-CLDN2 mAb 1A2, or rat IgG (10 μ g/ml) for 48 hours. TEER values were measured at -24 hours (before treatment with TNF- α , white bars), 0 hour (before treatment with anti-CLDN2 mAb, gray bars), and 48 hours

IBDs (de Souza and Fiocchi, 2016). However, therapeutic agents targeting the intestinal barrier function have not been fully developed previously, in part due to a delay in understanding the mechanism underlying the impairment of the intestinal barrier function. In the present study, we hypothesized that CLDN2, a characteristic component of leaky TJ seals, might be an effective target through which to increase TJ integrity for IBD therapy. We consequently generated an mAb that recognized the first extracellular loop domain of CLDN2; the mAb restored the TJ seal function in cultures of TNF- α -treated cells.

Infliximab and adalimumab, commercially available anti-TNF- α mAbs, prevent the intestinal barrier dysfunction due to TNF- α treatment, which increases the expression of CLDN2 (Fischer et al., 2013). Butyrate, a short-chain fatty acid, prevents chemical-induced colitis, accompanied by down-regulation of CLDN2 (Vieira et al., 2012). Berberine, a plant alkaloid, is a traditional Eastern medicine for the treatment of diarrhea and gastroenteritis (Rosenthal et al., 2010); like butyrate, berberine downregulates CLDN2, the effect that is associated with the antidiarrheal properties of berberine. These findings strongly support our data regarding the use of an anti-CLDN2 mAb to treat the TNF- α -induced dysfunction of TJ seals in the intestinal epithelium of patients with IBDs. However, butyrate downregulated 161 genes, including CLDN2 (Vieira et al., 2012), and berberine inhibited the PI3K-Akt signaling pathway, which regulates CLDN2 expression (Rosenthal et al., 2010). Therefore, butyrate and berberine are not specific inhibitors of CLDN2, and, as such, their use for IBD therapy might cause unexpected adverse effects. In addition, CLDN2 was detectable in the colons of IBD patients but not in the colons of healthy people (Heller et al., 2005; Zeissig et al., 2007; Oshima et al., 2008). Accordingly, one key therapeutic benefit of an anti-CLDN2 mAb is its apparent specificity.

CLDN2 forms paracellular channels for cations and water (Amasheh et al., 2002, 2010). Incorporation of CLDN2 into TJ strands does not change their number but rather makes them discontinuous, resulting in the formation of TJ seals that are leaky to solutes (Furuse et al., 2001). CLDN2 forms homodimers with adjacent CLDN2 or heterodimers with CLDN3, but CLDN2 does not interact with CLDN1 (Furuse et al., 1999). The *trans*-interaction in CLDN2 homodimers is mediated by the first extracellular domains of each unit through electrostatic interactions with D65 and weaker interactions with negatively charged π electrons of the Y67 aromatic residue (Lim et al., 2008). Our anti-CLDN2 mAb 1A2 recognized the first extracellular domain of CLDN2. Given these findings, the binding of an anti-CLDN2 mAb to CLDN2 might prevent *cis*- and *trans*-

(after treatment with anti-CLDN2 mAb, black bars). Data are shown as the mean $\pm$ S.D. ($n = 3$). * $P < 0.05$ (Tukey's test). (C) Additive effects of anti-TNF- α mAb and anti-CLDN2 mAb on TJ integrity disrupted by TNF- α treatment. When TEER values had reached a plateau (approximately 10 days after cells were seeded on the Transwell membrane), the cells were treated with vehicle (medium) or TNF- α (10 ng/ml) for 24 hours. Then the cells were treated with anti-CLDN2 mAb 1A2 or rat IgG (10 μ g/ml) and vehicle (PBS) or anti-TNF- α mAb [infliximab (Inf) or adalimumab (Ada), 10 μ g/ml] for 48 hours. TEER values were measured before and after TNF- α treatment (-24 and 0 hours, respectively) and after anti-CLDN2 mAb treatment (48 hours). Data are shown as the mean $\pm$ S.D. ($n = 3$). * $P < 0.05$ (Tukey's test).

interactions of CLDN2, thus attenuating the formation of leaky TJ seals.

CLDN2 is expressed in normal tissues, including liver, kidney, and small intestine (specifically the crypt-villus axis in humans), but not in the human colon (Furuse et al., 1998a; Enck et al., 2001; Kiuchi-Saishin et al., 2002; Lameris et al., 2013). CLDN2-deficient mice are normal in appearance, activity, growth, and behavior and lack functional and histologic abnormalities of liver, kidney, and small intestine (Muto et al., 2010; Tamura et al., 2011). Therefore, CLDN2 might be a safe target for IBD therapy.

In conclusion, we have generated an mAb that recognizes the first extracellular loop domain of CLDN2; this anti-CLDN2 mAb ameliorated TNF- α -induced loss of intestinal barrier function in vitro. CLDN2 binders might be a potent therapeutic option for IBD therapy to enhance the intestinal mucosal barrier.

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Authorship Contributions

Participated in research design: Takigawa, Iida, Suzuki, Tada, Kunisawa, and Kondoh.

Conducted experiments: Takigawa, Iida, Nagase, and Suzuki.

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Contributed to the writing of the manuscript: Takigawa, Iida, Suzuki, Watari, Tada, Okada, Doi, Fukasawa, Yagi, Kunisawa, and Kondoh.

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