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ORIGINAL ARTICLE

Efficacy and safety evaluation of claudin-4-targeted antitumor therapy using a human and mouse cross-reactive monoclonal antibody

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Abstract

Claudin-4 (CLDN-4), a tight-junction protein, is overexpressed in various malignant tumors, including gastric, colorectal, pancreatic, and breast cancers. However, CLDN-4 is also expressed in normal tissues, including the liver, pancreas, kidney, and small intestine. Whether CLDN-4 is an effective and safe target for cancer therapy has been unclear owing to the lack of a binder with both CLDN-4 specificity and cross-reactivity to human and murine cells. In this study, we successfully generated a rat anti-CLDN-4 monoclonal antibody (5D12) that was specific to, and cross-reactive with, human and mouse CLDN-4. 5D12 recognized the second extracellular domain of human CLDN-4 in a conformation-dependent manner. A human–rat chimeric IgG1 of 5D12 (xi-5D12) activated the FcγIIIa receptor, indicating the activation of antibody-dependent cellular cytotoxicity in CLDN-4-expressing cells. Moreover, xi-5D12 significantly suppressed tumor growth in mice bearing human colorectal and gastric tumors without apparent adverse effects, such as weight loss or liver and kidney damage. These results suggest that CLDN-4 is a potent target for cancer therapy and that an anti-CLDN-4 antibody is a promising candidate anticancer agent.

Abbreviations

ADCC, antibody-dependent cellular cytotoxicity; ADP, antibody-dependent phagocytosis; C-CPE, C-terminal binding domain of *Clostridium perfringens* enterotoxin; CLDN, claudin; CPE, *Clostridium perfringens* enterotoxin; FcγR, Fcγ receptor; hCLDN, human claudin; mCLDN, mouse claudin; NFAT, nuclear factor of activated T cells.

Introduction

Most human cancers are carcinomas, which arise from epithelial cells. In normal epithelial cells, tight junctions between adjacent cells regulate the permeation of small ions, solutes, and large molecules such as proteins (Tsukita et al. 2001; Nagase et al. 2013; Van Itallie and Anderson 2013). Tight junctions normally are located on the apical side of the lateral membrane to maintain cell polarity, but various carcinomas display abnormal expression of tight-junction proteins and resultant disruption of cell polarity (Singh et al. 2010; Beyer et al. 2012). Claudins (CLDNs) are well-known tight-junction proteins that have four transmembrane domains and two extracellular domains (approximate molecular weight, 23 kDa) (Tsukita et al. 2001). The CLDN family consists of 27 members, which are expressed in a tissue-specific manner and frequently are either overexpressed or downregulated in carcinomas (Ding et al. 2013; Runkle and Mu 2013). In particular, CLDN-4 is overexpressed most frequently in colon, gastric, ovarian, breast, and pancreatic cancers (Facchetti et al. 2007; Ding et al. 2013).

CLDN-3 and CLDN-4 are receptors for the C-terminal fragment of *Clostridium perfringens* enterotoxin (C-CPE) (Fujita et al. 2000). Proof of concept for CLDN-targeted cancer therapy was achieved by using the *C. perfringens* enterotoxin (CPE) itself and C-CPE-fused proteins, including tumor necrosis factor and protein synthesis inhibitory factor (Gao and McClane 2012). However, CPE and C-CPE have limited clinical application because they are immunogenic proteins. Moreover, CPE interacts with CLDN-3, -4, -6, -7, -8, and -14 (Fujita et al. 2000). Therefore, a novel CLDN-4-specific binder must be developed and its safety confirmed to move CLDN-4-targeting into the clinical realm of cancer therapy.

Antibodies are well-known canonical binders, and monoclonal antibodies against membrane proteins that are overexpressed, mutated, or selectively expressed in tumor tissue show promise as therapeutic or diagnostic reagents. However, antibodies against the extracellular domains of CLDN family members are difficult to develop because of their low immunogenicity: they have small extracellular loop domains (~50 amino acids in the first loop and 18 in the second loop) and high interspecies similarity (~90% identity) (Mineta et al. 2011).

We recently developed monoclonal antibodies against human CLDN-4 (hCLDN-4; named 5A5 and 4D3) by immunizing rats with a CLDN-4-encoding plasmid vector (Li et al. 2014a; Kuwata et al. 2015). 5A5 and 4D3 bind to hCLDN-4 only and not to murine CLDN-4 (mCLDN-4); 5A5 also recognizes hCLDN-3. Because of its lack of murine cross-reactivity, the safety of 5A5 and 4D3 in a CLDN-4-targeting strategy could not be evaluated in murine models, and whether CLDN-4 is a potent target for cancer therapy remained unclear. In the current study, we generated a human–mouse cross-reactive and CLDN-4-specific monoclonal antibody (named 5D12) and assessed its antitumor activity and adverse effects.

Materials and Methods

Animals

Female Balb/c mice (6 weeks old), female Balb/c nu/nu mice (6 weeks old), and male Wistar rats (6 weeks old) were purchased from Shimizu Laboratory Supplies (Kyoto, Japan). All animals were maintained under controlled conditions of a 12:12-h light:dark cycle and 23 ± 1.5°C. Animal experiments were performed according to the ethics guidelines of the Graduate School of Pharmaceutical Science (Osaka University, Osaka, Japan).

Cells

L cells stably expressing mouse CLDN-1, -3, or -4 (L/mCLDN cells) were kindly provided by Dr. S. Tsukita (Kyoto University, Kyoto, Japan). HT-1080 cells stably expressing hCLDN-1, -2, -3, -4, -5, -6, -7, or -9 (HT-1080/hCLDN cells) were developed as described previously (Li et al. 2014a). LoVo, Colo205, and HT-29 human colorectal cancer cells; MKN74 and MCF-7 human gastric cancer cells; Phoenix-A packaging cells; and P3U1 mouse myeloma cells were purchased from ATCC (Manassas, VA). Jurkat cells expressing the human Fcγ receptor (FcγR) IIIa or IIa and carrying a luciferase reporter under the control of the nuclear factor of activated T cells (NFAT) promoter (Jurkat/FcγRIIIa/NFAT-Luc or Jurkat/FcγRIIa/NFAT-Luc) were used to assess the activation of FcγRIIIa or FcγRIIa, respectively (Tada et al. 2014). L/mCLDN cells, HT-1080/hCLDN cells, and Phoenix-A cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum (v/v) (Nichirei Biosciences, Tokyo, Japan), 100 U/mL penicillin, 100 μg/mL streptomycin (Nacalai Tesque, Kyoto, Japan), and 500 μg/mL G418 (Nacalai Tesque). Jurkat/FcγRIIIa/NFAT-Luc, Jurkat/FcγRIIa/NFAT-Luc, Colo205, HT-29, MKN74, MCF-7, and P3U1 cells were maintained in RPMI1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin. LoVo cells were maintained in modified HAM's F12 supplemented with 20% heat-inactivated fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin. All cells were incubated at 5% CO_2 in air at 37°C.

Generation of anti-CLDN4 monoclonal antibodies

Male Wistar rats were immunized every 2 weeks for 2 months with a eukaryotic expression vector encoding hCLDN-4, according to proprietary GENOVAC technology (GENOVAC GmbH, Freiburg, Germany). To generate hybridoma cells, lymphocytes were harvested 7 days after the final immunization and fused with P3U1 cells by using polyethylene glycol 1000 (Roche Diagnostics, Basel, Switzerland). Hybridoma cells producing the antibody that reacted with both human and mouse CLDN-4 were screened for the ability of their supernatant to bind to both hCLDN-4 and mCLDN-4 transfectants but not to parental cells. The monoclonal antibody produced by the cloned hybridoma cells was named 5D12. The subclass of 5D12 was determined by using a rat immunoglobulin isotyping enzyme-linked immunosorbent assay kit (BD Biosciences, Franklin Lakes, NJ).

Flow cytometric analysis

To analyze the binding specificity of 5D12 to various CLDNs, L/mCLDN cells, HT-1080/hCLDN cells, LoVo cells, and MKN74 cells were detached from plates by using 0.05% trypsin containing 0.02% EDTA, incubated with 5D12 (5 μg/mL), and then stained with fluorescence-conjugated goat anti-rat IgG (BD Biosciences). The fluorescence of the stained cells was analyzed by flow cytometry (FACSCalibur, BD Biosciences).

Surface plasmon resonance analysis

To determine the binding kinetics of 5D12 to hCLDN-4, surface plasmon resonance analysis (Biacore T200, GE Healthcare, Little Chalfont, UK) was performed as described previously (Li et al. 2014a). Briefly, anti-rat IgG (BD Biosciences) was immobilized on a CM5 sensor chip to approximately 5000 resonance units by using *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide and *N*-hydroxysuccinimide as activating reagents; remaining reactive groups were blocked with ethanolamine. The 5D12 antibody was immobilized on a CM5 sensor chip by using anti-rat IgG. The subsequent binding experiment used His-tagged hCLDN-4 (purified by using an Sf-9 cell expression system) as an analyte at concentrations ranging from 10 to 500 nmol/L (Uchida et al. 2010). Within a single binding cycle, the analyte was injected sequentially in order of increasing concentration over both the ligand and the reference surfaces. The reference surface, an unmodified flow cell, was used to correct for systematic noise and instrumental drift. The sensorgrams were globally fitted by using a 1:1 binding model to determine the k_a, k_d, and K_D values (Biacore T200 Evaluation Software, GE Healthcare).

Preparation of HT-1080 cells expressing various hCLDN-4 mutants

To analyze the epitope of 5D12, HT-1080 cells expressing various mutant hCLDN-4 proteins were prepared as follows: cDNAs of two hCLDN-4 mutants (T33S/V41I/T45N and L151V/A153P/S154E/G155A) were generated by site-directed mutagenesis of hCLDN-4-encoding pCX4pur (Akagi et al. 2000). The resulting vectors were transfected into Phoenix-A cells by using X-treme GENE HP DNA transfection reagent (Roche Diagnostics), and the retrovirus-containing supernatant was harvested 48 h after transfection. The retrovirus-containing supernatant was mixed with 8 μg/mL Polybrene (Sigma-Aldrich, Melbourne, Australia) and used to transduce HT-1080 cells. Stably transduced HT-1080 cells expressing each of the mutated hCLDN-4s were selected in 5 μg/mL puromycin (Life Technologies, Gaithersburg, MD).

Dot blot analysis

Samples of His-tagged hCLDN-4 with or without sodium dodecyl sulfate and 2-mercaptethanol (each containing 50 μg protein in 2 μL of phosphate-buffered saline) were spotted onto polyvinylidene fluoride membrane (GE Healthcare). After blocking of the membranes with Tris-buffered saline containing 5% skim milk and 0.05% Tween-20, the membranes were treated with 1 μg/mL of 5D12 or 100-fold-diluted mouse anti-His tag antibody (Life Technologies) as a primary antibody at 4°C for 12 h and then with 1000-fold-diluted horseradish-peroxidase–conjugated anti-rat IgG (Jackson ImmunoResearch, West Grove, PA) or horseradish-peroxidase–conjugated anti-mouse IgG (Jackson ImmunoResearch), respectively, as a secondary antibody at room temperature for 1 h. After the membrane had been washed with Tris-buffered saline containing 0.05% Tween-20, the antibody-reacted dots were detected by using a chemiluminescence imaging system (ECL Western Blotting Detection Reagent and LAS-4010 ImageQuant, GE Healthcare).

Generation of human–rat chimeric 5D12 (xi-5D12)-producing myelomas

The cDNAs encoding the heavy-chain and light-chain variable domains of 5D12 were amplified by PCR and then transferred into the pFUSE-CHIg-hG1 and pFUSE2-CLIg-hk vectors, which contain the human γ1 and κ constant domains, respectively (InVivoGen, San Diego, CA). The resulting human–rat chimeric heavy and light chains

of 5D12 were transferred into pCX4br (Akagi et al. 2000) and pCX4pur, respectively. Phoenix-A cells were cotransfected with each of these vectors by using X-treme GENE HP DNA transfection reagent, and the retrovirus-containing supernatant was harvested 48 h after transfection. The retrovirus-containing supernatant was mixed with 8 μg/mL of Polybrene and used to transduce P3U1 cells. Stably transduced xi-5D12–producing P3U1 cells were selected in 2 μg/mL of puromycin and 10 μg/mL of blastcidin (Life Technologies).

Preparation and purification of 5D12 and xi-5D12

Hybridoma cells producing 5D12 and P3U1 cells producing xi-5D12 were inoculated intraperitoneally into pristane-injected female Balb/c nu/nu mice, resulting in the production of ascitic fluid containing antibodies. Antibodies were purified from the ascitic fluid by using Protein G Sepharose 4 Fast-Flow columns (GE Healthcare). The purified antibodies then dialyzed against in phosphate-buffered saline and stored at −30°C.

Measurement of the interaction between xi-5D12 and FcγRIIIa/IIa

To assess whether xi-5D12 induced antibody-dependent cellular cytotoxicity (ADCC) and antibody-dependent phagocytosis (ADP), CLDN-expressing cells (1.0×10^4 cells/well) were seeded onto white 96-well plates (Thermo Fisher Scientific, Waltham, MA). At 24 h after seeding, the medium was changed to Opti-MEM I Reduced Serum Medium (Life Technologies) containing Jurkat/FcγRIIIa/NFAT-Luc cells (1.0×10^5 cells/well) and various concentrations of 5D12, control rat IgG (BD Biosciences), xi-5D12, or control human IgG1 (Eureka Therapeutics, Emeryville, CA). After a 5-h incubation at 5% CO_2 in air at 37°C, luciferase activity was measured by adding ONE Glo (Promega, Madison, WI) and measuring luminescence on a plate reader (TriSTAR LB 941, Berthold Technologies, Tokyo, Japan).

Distribution of 5D12 in wild-type and tumor xenograft mice

To assess the biodistribution of 5D12, 5D12 and control rat IgG were labeled with the fluorescent dye CF750 by using a XenoLight CF750 labeling kit (Caliper Life Science, Hopkinton, MA) in accordance with the manufacturer's instructions. To prepare the tumor xenograft model, Balb/c nu/nu mice were injected subcutaneously in the left flank with LoVo or MKN74 cells (1×10^7 cells in 100 μL phosphate-buffered saline). When tumor size reached 200 mm^3, mice were used for the distribution assay. Wild-type Balb/c mice or tumor-bearing mice were injected intravenously with either CF750-labeled 5D12 or control rat IgG (20 μg per mouse). At various times after injection, the tissues were harvested, and the distribution of the CF750-labeled monoclonal antibodies was visualized by using an in vivo imaging system (Maestro EX, version 2.10.0, Cambridge Research and Instrumentation, Woburn, MA).

Evaluation of antitumor activity

To study the antitumor activity of xi-5D12, mice were injected intravenously with either xi-5D12 or control human IgG1 (20 μg per mouse) twice weekly for 4 weeks, beginning after tumor inoculation. Body weight and tumor size were measured before each injection. The tumor volume (in mm^3) was calculated as tumor length $\times$ (tumor width)^2/2.

Safety study of xi-5D12

To evaluate the safety of xi-5D12, Balb/c mice were intravenously injected with either xi-5D12 or control human IgG1 at 20 μg per mouse twice weekly for 4 weeks. At 24 h after the eighth (final) injection, blood was drawn, and serum markers of liver damage (aspartate aminotransferase and alanine aminotransferase) and kidney damage (blood urea nitrogen) were evaluated by using test kits (Wako Biomedicals, Tokyo, Japan).

Statistical analysis

Data were analyzed by using Student's *t*-test followed by post hoc pairwise comparison. The statistical significance for all comparisons was set at $P < 0.05$.

Results

Generation of a cross-species monoclonal antibody against claudin-4

Attempts to generate a monoclonal antibody that recognizes a multispanning membrane protein in a steric structure-dependent manner have had limited success, partly because preparing a protein antigen is difficult. However, DNA immunization circumvents protein preparation, and because it breaks immune tolerance, DNA immunization is a means for creating antibodies that recognize integral membrane proteins and evolutionarily conserved proteins (Steitz et al. 2000; Chambers and Johnston 2003). Therefore, to generate a monoclonal antibody that was cross-reactive between human and mouse CLDN-4, rats were immunized with a plasmid vector encoding hCLDN-4.

Hybridomas were screened by using both hCLDN-4- and mCLDN-4-expressing cells, successfully resulting in the preparation of a hybridoma that produced a human–mouse anti-CLDN-4 monoclonal antibody (5D12). The subclass of 5D12 was IgG2a. 5D12 bound to HT-1080/hCLDN-4 cells and L/mCLDN-4 cells but not to HT-1080 cells expressing other hCLDNs (CLDN-1, -2, -3, -5, -6, -7, or -9) or to L cells that expressed mCLDN-1 or -3 (Fig. 1). Surface plasmon resonance analysis performed as described previously (Uchida et al. 2010) revealed the binding kinetics of 5D12 to recombinant hCLDN-4 as: k_a, 2.38 (1/Ms, $\times 10^4$); k_d, 0.34 (1/s, $\times 10^{-4}$); and K_D, 1.41 nmol/L.

Determination of the binding specificity of 5D12 to hCLDN-4

Next, the epitope of CLDN-4 recognized by 5D12 was determined. The amino acid sequences of the extracellular domains are highly conserved between hCLDN-3 and hCLDN-4, and only residues 33, 41, and 45 in the first extracellular domain and residues 151, 153, 154, and 155 in the second extracellular domain differ between these proteins (Fig. 2A). Therefore, HT-1080 cells were prepared that expressed amino acid-substituted hCLDN-4s: T33S/V41I/T45N, which contained the first extracellular domain of CLDN-3 and the second of CLDN-4, and L151V/A153P/S154E/G155A, which included the first extracellular domain of CLDN-4 and the second of CLDN-3 (Fig. 2B). An antibody that recognized both CLDN-3 and -4 (5A5 [Li et al. 2014a]) bound to both cell lines. In contrast, 5D12 bound to the T33S/V41I/T45N but not to L151V/A153P/S154E/G155A, indicating that 5D12 recognized the second extracellular domain of CLDN-4 (Fig. 2C). To investigate whether 5D12 recognized the conformation of CLDN-4, dot blot analyses were performed. Although anti-His antibody recognized both native and denatured proteins (Fig. 2D), 5D12 interacted with the native protein only (Fig. 2E). These results suggest that 5D12 recognizes the conformation of

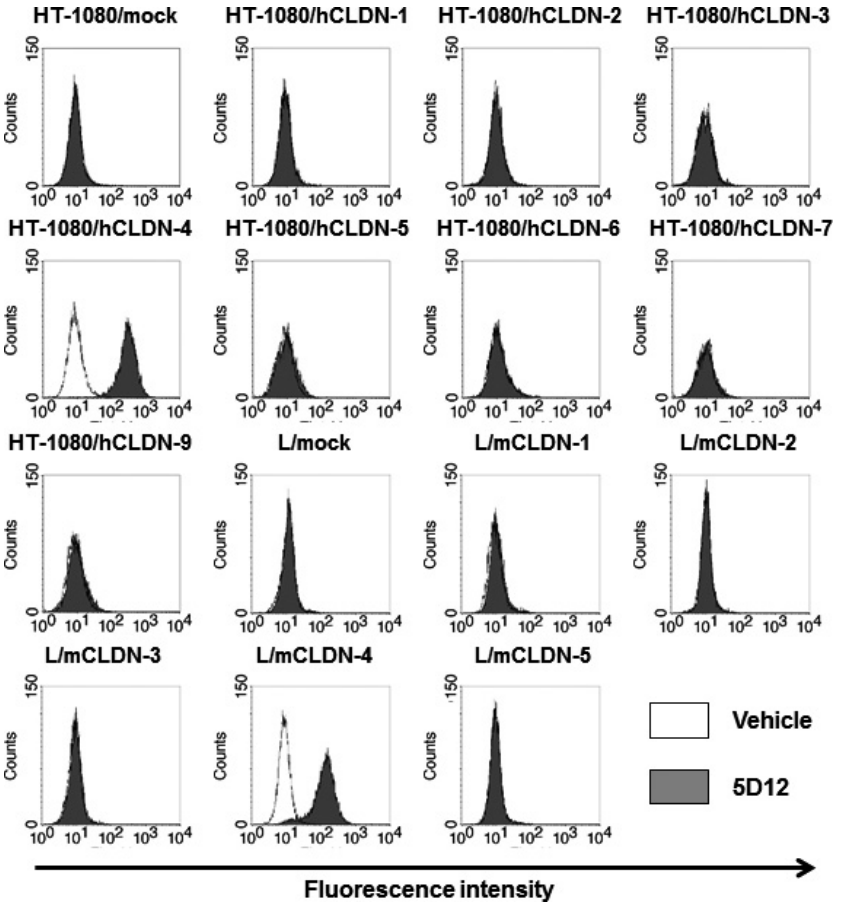

Figure 1. Claudin specificity of 5D12. Human (HT-1080) and murine (L) cells stably expressing various claudins (CLDNs) were incubated with 5 μg/mL 5D12 or vehicle only and then reacted with fluorescence-conjugated goat anti-rat IgG (H + L). The fluorescence intensity of antibody-treated cells was detected by flow cytometry. Open and filled histograms represent vehicle- and 5D12-treated cells, respectively.

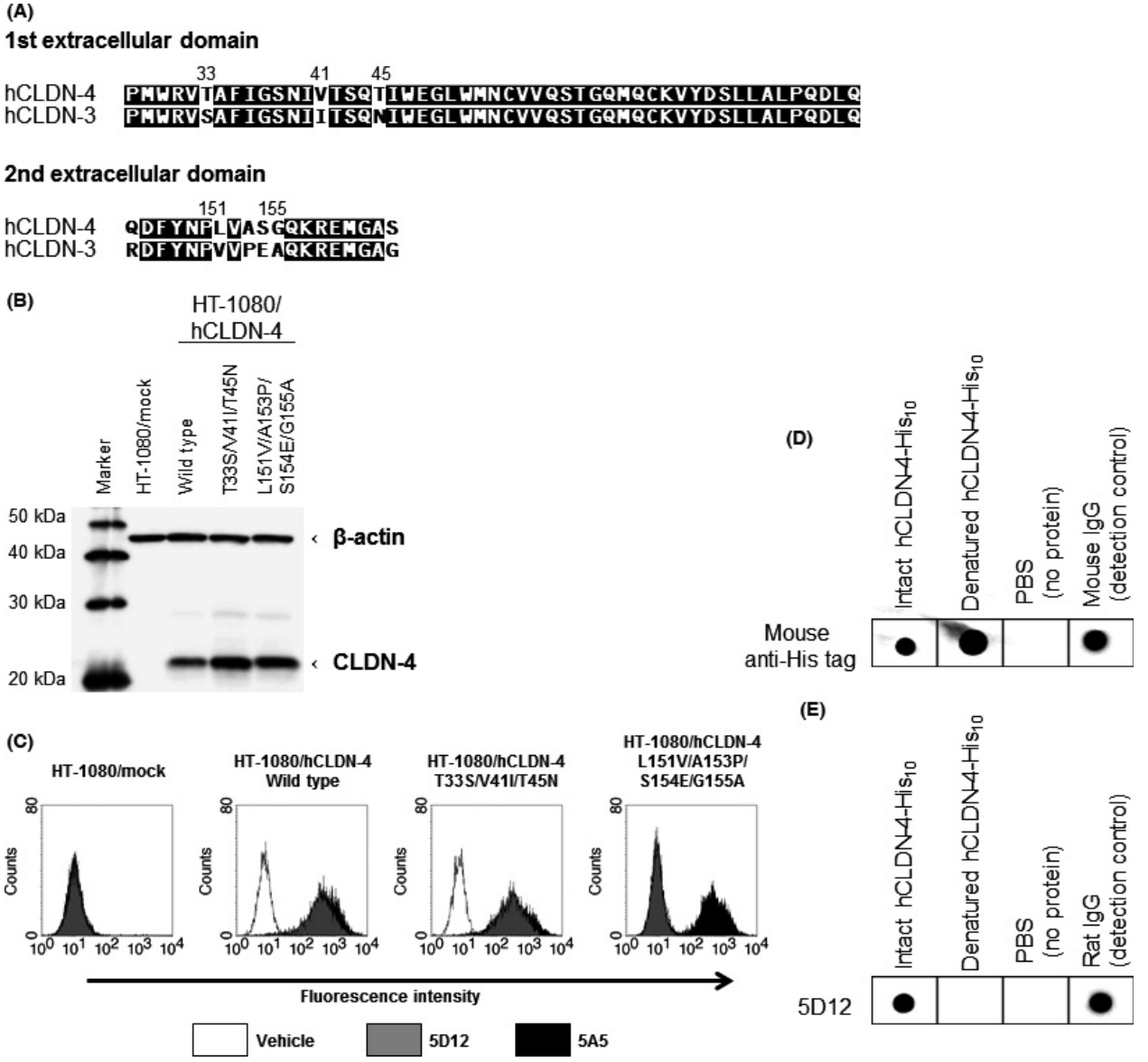

Figure 2. Epitope analysis of 5D12. (A) Sequence alignment of the first (top) and second (bottom) extracellular domains of hCLDN-4 and hCLDN-3. Amino acids perfectly conserved between hCLDN-4 and hCLDN-3 are highlighted in black. Amino acids in the white regions of hCLDN-4 were exchanged for the corresponding hCLDN-3 amino acid to construct the two domain-swapped hCLDN-4 molecules. (B) Expression of the domain-swapped hCLDN-4s in HT-1080 cells was evaluated by western blotting. All hCLDN-4 constructs were detected by an antibody that recognizes the intracellular domain of CLDN-4, and β-actin expression levels were used as a loading control. (C) HT-1080 cells expressing either wild-type hCLDN-4 or a domain-swapped hCLDN-4 (T33S/V41I/T45N and L151V/A153P/S154E/G155A) were incubated with 5 μg/mL 5D12 or 5A5 and then reacted with fluorescence-conjugated goat anti-rat IgG (H + L). The fluorescence intensity of antibody-treated cells was detected by flow cytometry. White, gray, and black histograms represent vehicle-, 5D12-, and 5A5-treated cells, respectively. (D and E) Effect of conformational structure of hCLDN-4 on the interaction between 5D12 and hCLDN-4. Either native (intact) or denatured His-tagged recombinant hCLDN-4 was spotted onto polyvinylidene fluoride membrane; spots were detected by using either (D) 5D12 or (E) anti-His tag antibody; mouse and rat IgG served as spot controls and PBS served as the no-protein control. CLDNs, claudins PBS, phosphate-buffered saline.

hCLDN-4. 5D12 bound to various cell lines that endogenously expressed hCLDN4, including colon carcinomas (LoVo cells, Colo205 cells, and HT-29 cells) and gastric carcinomas (MKN74 cells and MCF-7 cells) (Fig. 3).

Biodistribution of 5D12

The biodistribution of a therapeutic antibody is important in evaluating its efficacy and safety. To investigate

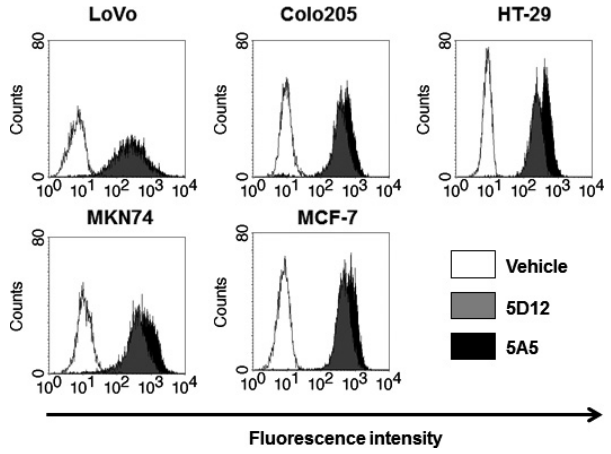

Figure 3. Binding of 5D12 to carcinomas that endogenously express hCLDN-4. The hCLDN-4-expressing cells indicated were incubated with 5 μg/mL 5D12 or 5A5 and then reacted with fluorescence-conjugated goat anti-rat IgG (H + L). The fluorescence intensity of antibody-treated cells was detected by flow cytometry. White, gray, and black histograms represent vehicle-, 5D12-, and 5A5-treated cells, respectively. CLDNs, claudins.

the biodistribution of 5D12, CF750-conjugated 5D12 or rat IgG was intravenously injected into mice, and distribution to the heart, lung, liver, spleen, kidney, intestine, and pancreas was analyzed at 10 min to 72 h after injection. 5D12 distributed to those tissues as rat IgG did (Fig. 4A). To investigate whether 5D12 was distributed to tumor tissues, the labeled antibodies were injected into mice bearing human colorectal cancer (LoVo) or human gastric cancer (MKN74) cells. 5D12 accumulated in tumor tissues more than rat IgG did (Fig. 4B). Together, these results suggest that the 5D12 anti-CLDN-4 antibody is an in vivo tumor-targeting antibody.

Preparation of human–rat chimeric 5D12

Most therapeutic antibodies for cancer therapy are human IgG1 molecules and thus activate ADCC. We therefore generated a human-rat chimeric 5D12 (xi-5D12) (Fig. S1). Like 5D12, xi-5D12 bound to both hCLDN-4 and mCLDN-4 (Fig. S2).

Human FcγRIIIa is considered to be the most important receptor for ADCC in humans (Houot et al. 2011; Shuptrine et al. 2012), and the activation of FcγRIIa leads to ADP (Jung et al. 2013). To investigate whether xi-5D12 activates FcγRIIa and FcγRIIIa, reporter assays using Jurkat/FcγRIIa/NFAT-Luc cells or Jurkat/FcγRIIIa/NFAT-Luc cells were performed (Tada et al. 2014). In this assay, the activation of FcγR on the cell surface drives the expression of a luciferase gene under the control of a NFAT-response element (Tada et al. 2014). In the presence of target cells, including mCLDN-4/L cells, hCLDN-4/HT-1080 cells, LoVo cells, and MKN74 cells, luciferase activity was dose dependently increased in Jurkat/FcγRIIa/NFAT-Luc and Jurkat/FcγRIIIa/NFAT-Luc cells by treatment with xi-5D12 in (Fig. 5A and B). These results suggest that xi-5D12 induces ADCC and ADP.

Antitumor activity and safety evaluation of xi-5D12

To assess the antitumor activity of xi-5D12, LoVo- and MKN74-bearing mice were intravenously injected with 1 mg/kg of xi-5D12 or human IgG1 (control) twice weekly until day 24 (eight doses total). Compared with human IgG1, injection of xi-5D12 suppressed tumor growth in LoVo- and MKN74-bearing mice (Fig. 6A). By day 28, xi-5D12-treatment had resulted in 50.2% and 50.3% inhibition of tumor growth in LoVo- and MKN74-bearing mice, respectively, whereas the control human IgG1 treatment had no effect on tumor growth. Furthermore, neither the LoVo- or MKN74-bearing mice showed any weight loss during the treatment period (Fig. 6B).

To confirm whether xi-5D12 induced adverse effects, Balb/c mice were intravenously injected with 1 mg/kg of xi-5D12 or human IgG1 twice weekly until day 24 (thus mirroring the therapeutic dosing regimen used in the antitumor assay). Repeated injection of mice with xi-5D12 caused no loss of body weight and no significant changes in serum markers of liver damage (alanine aminotransferase and aspartate aminotransferase) or kidney damage (blood urea nitrogen) 24 h after the last injection (Fig. 7A–C).

Discussion

Members of the CLDN family have received much attention as potential therapeutic targets (Cereijido et al. 2007; Nagase et al. 2013; Runkle and Mu 2013; Haseloff et al. 2014). In particular, CLDN-4 is a potential target for antitumor drugs because it is highly expressed in a wide spectrum of cancers, including pancreatic, ovarian, breast, gastric, and colorectal cancers (Facchetti et al. 2007; Ding et al. 2013). Several monoclonal antibodies against various extracellular domains of hCLDN-4 have shown antitumor activity in vivo, and one of them enhanced cisplatin cytotoxicity by increasing cellular permeability, leading to increased intracellular cisplatin concentrations. However, these antibodies lacked cross-reactivity to the CLDN-4 of other animals (Suzuki et al. 2009; Li et al. 2014a; Kuwata et al. 2015). The lack of antibodies with high CLDN-4 specificity and cross-reactivity to mCLDN-4 has prevented us from obtaining efficacy and safety profiles by using the same CLDN-4 binder. In present study, we successfully generated a human–mouse cross-reactive

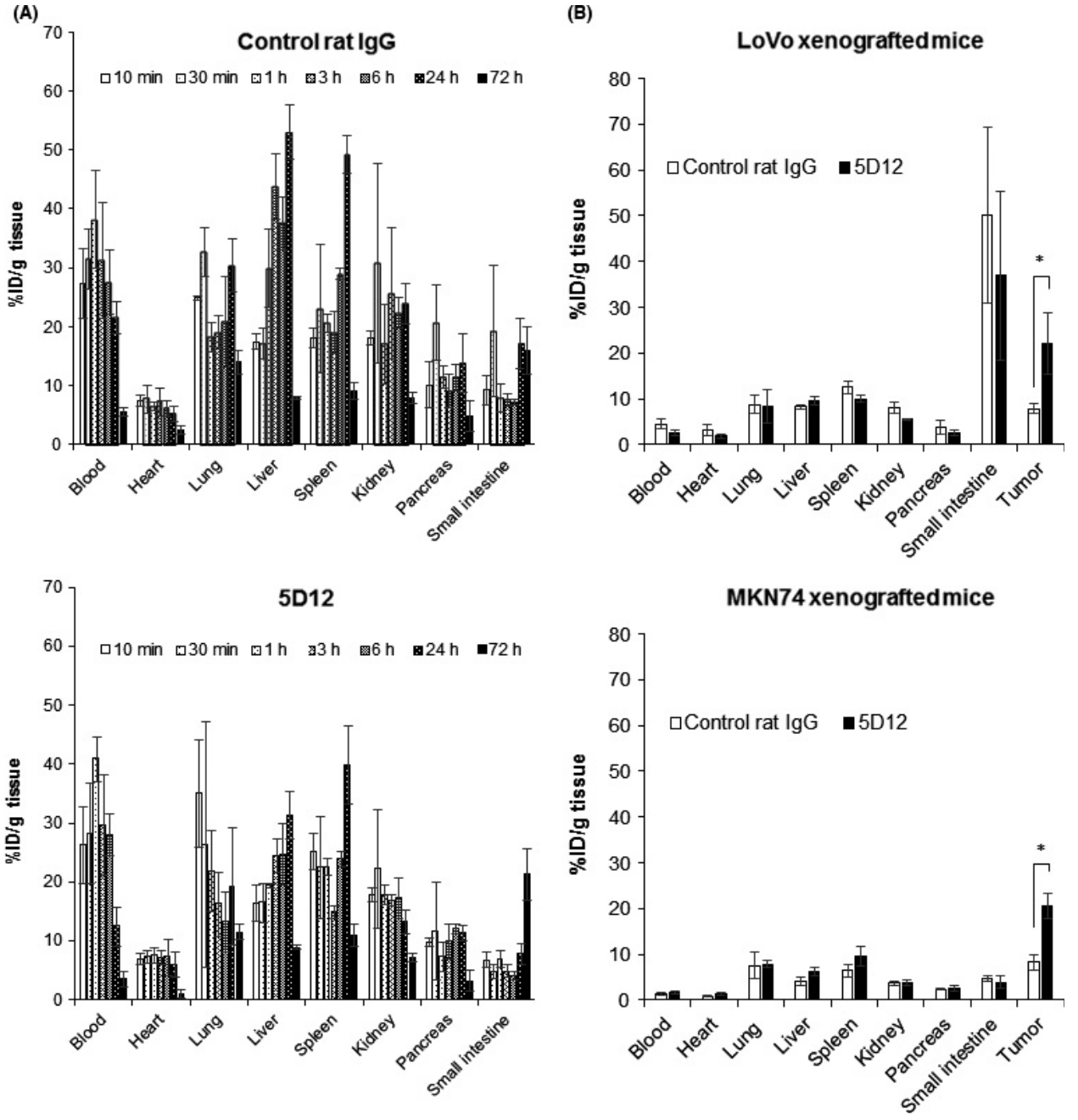

Figure 4. Biodistribution of xi-5D12 in wild-type and tumor xenograft mice. (A) Tissue distribution of xi-5D12 in wild-type mice. Mice were intravenously injected with CF750-labeled 5D12 (upper panel) or rat IgG (control; lower panel) at 20 μg per mouse, and tissues were collected at the indicated times after intravenous injection. (B) Tissue distribution of xi-5D12 in mice bearing tumors. Mice bearing LoVo (upper panel) or MKN74 (lower panel) cells were intravenously injected with CF750-labeled 5D12 or rat IgG (control) at 20 μg per mouse, and tissues were collected 72 h after intravenous injection. The amount of antibody that accumulated in each tissue was calculated as a percentage of the injected dose per gram of tissue (%ID/g tissue). Data are means ± SD (n = 3). *P < 0.05 versus control human IgG1.

CLDN-4-specific monoclonal antibody, 5D12, and we studied its physicochemical properties, antitumor activity against colorectal and gastric carcinomas, and adverse effects in mice.

We found that 5D12 recognizes the amino acid sequence around L151–G155 in the second extracellular domain of hCLDN-4 (Fig. 2B). Interestingly, this region is conserved between not only humans and mice but

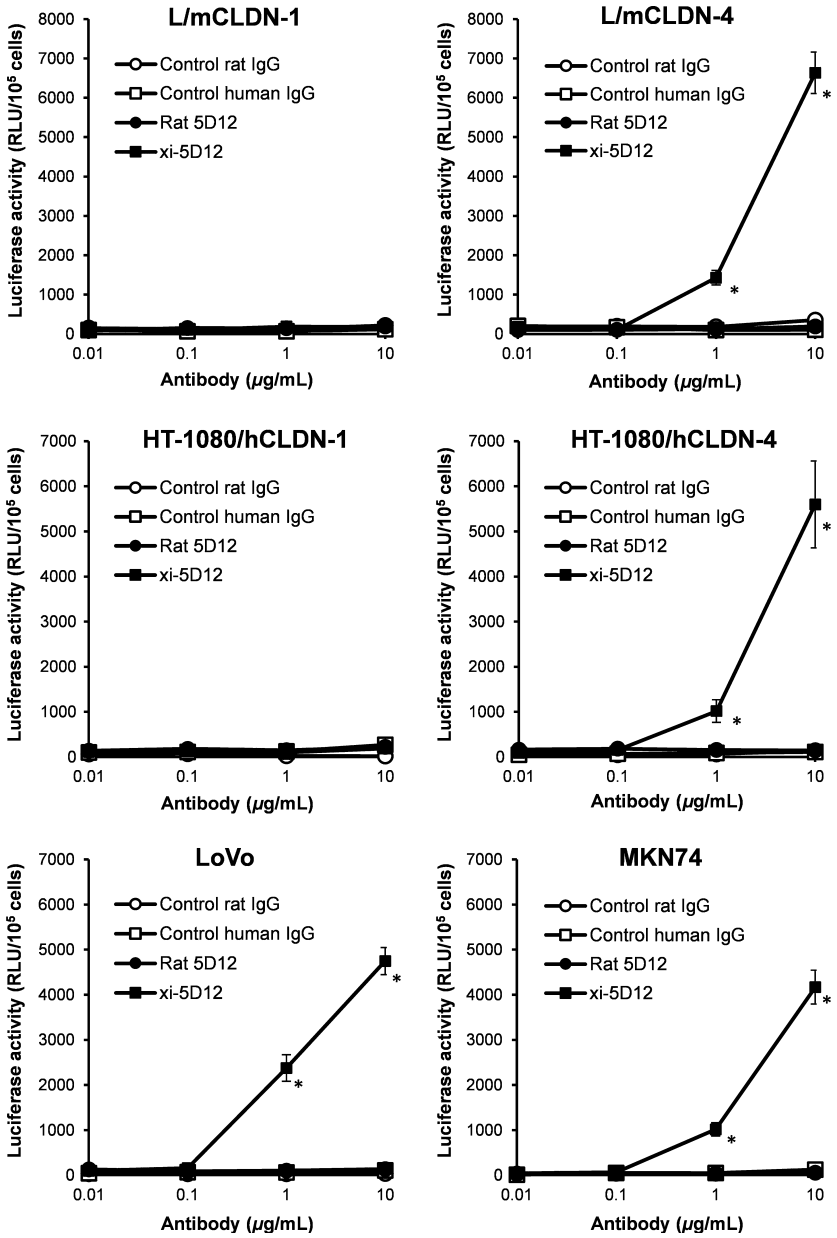

Figure 5. Estimated ADCC activity of xi-5D12 according to the FcγR reporter assay. The activity of xi-5D12 in (A) ADCC and (B) ADP was analyzed by using Jurkat/FcγIIIa/NFAT-Luc cells and Jurkat/FcγIIa/NFAT-Luc cells, respectively. L/mCLDN-1, L/mCLDN-4, HT-1080/hCLDN-1, HT-1080/hCLDN-4, LoVo, and MKN74 cells were seeded at 1×10^4 cells/well in white 96-well plates. After 24 h of incubation, Jurkat/FcγIIIa/NFAT-Luc or Jurkat/FcγIIa/NFAT-Luc cells were added at 1×10^5 cells/well in the presence of the indicated concentrations of either control rat IgG, control human IgG, 5D12, or xi-5D12. After 5 h of incubation, the luciferase activity in each well was measured. Data are means ± SD ($n = 3$). *$P < 0.05$ versus control human IgG1. CLDNs, claudins; ADCC, antibody-dependent cellular cytotoxicity; ADP, antibody-dependent phagocytosis.

many other animals as well, including Norway rats, pigs, and rhesus monkeys. In addition, this region lies within the highly variable region that is poorly conserved among members of the CLDN family (Suzuki et al. 2014). Thus, because this epitope is highly specific for CLDN-4 among the many CLDN family members, 5D12 binds specifically to CLDN-4.

Like other membrane proteins, CLDNs are difficult to crystalize; detailed structures are available for mouse CLDN-15 (Suzuki et al. 2014) and CLDN-19 (Saitoh et al. 2015) only. When the high-resolution 3D structure of CLDN-4 is determined, this information can be used as a guide for designing new small-molecule anticancer drugs. Although it might be possible to purify CLDN-4

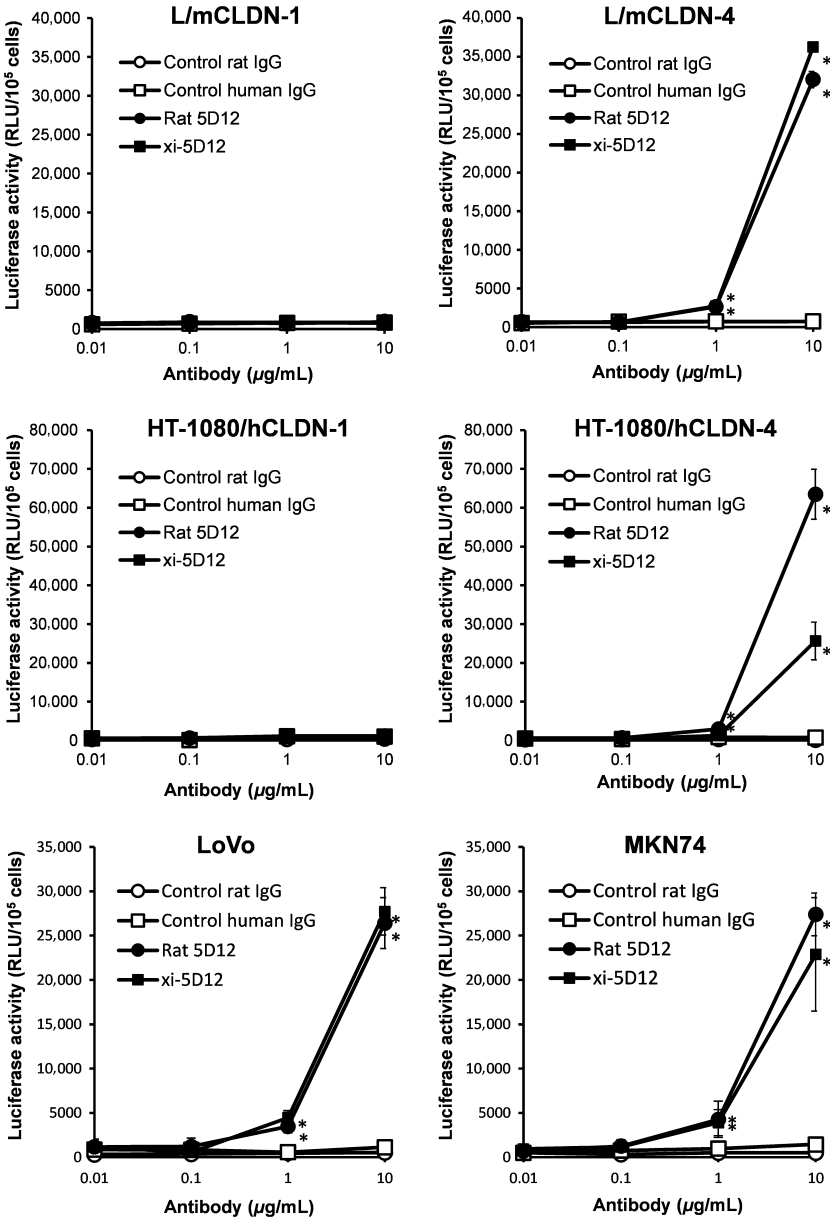

Figure 5. Continued.

by using the Sf9 system (Uchida et al. 2010), its crystallization would remain difficult. Some antibodies cocrystalize with membrane proteins by increasing the polar surface area for protein–protein interaction within the crystal lattice (Iwata et al. 1995; Day et al. 2007). Because 5D12 recognizes the extracellular domain of CLDN-4 in a conformation-dependent manner (Fig. 2E) with high affinity (K_D < 10 nmol/L), it might be used to fix the native structure of CLDN-4 and thus serve as a cocrystallizer.

In addition to its expression in various tumors, CLDN-4 is highly expressed in normal intestine and kidney in mice (Kiuchi-Saishin et al. 2002) and humans (Kirk et al. 2010). The results of our biodistribution study showed no significant difference in biodistribution in the intestine and the kidney between rat IgG and 5D12. We speculate that this result is partly due to differences in the subcellular localization of CLDN-4. CLDN-4 reportedly is restricted to the apical surfaces of intestinal cells (Rahner et al. 2001) and to the tight junctions between kidney cells (Kiuchi-Saishin et al. 2002). Given its size as an IgG molecule (height, 14.5 nm; width, 8.5 nm; thickness, 4.5 nm; Amit et al. 1986), it is unlikely that 5D12 is able to bind to CLDN-4 that is incorporated into tight

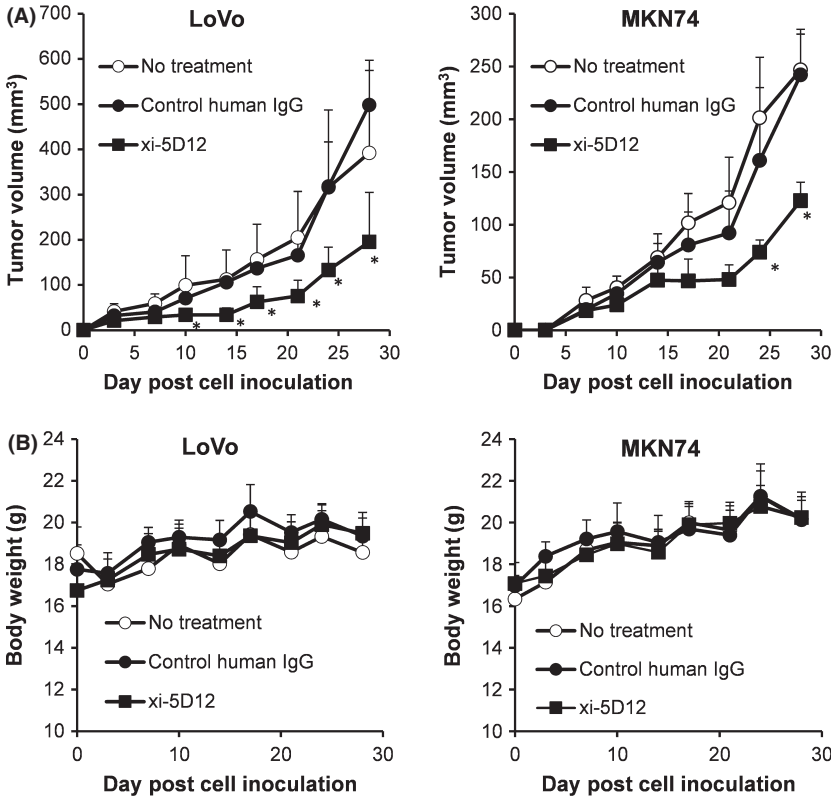

Figure 6. Antitumor effect of xi-5D12 in mice bearing tumor xenografts. (A) Tumor size and (B) body weight of xenografted mice were monitored through treatment. On day 0, LoVo cells (left) or MKN74 cells (right) were subcutaneously injected into the left flanks of Balb/c nu/nu mice. Beginning on day 0, either xi-5D12 or human IgG (control) was intravenously injected at 20 μg per mouse twice weekly for 4 weeks. The tumor volume (in mm^3) was calculated as (length $\times$ $width^2$)/2. Data are means $\pm$ SD ($n = 5$). *$P < 0.05$ versus control human IgG1.

junctions or to pass to the apical surface from the blood vessel side of cells, owing to the small pore size of tight junctions approximately 4 nm (Van Itallie et al. 2008). Treatment of human epithelial cells with 5D12 did not decrease TJs integrity (our unpublished data). However, because of the lack of cellular polarization in tumors (Singh et al. 2010), tight-junction proteins, including CLDN-4, are exposed throughout the entire cell surface. Our previous study revealed that the cytotoxicity of C-CPE–conjugated toxin was more pronounced in subconfluent cells, in which tight junctions were not yet fully developed, than in confluent cells, which had complete tight junctions (Saeki et al. 2009). Therefore, 5D12 likely can access the CLDN-4 in tumors more easily than that in healthy epithelial cells. Indeed, xi-5D12 accumulated in tumor tissues, and xi-5D12 treatment did not induce adverse effects, such as weight loss and liver and kidney injury. Together, these findings suggest that xi-5D12 might be a safe anticancer drug.

Regarding the targeting of CLDN-4, we previously generated two types of binder: C-CPE (Uchida et al. 2010) and monoclonal antibodies, 5A5 and 4D3 (Li et al. 2014a; Kuwata et al. 2015). However, C-CPE and 5A5 bound to CLDN-3 as well as CLDN-4, and 5A5 and 4D3 failed to recognize murine CLDN-4. Our study using C-CPE (a CLDN-3/CLDN-4 binder) revealed that it preferentially accumulated in the livers of mice and led to hepatic toxicity when it was fused with a cytotoxic agent (Li et al. 2014b). In contrast, our newly generated 5D12 monoclonal antibody lacked preferential hepatic accumulation and overt hepatic toxicity. Consistent with our results, many reports indicate that CLDN-3 – but not CLDN-4 – is expressed in the liver (Rahner et al. 2001). Because the C-CPE-fused toxin lacked obvious toxicity except for hepatic toxicity, 5D12 might be an even less toxic CLDN-4–targeting antibody than 5A5 and other CLDN-3/CLDN-4–binding antibodies (Kato-Nakano et al. 2010). However, a CLDN-3/CLDN-4 binder is more useful than a CLDN-4–specific binder as a diagnostic reagent, because many cancers show increased expression of both CLDN-3 and -4 (Kwon 2013). Consistent with this feature, 5A5 might bind more strongly to Colo205, HT-29, and

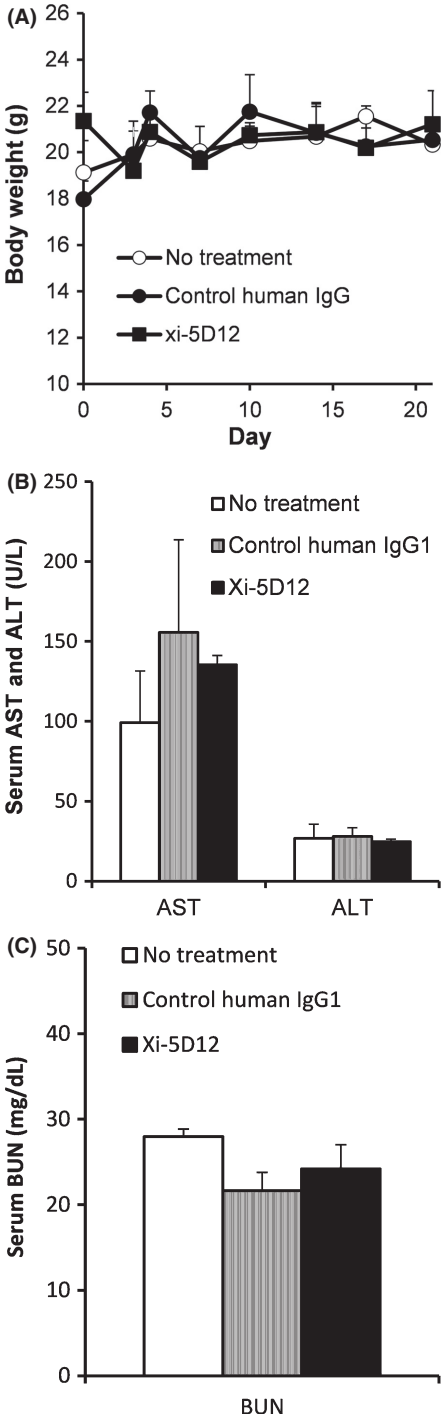

Figure 7. Safety profiles of a therapeutic regimen for xi-5D12 in wild-type mice. Balb/c mice were intravenously injected with either xi-5D12 or human IgG (control) at 20 μg per mouse twice weekly for 4 weeks. (A) The body weights of the mice were monitored throughout treatment. At 24 h after the last injection, blood was withdrawn, and serum markers of (B) liver damage (aspartate aminotransferase [AST] and alanine aminotransferase [ALT]) and (C) kidney damage (blood urea nitrogen [BUN]) were measured. Data are means ± SD (n = 3).

MCF-7 cells than would 5D12, although the binding strength of 5A5 against HT-1080/hCLDN-4 is similar to that of 5D12 (Fig. 3).

CLDN-4 facilitates the building of tight junctions through CLDN–CLDN interactions; CLDN-4 also helps to maintain homeostasis via interactions between CLDNs and currently undefined proteins or through CLDN-associated signal transduction (Van Itallie and Anderson 2013). The extracellular domain of EphA2, an ephrin receptor, might bind to the first extracellular domain of CLDN-4, thus enhancing paracellular permeability via phosphorylation of the cytoplasmic tail of CLDN-4 without altering its expression level (Tanaka et al. 2005); 5D12 might hamper the interaction between CLDN-4 and EphA2 through its steric effect. In addition, CLDN-4 is expressed in M cells, which facilitate the uptake of mucosal antigens (Suzuki et al. 2012), and Aire-expressing medullary thymic epithelial cells, which facilitate the display of tissue-restricted antigens to thymocytes for negative selection (Hamazaki et al. 2007). Because the function of CLDN-4 in these cells is currently unclear, we cannot rule out the possibility that 5D12 might induce additional effects in vivo via as-yet undefined mechanisms.

In conclusion, we generated 5D12, a novel antibody-based human–mouse CLDN-4–specific binder. To our knowledge, this study is the first to report both the antitumor efficacy and safety profiles of a CLDN-4–specific binder in experimental animals. Our findings likely have important implications for the design and development of CLDN-4 binders as anticancer drugs, targeting ligands, and diagnostic agents.

Acknowledgements

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Author Contributions

Hashimoto, Tada, Yagi, and Kondoh participated in research design; Hashimoto, Kawahigashi, Hata, and Li conducted experiments; Watari, Tada, Ishii-Watabe, and Fukasawa contributed new reagents or analytic tools; Hashimoto, Tada, Ishii-Watabe, Kuniyasu, and Kondoh performed data analysis; Hashimoto, Tada, Okada, Doi, Fukasawa, Kuniyasu, and Kondoh wrote or contributed to the writing of the manuscript.

Disclosures

None declared.

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Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article:

Figure S1. Purity of xi-5D12. Prepared xi-5D12 was subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis under nonreducing conditions and stained with Coomassie Brilliant Blue.

Figure S2. Claudin specificity of xi-5D12. Human (HT1080) and murine (L) cells stably expressing various CLDNs were incubated with 5 μg/mL xi-5D12 and then reacted with goat anti-human IgG (H + L) conjugated with fluorescein isothiocyanate. The fluorescence intensity of the vehicle (mock)- or antibody-treated cells was detected by flow cytometry. Open and filled histograms represent vehicle- and xi-5D12-treated cells, respectively.