

# World Journal of *Gastroenterology*

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**EDITORIAL**

- 2269 Gastroesophageal reflux disease and morbid obesity: To sleeve or not to sleeve?

*Rebecchi F, Allaix ME, Patti MG, Schlottmann F, Morino M*

**REVIEW**

- 2276 Advanced pancreatic ductal adenocarcinoma - Complexities of treatment and emerging therapeutic options

*Diwakarla C, Hannan K, Hein N, Yip D*

**MINIREVIEWS**

- 2286 Indoleamine 2,3-dioxygenase: As a potential prognostic marker and immunotherapeutic target for hepatocellular carcinoma

*Asghar K, Farooq A, Zulfiqar B, Rashid MU*

**ORIGINAL ARTICLE****Basic Study**

- 2294 Disruption of the TWEAK/Fn14 pathway prevents 5-fluorouracil-induced diarrhea in mice

*Sezaki T, Hirata Y, Hagiwara T, Kawamura YI, Okamura T, Takanashi R, Nakano K, Tamura-Nakano M, Burkly LC, Dohi T*

- 2308 CMA down-regulates p53 expression through degradation of HMGB1 protein to inhibit irradiation-triggered apoptosis in hepatocellular carcinoma

*Wu JH, Guo JP, Shi J, Wang H, Li LL, Guo B, Liu DX, Cao Q, Yuan ZY*

- 2318 Cullin 4A is associated with epithelial to mesenchymal transition and poor prognosis in perihilar cholangiocarcinoma

*Zhang TJ, Xue D, Zhang CD, Zhang ZD, Liu QR, Wang JQ*

- 2330 Notch signaling mediated by TGF- $\beta$ /Smad pathway in concanavalin A-induced liver fibrosis in rats

*Wang Y, Shen RW, Han B, Li Z, Xiong L, Zhang FY, Cong BB, Zhang B*

- 2337 MicroRNA-145 exerts tumor-suppressive and chemo-resistance lowering effects by targeting CD44 in gastric cancer

*Zeng JF, Ma XQ, Wang LP, Wang W*

**Case Control Study**

- 2346 Predictors for difficult cecal insertion in colonoscopy: The impact of obesity indices

*Moon SY, Kim BC, Sohn DK, Han KS, Kim B, Hong CW, Park BJ, Ryu KH, Nam JH*

**Retrospective Cohort Study**

- 2355 Impact of interferon-free antiviral therapy on lipid profiles in patients with chronic hepatitis C genotype 1b  
*Endo D, Satoh K, Shimada N, Hokari A, Aizawa Y*

**Retrospective Study**

- 2365 Transition after pediatric liver transplantation - Perceptions of adults, adolescents and parents  
*Junge N, Migal K, Goldschmidt I, Baumann U*
- 2376 Minimally invasive surgery for gastric cancer: A comparison between robotic, laparoscopic and open surgery  
*Parisi A, Reim D, Borghi F, Nguyen NT, Qi F, Coratti A, Cianchi F, Cesari M, Bazzocchi F, Alimoglu O, Gagnière J, Pernazza G, D'Imperio S, Zhou YB, Azagra JS, Facy O, Brower ST, Jiang ZW, Zang L, Isik A, Gemini A, Trastulli S, Novotny A, Marano A, Liu T, Annecchiarico M, Badii B, Arcuri G, Avanzolini A, Leblebici M, Pezet D, Cao SG, Goergen M, Zhang S, Palazzini G, D'Andrea V, Desiderio J*
- 2385 Clinical implication of FDG uptake of bone marrow on PET/CT in gastric cancer patients with surgical resection  
*Lee JW, Lee MS, Chung IK, Son MW, Cho YS, Lee SM*

**Observational Study**

- 2396 Safety and efficacy of tenofovir in chronic hepatitis B-related decompensated cirrhosis  
*Lee SK, Song MJ, Kim SH, Lee BS, Lee TH, Kang YW, Kim SB, Song IH, Chae HB, Ko SY, Lee JD*
- 2404 Can mean platelet volume play a role in evaluating the severity of acute pancreatitis?  
*Lei JJ, Zhou L, Liu Q, Xiong C, Xu CF*

**Prospective Study**

- 2414 Proposed criteria to differentiate heterogeneous eosinophilic gastrointestinal disorders of the esophagus, including eosinophilic esophageal myositis  
*Sato H, Nakajima N, Takahashi K, Hasegawa G, Mizuno K, Hashimoto S, Ikarashi S, Hayashi K, Honda Y, Yokoyama J, Sato Y, Terai S*
- 2424 Therapeutic experience of 289 elderly patients with biliary diseases  
*Zhang ZM, Liu Z, Liu LM, Zhang C, Yu HW, Wan BJ, Deng H, Zhu MW, Liu ZX, Wei WP, Song MM, Zhao Y*

**META-ANALYSIS**

- 2435 What is the quantitative risk of gastric cancer in the first-degree relatives of patients? A meta-analysis  
*Yaghoobi M, McNabb-Baltar J, Bijarchi R, Hunt RH*

**CASE REPORT**

- 2443 Hepatic angiosarcoma with clinical and histological features of Kasabach-Merritt syndrome  
*Wadhwa S, Kim TH, Lin L, Kanel G, Saito T*

**LETTERS TO THE EDITOR**

- 2448** Tumor biopsy and patient enrollment in clinical trials for advanced hepatocellular carcinoma

*Rimassa L, Reig M, Abbadessa G, Peck-Radosavljevic M, Harris W, Zagonel V, Pastorelli D, Rota Caremoli E, Porta C, Damjanov N, Patel H, Daniele B, Lamar M, Schwartz B, Goldberg T, Santoro A, Bruix J*



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## Basic Study

# Disruption of the TWEAK/Fn14 pathway prevents 5-fluorouracil-induced diarrhea in mice

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**Author contributions:** Sezaki T the majority of experiments, including acquisition of data, study concept and design, analysis and interpretation of data, and drafting the manuscript; Hirata Y, Hagiwara T, Okamura T, Takanashi R, Nakano K and Tamura-Nakano M acquisition of data; Kawamura YI acquisition of data, analysis and interpretation of data, and obtained funding; Burkly LC study concept and design, analysis and interpretation of data, and drafting the manuscript; Dohi T acquisition of data, study concept and design, analysis and interpretation of data, drafting the manuscript, and obtained funding.

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## Abstract

### AIM

To clarify the roles of TWEAK and its receptor Fn14 in 5-fluorouracil (5-FU)-induced diarrhea.

### METHODS

Diarrhea was induced in wild-type (WT), Fn14 knockout (KO), and IL-13 receptor (IL-13R) $\alpha$ 1 KO BALB/c mice using a single injection of 5-FU. Histological analysis, cytokine analysis, and flow cytometry was performed on ileal tissues and cells. Murine colon carcinoma-bearing mice were co-treated with an anti-TWEAK antibody and 5-FU. Embryonic fibroblast response to cytokines was also analyzed.

### RESULTS

5-FU induced high Fn14 expression in epithelial cells. The severity of 5-FU-induced diarrhea was lower in Fn14 KO mice compared with WT mice. Administration of anti-TWEAK antibody reduced 5-FU-induced diarrhea without affecting the antitumor effects of 5-FU *in vivo*. 5-FU-induced expression of IL-13, IL-17A, TNF- $\alpha$ , and IFN- $\gamma$  in the ileum was Fn14 dependent. The severity of 5-FU-induced diarrhea was lower in IL-13R $\alpha$ 1 KO mice, indicating major role for IL-13 signaling *via* IL-13R $\alpha$ 1 in pathogenesis. We found that IL-13R $\alpha$ 2, an IL-13 neutralizing/cell protective receptor, was strongly induced by IL-33 *in vitro* and *in vivo*. IL-13R $\alpha$ 2 was upregulated in the ileum of 5-FU-treated Fn14 KO mice. Thus, the deletion of Fn14 upregulated IL-13R $\alpha$ 2 expression, which reduced IL-13 expression and activity.

### CONCLUSION

Disruption of the TWEAK/Fn14 pathway affects several interconnected pathways, including those associated with IL-13, IL-33, and IL-13R $\alpha$ 2, to attenuate 5-FU-induced intestinal side effects.

**Key words:** Cancer chemotherapy; Interleukin-13; Interleukin-33; Interleukin-13 receptor  $\alpha$ 2

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**Core tip:** IL-13 signaling *via* IL-13 receptor (IL-13R) $\alpha$ 1 plays a central role in developing diarrhea, a major side effect of 5-fluorouracil (5-FU). Disruption of the TWEAK/Fn14 pathway alleviated diarrhea by downregulating expression of IL-13 and upregulating expression of IL-13R $\alpha$ 2, a decoy IL-13 receptor. The IL-13R $\alpha$ 2 was induced by IL-33 in mesenchymal cells of 5-FU-treated intestines *in vivo* and fibroblasts *in vitro*. IL-33 expression was independent of TWEAK/Fn14 signaling, and its cell protective function in 5-FU-treated mice was enhanced in the absence of Fn14. Disruption of the TWEAK/Fn14 pathway affects several interconnected pathways to attenuate 5-FU-induced intestinal side effects.

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## INTRODUCTION

The chemotherapeutic antimetabolite agent 5-fluorouracil (5-FU) is used to treat numerous cancer types. However, chemotherapy with 5-FU often is associated with severe gastrointestinal side effects, such as diarrhea and anorexia. These adverse effects can be difficult to manage and lead to discontinuation of chemotherapy. Therefore, strategies to reduce or prevent adverse effects are needed to improve the efficacy of this cancer treatment to allow the patient to take the full course of treatment.

Tumor necrosis factor (TNF)-like weak inducer of apoptosis (TWEAK) is a member of the TNF superfamily. TWEAK mRNA is commonly expressed in several types of leukocytes, but it has been detected in other cell types, including endothelial cells and astrocytes. Fibroblast growth factor inducible 14 (Fn14) is the smallest member of the TNF receptor superfamily and the only known signaling receptor for TWEAK<sup>[1]</sup>. Fn14 expression levels are relatively low in healthy tissues; however, Fn14 expression is strongly upregulated in injured tissues. TWEAK/Fn14 signaling can activate several downstream pathways, including the nuclear factor- $\kappa$ B (NF $\kappa$ B), mitogen-activated protein kinase, and phosphatidylinositol 3-kinase/protein kinase B pathways<sup>[2]</sup>. The TWEAK/Fn14 pathway is involved in the repair of injured tissue; however, excessive activation contributes to tissue damage and pathological tissue remodeling<sup>[2]</sup>. Thus, the TWEAK/Fn14 pathway is a potential drug target for various diseases, including those that affect intestines, kidneys, liver, joints, nervous system, skeletal muscles, and vasculature<sup>[1]</sup>.

This study focuses on the role of TWEAK/Fn14 in intestinal disease. Previously, we showed that Fn14 is expressed in injured epithelial cells (ECs). Treatment with a TWEAK-blocking antibody or knocking out TWEAK considerably ameliorated acute colitis in mice by reducing inflammation and limiting chemokine and matrix metalloproteinase expression in ECs<sup>[3]</sup>. Fn14 also mediates interleukin (IL)-13- and TNF- $\alpha$ -induced cell death in ECs<sup>[4]</sup>. TWEAK and TNF- $\alpha$  synergistically activate the canonical NF- $\kappa$ B pathway, which aggravates tissue damage in acute colitis<sup>[5]</sup>. In chronic colitis models, we showed that TWEAK and IL-13 cooperatively induce the expression of thymic stromal lymphopoietin in ECs. This expression is a

key mechanism in the development of fibrosis<sup>[6]</sup>. In the context of  $\gamma$ -irradiation-induced injury, both TWEAK and Fn14 KO mice had more regenerating microcolonies in intestinal crypts than wild-type (WT) mice<sup>[3]</sup>. However, to our knowledge, no studies have addressed if TWEAK/Fn14 is involved in chemotherapy-induced damage of normal intestinal mucosa.

IL-33 is a member of the IL-1 family<sup>[7]</sup>. It is expressed constitutively in ECs of healthy intestine and is localized to the nucleus. Nucleus-localized IL-33 functions as a transcription factor<sup>[8,9]</sup> where it contributes to mucosal protection in various colitis models<sup>[10-13]</sup>. After EC injury or death, IL-33 undergoes cleavage and is released into the extracellular space<sup>[14]</sup>. Released IL-33 induces the expression of T helper 2 cytokines, such as IL-5 and IL-13, in various cells, including T helper 2 cells, group 2 innate lymphoid cells, eosinophils, and basophils<sup>[15]</sup>. These responses play major roles in parasite clearance and allergic reactions. The mechanisms behind the dual role of IL-33 as a protector of mucosal barriers and an alarmin<sup>[15]</sup> are not fully understood.

In this report, we investigated the effect of blocking TWEAK/Fn14 signaling on chemotherapy-induced intestinal side effects. Disruption of the TWEAK/Fn14 pathway using genetic or pharmacological approaches reduced 5-FU-induced diarrhea without impacting the antitumor effect of 5-FU. We also propose a novel protective mechanism for IL-33 in mucosal tissues.

## MATERIALS AND METHODS

### Mice

Male BALB/c mice and Fn14 KO mice<sup>[16]</sup> with a BALB/c background that were 8-12-wk-old were purchased from Clea Japan, Inc. (Tokyo, Japan). IL-13 receptor (IL-13R)  $\alpha$ 1 KO mice were generated from BALB/c mice in our facility using a CRISPR/Cas9-mediated genome-editing system. All experimental protocols were approved by the institutional animal care and use committee of the National Center for Global Health and Medicine, Japan (#16086).

### Treatment with chemotherapeutic agents and diarrhea scores

An intraperitoneal injection of 5-FU (250 mg/kg body weight, Sigma, Tokyo, Japan) in phosphate-buffered saline pH 7.4 (PBS, Nacalai Tesque, Kyoto, Japan) was administered to mice on day 0. The mice were monitored for diarrhea once a day. To assess diarrhea severity, individual mice were photographed daily during defecation and scoring of the photos was performed blindly to guarantee the objectivity of the scoring system (Figure 1A). Severity was scored as follows: 0, normal; 1, wet and unformed stool; 2, watery stool.

### Real-time polymerase chain reaction

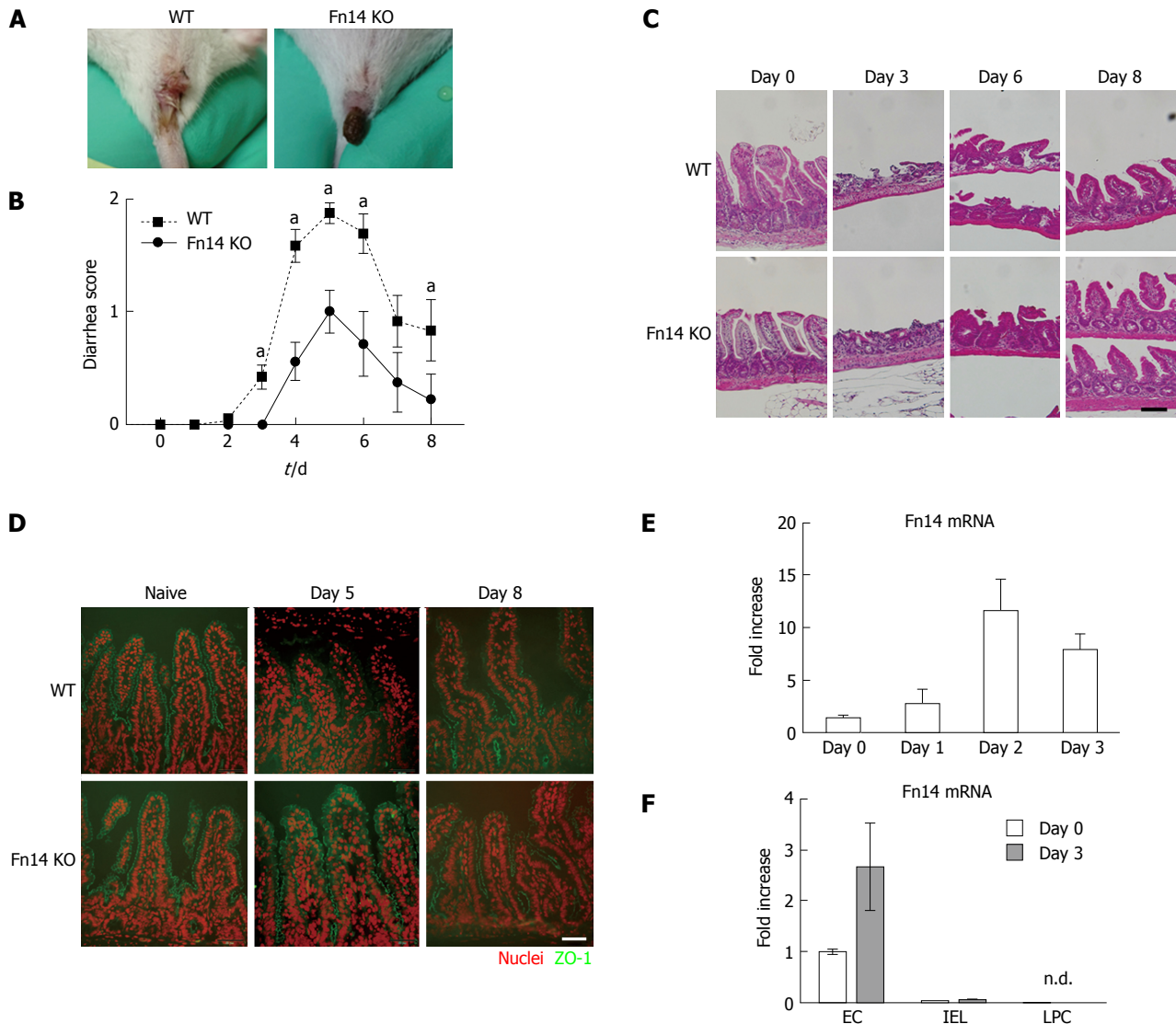
Total RNA of whole ileum was prepared using RNA-

Bee RNA isolation solvent (Tel-Tests, Inc, Friendswood, TX). RNA from purified lamina propria cells (LPCs) or ECs was extracted using the RNeasy micro kit (QIAGEN, Tokyo, Japan). Complementary DNA (cDNA) was synthesized from the RNA using High Capacity cDNA Reverse Transcription Kits (Life Technologies). The resulting cDNA samples were used to perform quantitative real-time PCR reactions using a 7900HT Fast Real-Time PCR System (Applied Biosystems, Warrington, England). Primers and probes for murine genes were purchased from Applied Biosystems. The following TaqMan Gene Expression Assays were used in this study: TWEAK (Mm02583406\_s), Fn14 (Mm00489103\_m1), IL-33 (Mm00505403\_m1), IL-13R $\alpha$ 1 (Mm00446726\_m1), IL-13R $\alpha$ 2 (Mm00515166\_m1), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (4352932E). All experiments were performed according to their respective manufacturer's protocols unless otherwise indicated. Results were normalized to GAPDH mRNA as an internal control. Threshold cycle numbers (Ct) were determined using Sequence Detector Software (version 2.3; Applied Biosystems) and transformed using the  $\Delta$ Ct/ $\Delta\Delta$ Ct method as described by the manufacturer.

### Histological analysis

Ileum tissues (the distal third of the small intestine) were opened longitudinally on filter paper and fixed overnight with Mildform (WAKO, Osaka, Japan). Tissues were rolled and embedded in paraffin. Paraffin-embedded tissues were sliced into 4- $\mu$ m thick sections and stained with hematoxylin and eosin. To detect proliferating cells, sections were autoclaved at 121 °C for 5 min in 10 mmol/L citrate (pH 6.0) and stained with anti-Ki67 antibody (Vector Laboratories, Burlingame, CA, United States) and Envision+ dual Link System -HRP (DAKO, Glostrup, Denmark). To detect apoptotic cells, TdT-mediated dUTP nick end labeling was applied using DeadEnd™ Colorimetric TUNEL System (Promega, Tokyo, Japan). For IL-33 and IL-13R $\alpha$ 2 staining, acetone-fixed frozen sections were used. Sections were incubated with anti-IL-33 antibody (1:20 dilution, R&D Systems) or anti-IL-13R $\alpha$ 2 antibody (1:50, R&D Systems) then incubated with FITC-conjugated anti-goat IgG antibody (1:200) or Alexa Fluor 488-conjugated anti-rat IgG antibody (1:300, Life Technologies), respectively. Zonula occludens-1 (ZO-1) immunohistochemistry was performed as described previously<sup>[4]</sup>. Briefly, cryosections were fixed with 4% paraformaldehyde, incubated with anti-ZO-1 antibody (1:25, Invitrogen, Yokohama, Japan) for 2h at RT, then incubated with FITC-conjugated anti-rabbit antibody (1:100, Santa Cruz Biotechnology, TX, United States). Images were collected using a BX50 fluorescence microscope (Olympus, Tokyo) with a DP72-cellSens Standard image capture system (Olympus, Tokyo). Sections were counterstained with 4',6-diamidino-2-phenylindole (DAPI, Sigma). When





**Figure 1** Fn14 deficiency ameliorated 5-FU-induced diarrhea and ileal damage. **A:** Representative images of the macroscopic appearance of an anus from a WT mouse with liquid diarrhea and an Fn14 KO mouse with normal fecal pellets following 5-FU administration; **B:** Diarrhea scores after injection of 5-FU (day 0). Number of mice was about 7-44 at each time point. Results of mouse groups of different observation-time points and endpoints were accumulated and summarized ( $P < 0.05$ ); **C:** Formalin-fixed paraffin-embedded ileal sections were prepared from samples collected at the indicated time point from 5-FU-treated WT or Fn14 KO mice and stained with hematoxylin and eosin. Scale bar = 100  $\mu$ m; **D:** Frozen ileal sections were prepared from samples collected at the indicated time point from 5-FU-treated WT or Fn14 KO mice ( $n = 3$  mice per group) and probed with anti-ZO-1 antibody. Green: ZO-1, Red: Nuclear staining. Scale bar = 50  $\mu$ m. Representative images are shown; **E:** Fold increase of Fn14 mRNA in total ileal mucosa after injection of 5-FU. Data are presented as the mean  $\pm$  SD; **F:** Epithelial cells (EC), intraepithelial lymphocytes (IEL), and lamina propria cells (LPC) were separated from the ileum collected on day 0 (naïve) or day 3 after 5-FU injection, and Fn14 mRNA expression was measured ( $n = 3$  mice per group). Data are presented as the mean  $\pm$  SD.

images with green signals were merged, the blue DAPI signal was converted to red for clarity. Image merging was performed using Adobe Photoshop CS5 version 12.1. To quantify the fluorescence signal, images were collected using a fixed exposure condition, and the signal was measured using ImageJ software (NIH). The IL-13R $\alpha$ 2-positive area in ileal tissue was normalized to the longitudinal length of the measured intestine in the image's field of view. In cell culture, IL-13R $\alpha$ 2-positive area was normalized to the number of nuclei (cells) in the measured area in the image's field of view. In some *in vitro* experiments, determination of IL-13R $\alpha$ 2-positive and -negative cells was definitive. Those data are presented as the percent of positive

cells for each experimental condition when more than 50 cells were observed.

#### Administration of anti-TWEAK antibody

Mice were inoculated subcutaneously in the left flank with  $1 \times 10^7$  CT26 murine colon carcinoma cells 7 d prior to day 0. Tumor volume (calculated as volume =  $0.52 \times \text{length} \times \text{width}^2$ ) was monitored throughout the experiment. Starting at 3 d prior to day 0, treatment with murine anti-TWEAK antibody (10 mg/kg; P2D10; Biogen, Cambridge, MA, United States) or control antibody (10 mg/kg, anti-hen egg lysozyme, mIgG2a, Biogen) was initiated. Antibodies were intraperitoneally injected every other day throughout the study

period. On day 0, 250 mg/kg 5-FU was injected intraperitoneally.

### Cell culture

The murine colon carcinoma cell line CT26 was obtained from American Type Culture Collection. CT26 cells were maintained in RPMI1640 medium (Sigma, Tokyo, Japan) with 10% fetal bovine serum. Mouse embryonic fibroblasts (MEFs) were cultured from WT BALB/c mice. MEFs were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. For IL-13R $\alpha$ 2 induction in MEFs, 8-well chamber slides (Matsunami glass, Kishiwada, Japan) were inoculated with  $3 \times 10^3$  cells per well and cultured with IL-13 (40 ng/mL, R&D Systems, Minneapolis, MN) or IL-33 (20 ng/mL, R&D Systems) for 3 d. Cells were fixed with methanol at -20 °C for 20 min, blocked with BLOCKACE (DS Pharma Biomedical, Osaka, Japan), incubated with anti-IL-13R $\alpha$ 2 antibody (R&D Systems, Minneapolis, MN) for 1 h, then incubated with Alexa Fluor 488-conjugated anti-rabbit IgG antibody (Life Technologies, Tokyo, Japan) for 1 h. Nuclei were visualized using DAPI staining. Images were collected using a BX50 fluorescent microscope (OLYMPUS, Tokyo, Japan).

### Separation of ECs, intraepithelial lymphocytes, and LPCs

To dissociate ECs, ileal tissues were rinsed with PBS, suspended in 2 mmol/L EDTA in PBS, and stirred for 20 min at 37 °C. Single cell suspensions were stained using phycoerythrin (PE)-conjugated anti-EpCAM antibody (BioLegend, San Diego, CA, United States) and PE-Cy7-conjugated anti-CD45 antibody (BioLegend). Cells were sorted using a MoFlo XDP cell sorter (Beckman Coulter, Tokyo, Japan). ECs were EpCAM<sup>+</sup>CD45<sup>-</sup>, and intraepithelial lymphocytes (IELs) were EpCAM<sup>-</sup>CD45<sup>+</sup>. To prepare LPCs, ileal tissues were rinsed with PBS, suspended in 2 mmol/L EDTA in PBS, and stirred for 20 min at 37 °C to dissociate the ECs. The non-dissociated cells were digested with a Lamina propria dissociation kit (Miltenyi Biotec, Tokyo, Japan) according to the manufacturer's protocol to obtain LPCs.

### Immunoblotting

Cells were washed with PBS and lysed in 1% sodium dodecyl sulfate (Sigma) with a proteinase inhibitor cocktail (GE Healthcare, Little Chalfont, England) and a phosphatase inhibitor cocktail (Roche Diagnostics, Tokyo, Japan). Samples were electrophoresed and transferred onto PVDF membranes (Millipore). After blocking with 10% bovine serum albumin (Wako, Osaka, Japan), the membrane was incubated with anti-p-STAT6 antibody (Cell Signaling Technology, Tokyo, Japan) or anti-GAPDH antibody (Cell Signaling Technology) then incubated with HRP-conjugated anti-rabbit IgG antibody (Jackson ImmunoResearch, West Grove, PA, United States). The signal was detected

with an ECL prime kit (GE Healthcare, Little Chalfont, United Kingdom) and imaged using a LAS 4000 analyzer (FUJIFILM, Tokyo, Japan).

### Measurement of cytokines

Ileum was collected on day 0 (naïve tissue) and days 3, 5, and 8 after the administration of 5-FU or PBS. Tissues were homogenized with a Bio-Plex cell lysis kit (BIO-RAD, Hercules, CA, United States) and stored at -80 °C until needed. Cytokines were quantified with the Bio-Plex Pro Mouse Cytokine 23-Plex and Bio-Plex Pro Mouse Th17 panels using a Bio-Plex 3D Suspension Array System (BIO-RAD) according to the manufacturer's instructions.

### Detection of IL-13-positive cells and flow cytometry

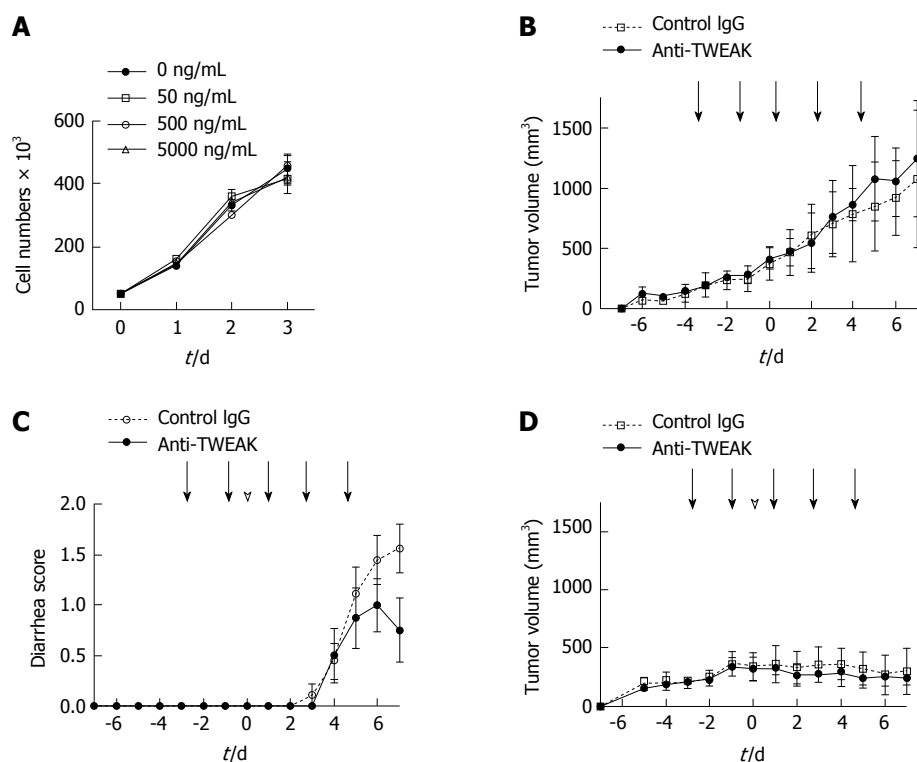
Ileum-derived LPCs ( $1 \times 10^6$  cells/assay) were incubated for 44 h in RPMI1640 with 5% fetal bovine serum and IL-33 (40 ng/mL, R&D Systems) or vehicle (PBS). Protein Transport Inhibitor (0.33  $\mu$ L, BD GolgiStop™, BD Biosciences, NJ) was added, and the cells were incubated for an additional 4 h. Cells were incubated with allophycocyanin (APC)-conjugated anti-lineage antibody cocktail (BD Biosciences). Following surface marker labeling, cells were treated with a Fixation/Permeabilization Solution Kit (BD Biosciences) and incubated with PE-conjugated anti-IL-13 antibody (eBioscience). Labeled cells were analyzed using the MoFlo XDP cell sorter.

### Preparation of ileum homogenate and depletion of IL-33

Ileum tissue was collected from WT or Fn14 KO mice two days after 5-FU administration, and 28 mg was minced in 1 mL of MEF culture medium (described above) with Antibiotic-Antimycotic Mixed Solution (Nacalai tesque, Kyoto, Japan). The sample was centrifuged at  $300 \times g$  for 5 min. The supernatant fraction was collected as ileum homogenate. The homogenate was mixed in a 1:9 ratio with MEF culture medium. IL-33 was depleted from the homogenate by adding 2  $\mu$ g of anti-IL-33 antibody or total goat IgG (Jackson ImmunoResearch, West Grove, PA, United States) to 1 mL of homogenate and incubating for 1 hour at 4 °C. Antibodies were removed by adding 20  $\mu$ L of Protein G PLUS-Agarose (resuspended volume, Santa Cruz biotechnology) and incubating for 1 h at 4 °C. After centrifugation, the supernatant fraction (*i.e.*, the IL-33-depleted homogenate) was used for cell culture.

### Statistical analysis

All statistical analyses were performed in the Prism 5 software for Mac OS X, version 5.0d. The data were expressed as the mean  $\pm$  SD.  $P < 0.05$  was considered to be statistically significant. Results of diarrhea score, cytokine measurement and IL-13R $\alpha$ 2 protein expression in time course experiments were analyzed with Two-way ANOVA adjusted with



**Figure 2** Blocking the TWEAK/Fn14 pathway prevents 5-FU-induced diarrhea in tumor-bearing mice. **A:** CT26 cells were cultured with various doses of anti-TWEAK antibody. Cell numbers were counted at the indicated time using the trypan-blue dye exclusion method. Triplicate cultures were performed for each condition. Data are shown as a mean  $\pm$  SD; **B:** CT26 cells were inoculated in WT mice on day -7 ( $n = 3$  per group). Mice were injected with anti-TWEAK antibody or control IgG from day -3, and injections were repeated every other for the remainder of the study period (indicated as arrows). 5-FU was not administered. C26 tumor volume was monitored. Data are shown as mean  $\pm$  SD; **C:** Diarrhea score of CT26 tumor-bearing WT mice treated with anti-TWEAK antibody ( $n = 8$ ) or control IgG ( $n = 9$ ). Experiment was performed as described in panel B with the addition of a single injection of 5-FU on day 0 (blank arrowhead); **D:** Tumor volume of measurements from the experiment described in (C).

Bonferroni correction. IL-13R $\alpha$ 2 protein expression after stimulation with IL-33 *in vivo* and *in vitro* in comparison of WT and Fn14 KO was analyzed with Mann Whitney *U* test. IL-13R $\alpha$ 2 protein expression in various conditions of *in vitro* culture was compared with one-way ANOVA adjusted with Bonferroni correction.

## RESULTS

### Fn14 KO mice are resistant to the side effects of 5-FU

All WT mice developed severe diarrhea on day 3 after 5-FU administration, and it persisted throughout the study period. By contrast, Fn14 KO mice developed milder diarrhea than WT mice (Figure 1A and B). Despite the difference in diarrhea severity between WT and Fn14 KO mice, histological analysis showed no difference in the small and large intestine between the two groups. Both mouse groups showed decreased numbers and height of ileal villi (Figure 1C), and there was no difference in the number of apoptotic or proliferating cells in the ileum and colon (Supplementary Figure 1). To investigate the mechanism for developing diarrhea, we measured the expression of transporter genes related to diarrhea, such as cystic fibrosis transmembrane conductance regulator (CFTR), Na-K-Cl cotransporter 1 (NKCC1),

and downregulated in adenoma (DRA) (Supplementary Figure 2). 5-FU decreased CFTR expression and increased NKCC1 and DRA expression up to 3-fold; however, there were no differences between gene expression in WT and Fn14 KO mice. We concluded that expression of these molecules did not play a major role in 5-FU-induced diarrhea and hypothesized that the diarrhea was related to epithelial cell damage. On day 8 after 5-FU administration, both mouse groups had villi shortening. However, expression of ZO-1, a tight junction protein, persisted at days 5 and 8 in the villi of Fn14 KO mice, whereas its expression decreased in the villi of WT mice, especially in the upper part (Figure 1D). Thus, ZO-1 expression in the villi was parallel to diarrhea score. These results suggested that 5-FU can damage tight junctions and the epithelial cell barrier, which increases paracellular permeability and results in diarrhea.

Next, we measured Fn14 expression in the ileum. In WT mice, 5-FU administration strongly induced Fn14 mRNA expression in the ileum by day 2 (Figure 1E). 5-FU-induced Fn14 mRNA expression was observed in ECs but not IELs or LPC (Figure 1F). Collectively, these results indicate that Fn14 expression in ECs increases chemotherapeutic-induced diarrhea and disruption of the TWEAK/Fn14 pathway could potentially prevent 5-FU-induced diarrhea.

### Blocking the TWEAK/Fn14 pathway prevents 5-FU-induced diarrhea in tumor-bearing mice

We used a mouse model bearing CT26 murine colon carcinoma tumors to investigate the effect of blocking the TWEAK/Fn14 signal pathway on 5-FU anticancer therapy. While CT26 expressed considerable levels of Fn14, addition of a TWEAK-neutralizing antibody *in vitro* did not change the cell growth rate (Figure 2A). The growth of CT26 tumors in the left inguinal region of WT mice was not altered by repeated anti-TWEAK or control antibody administration (Figure 2B). These results suggest that TWEAK/Fn14 signal disruption does not affect CT26 tumor growth *in vitro* or *in vivo*. Then, we treated CT26-tumor-bearing mice with a combination of 5-FU and anti-TWEAK antibody or a control antibody. Control antibody-treated mice had severe continuous 5-FU-induced diarrhea, whereas anti-TWEAK antibody treatment significantly suppressed 5-FU-induced diarrhea (Figure 2C,  $P = 0.0384$ ). Importantly, tumor growth inhibition by 5-FU was effective with both control and anti-TWEAK antibody administration (Figure 2D). These results indicate that the anti-TWEAK antibody could be used to prevent 5-FU-induced diarrhea in anticancer chemotherapy.

### IL-13-mediated diarrhea in Fn14 KO mice

To investigate the mechanism by which diarrhea is prevented with Fn14 deficiency, we measured the expression levels of 5-FU-induced cytokines in the ileum. IL-13, TNF- $\alpha$ , IFN- $\gamma$ , and IL-17A protein levels tended to increase on day 5 after 5-FU administration in WT mice compared with naïve ileum. These levels were higher than those in Fn14 KO mice (Figure 3). Previously, we showed that IL-13 is a pivotal mediator of intestinal epithelia damage following  $\gamma$ -irradiation<sup>[17]</sup>. Additionally, we showed that IL-13 works in concert with TNF- $\alpha$  to induce apoptosis in intestinal ECs in an Fn14-dependent manner<sup>[4]</sup>. To assess the role of IL-13 in 5-FU-induced diarrhea, we disrupted the IL-13R $\alpha$ 1 gene to generate IL-13R $\alpha$ 1 KO mice (Figure 4). Similar to the phenotype observed in Fn14 KO mice, the diarrhea score following 5-FU injection was lower in IL-13R $\alpha$ 1 KO mice through day 6. However, on days 7 and 8, there were no significant differences between the diarrhea score of IL-13R $\alpha$ 1 KO and WT mice (Figure 4). These results indicate that IL-13 is a major mediator of 5-FU-induced severe diarrhea, and its impact peaks around days 5-6.

### Abrogation of IL-33-induced IL-13 expression in Fn14 KO mice

Because IL-33 is a potent inducer of IL-13, we initially hypothesized that IL-33 expression in ECs was Fn14 dependent. However, 5-FU induced IL-33 protein expression in the ileum of both WT and Fn14 KO mice by day 3 (Figure 5A). IL-33 was sustained at high levels in Fn14 KO ileum on day 5 (Figure 5A). IL-33 mRNA levels were elevated in ECs of Fn14 KO mice

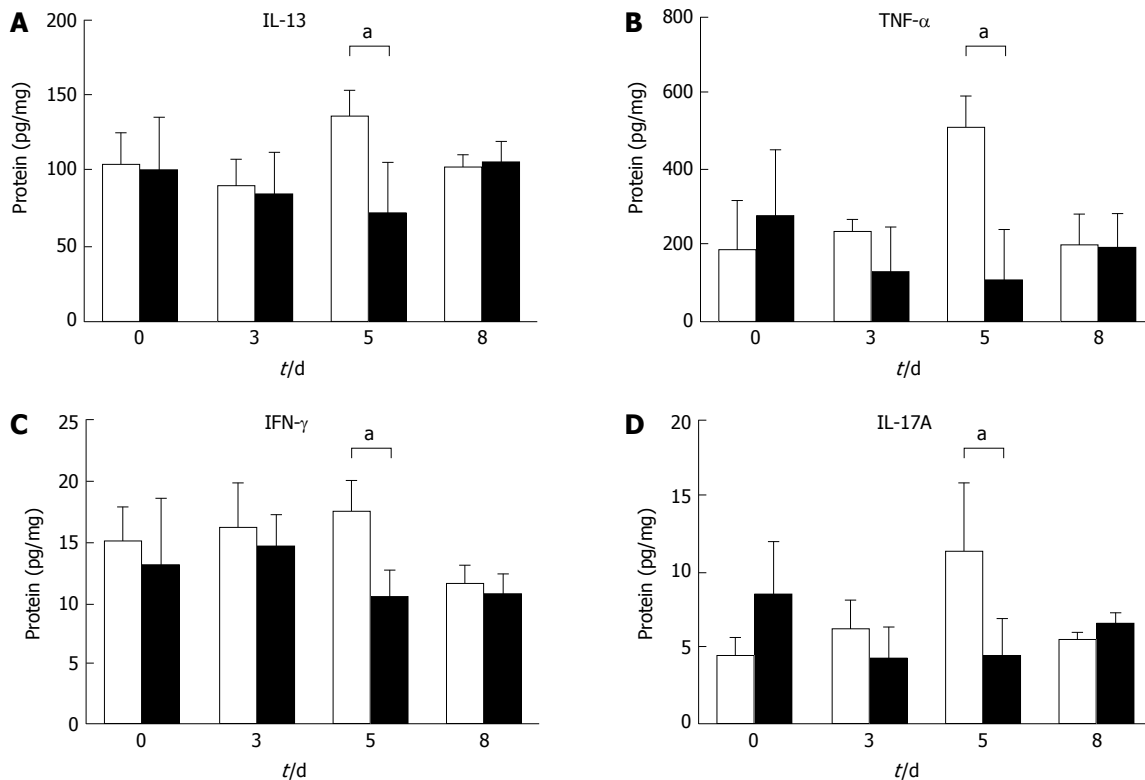
but not detected in LPCs, which is consistent with our observations of IL-33 protein levels (Figure 5B). Immunostaining with anti-IL-33 antibody confirmed that the number of IL-33-positive ileal ECs in Fn14 KO mice was greater than that in WT mice on day 5 after 5-FU administration (Figure 5C). IL-33 localized to the cytosol in WT mice but was found in both the cytosol and nucleus in Fn14 KO mice on day 5. The IL-33 staining pattern remained the same in Fn14 KO mice on day 8 (Figure 5C). These results indicate that 5-FU induced IL-33 in both WT and Fn14 KO mice but at much higher levels and with different subcellular localization in Fn14 KO mouse.

Because IL-13 expression in Fn14 KO mice was low compared with that in WT mice despite higher IL-33 expression (Figure 3A), we investigated whether IL-33-induced IL-13 expression was impaired in Fn14 KO LPCs. *In vitro* stimulation of WT LPCs with recombinant IL-33 induced IL-13 expression in lineage-negative (Lin-) fractions as expected. By contrast, this induction was attenuated in Fn14 KO LPCs (Figure 5D). These results indicate that IL-33-induced expression of IL-13 is dependent on an Fn14-mediated signal pathway. However, WT LPCs do not express Fn14 (Figure 1F). Thus, in Fn14 KO mice, IL-33 signaling in LPCs is reduced likely due to an Fn14 deficiency in an Fn14-expressing cell type, such as ECs. Overall, these results suggest that the presence of Fn14 in ECs affects IL-33-induced IL-13 expression in LPCs through an unknown mechanism.

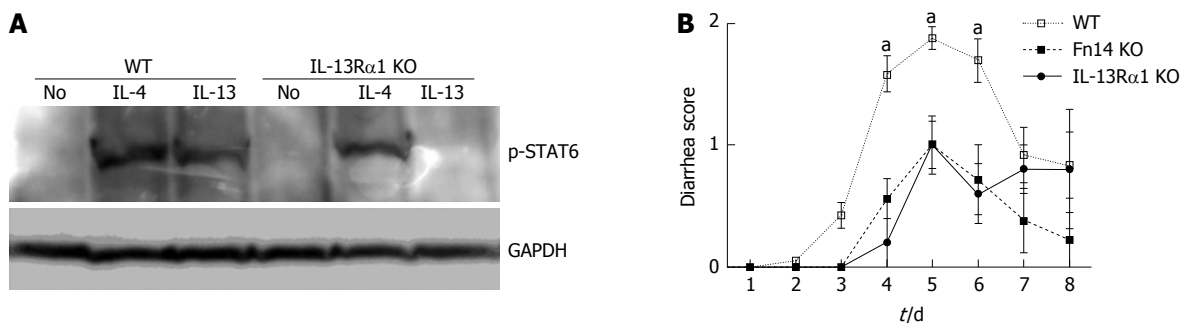
### Enhancement of 5-FU-induced IL-13R $\alpha$ 2 expression in Fn14 KO mice

We observed that IL-13 expression was higher in WT mice than Fn14 KO mice on day 5. However, when comparing WT mice at day 5 following 5-FU administration with naïve mice (Day 0), induction of IL-13 expression was modest (Figure 3). Therefore, we hypothesized that there was an additional mechanism to reduce IL-13 activity in the absence of Fn14. This mechanism could potentially explain the dramatic diarrhea reduction in Fn14 KO mice. Previously, we showed that expression of IL-13R $\alpha$ 2, the IL-13 neutralizing receptor, promoted intestinal EC regeneration following  $\gamma$ -irradiation<sup>[17]</sup>. Thus, we hypothesized that IL-13R $\alpha$ 2 expression increased in Fn14 KO mice and contributed to the reduction of IL-13-induced diarrhea. As expected, on day 5 following 5-FU administration, IL-13R $\alpha$ 2 mRNA expression transiently increased 5-fold in the ileum of Fn14 KO mice but not WT mice (Figure 6A). IL-13R $\alpha$ 1 was not upregulated in either the WT or Fn14 KO ileum (Figure 6A). Consistent with the mRNA expression, immunostaining for IL-13R $\alpha$ 2 confirmed a transient but strong upregulation of protein expression in mesenchymal cells of Fn14 KO mice on day 5 but not in those of WT mice (Figure 6B and C). These results indicate that increased IL-13R $\alpha$ 2 expression in Fn14 KO mice may efficiently neutralize IL-13. Reduced





**Figure 3** 5-FU induced inflammatory cytokines in the ileum and the role of IL-13 in 5-FU-induced diarrhea. A-D: Cytokine levels were measured from homogenate of the ileum following injection of 5-FU. Open bar, WT mice; solid bar, Fn14 KO mice ( $n = 4$  mice per time point). Data are shown as mean  $\pm$  SD ( $^{\#}P < 0.05$ ).



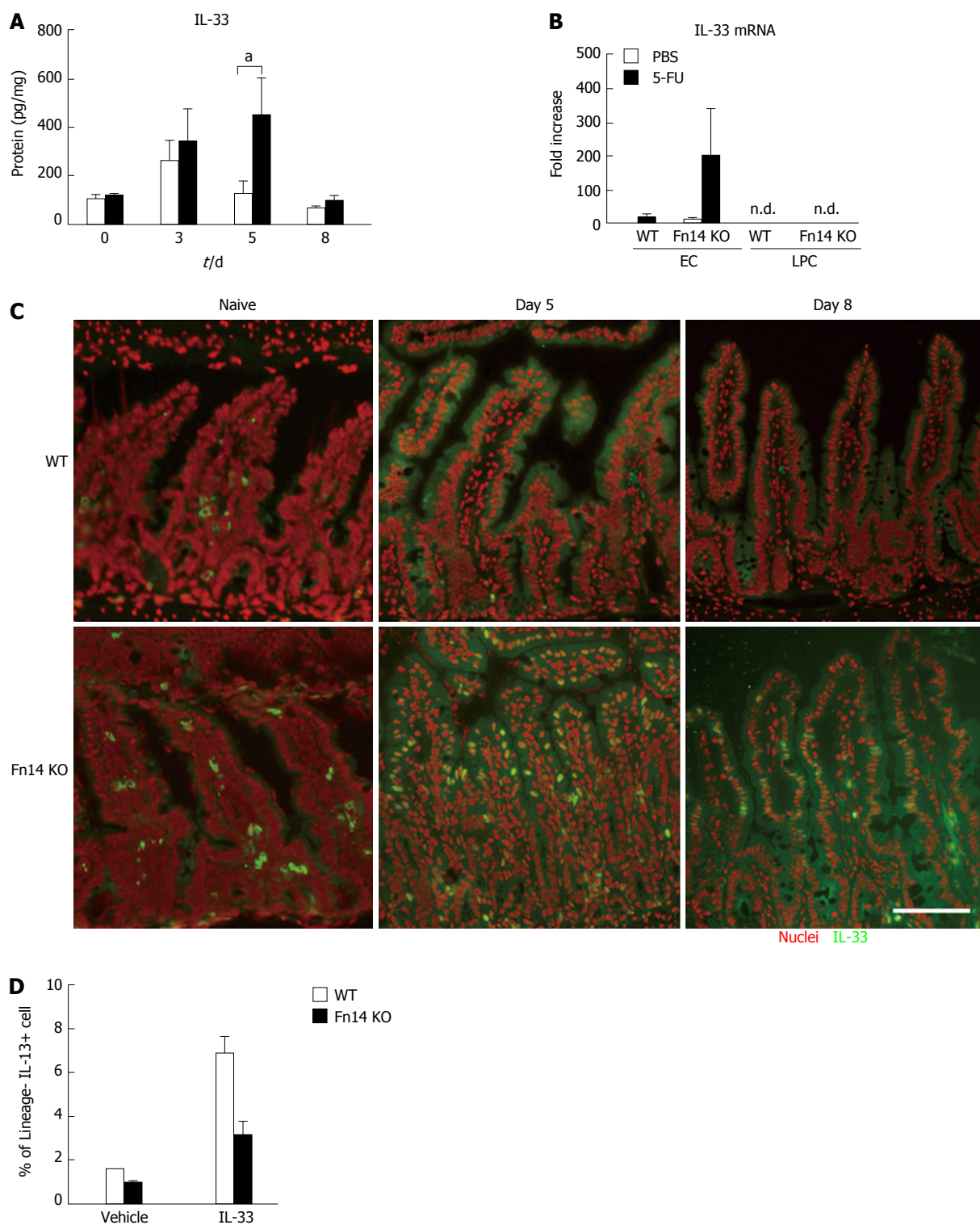
**Figure 4** Lack of response to IL-4 and IL-13 in IL-13Rα1 KO mice. A: Bone marrow cells from WT or IL-13Rα1 KO mice were stimulated with IL-4 or IL-13 for 30 min, and phosphorylated (p)-STAT6 was detected using western blotting. GAPDH was used as a loading control; B: Diarrhea scores of WT ( $n > 8$ , at each time point), Fn14 KO ( $n > 9$ ), and IL-13Rα1 KO mice ( $n > 5$ ) after injection of 5-FU. Number of mice was about 5-44 at each time point. Results of mouse groups of different observation-time points and endpoints were accumulated and summarized ( $^{\#}P < 0.05$ ).

IL-13 expression by Fn14-deficient cells combined with IL-13 neutralization through IL-13Rα2 could account for the attenuated diarrhea observed in 5-FU-treated KO mice.

#### Fn14-independent induction of IL-13Rα2 expression by IL-33

IL-13 is known to induce IL-13Rα2 expression; however, IL-13 protein levels did not increase in Fn14 KO mice following 5-FU administration. Because IL-33 was highly expressed in Fn14 KO mice after 5-FU administration, we investigated if IL-33 induces IL-13Rα2 expression. Administration of recombinant IL-33 to mesenchymal cells of the ileum induced IL-

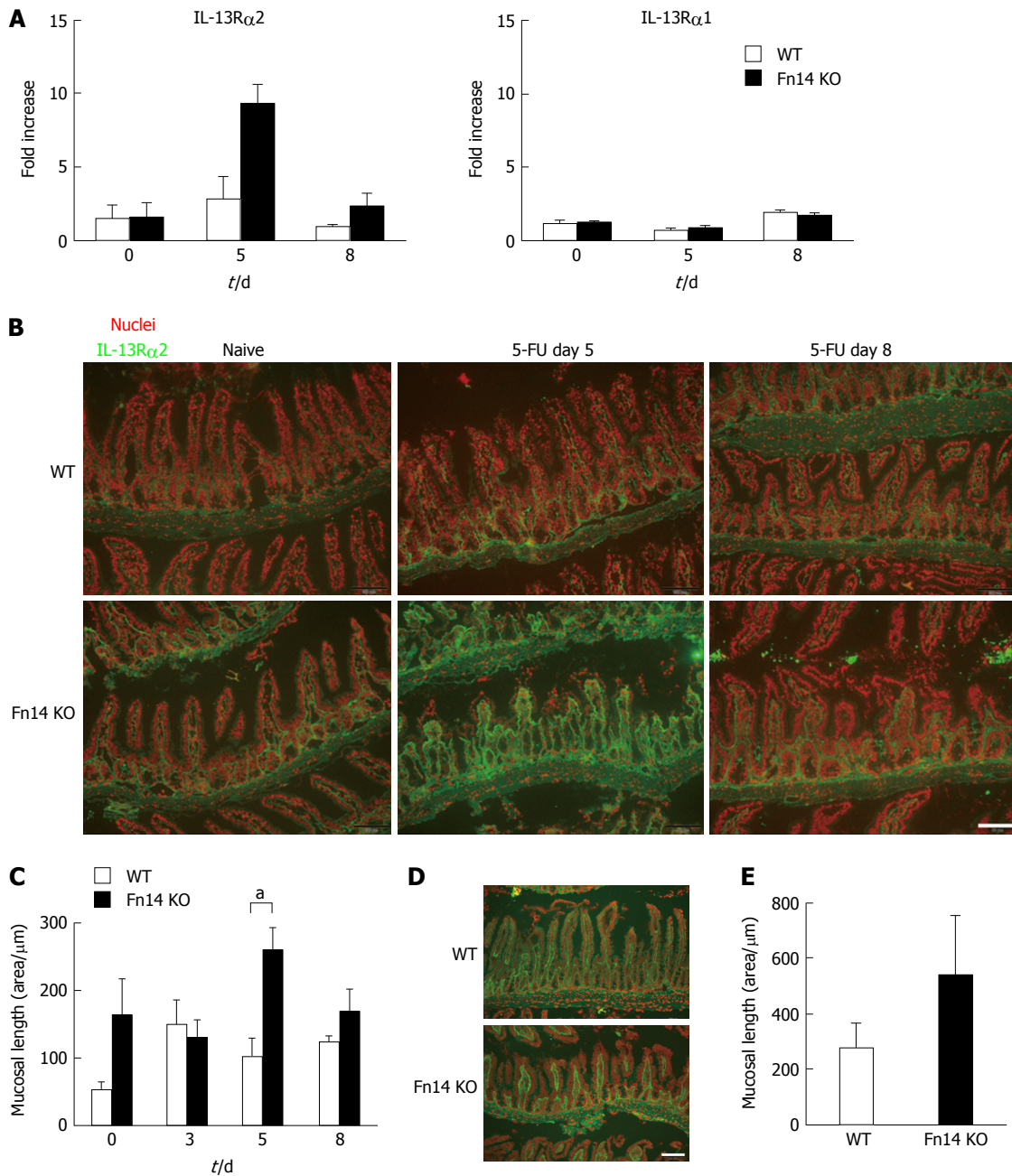
13Rα2 expression at a similar level in both WT and Fn14 KO mice (Figure 6D and E). Therefore, the high levels of IL-33 produced by ECs of Fn14 KO mice following 5-FU administration could have induced IL-13Rα2 upregulation. This mechanism was confirmed with *in vitro* assays using MEFs. *In vitro* stimulation of MEFs showed that recombinant IL-33 alone and in combination with recombinant IL-13 induced IL-13Rα2 expression in both WT and Fn14 KO cells (Figure 7A). To confirm that IL-33 was active in 5-FU-treated tissue, WT MEFs were cultured with ileal tissue homogenate from WT or Fn14 KO mice collected 2 d after 5-FU treatment. At this time point, WT and Fn14 KO mice have similar IL-33 levels in their tissues. Both



**Figure 5** IL-33-induced IL-13 expression was Fn14 dependent. **A:** IL-33 levels were measured from homogenate of the ileum after injection of 5-FU ( $n = 4$  mice per time point). Open bar, WT mice; solid bar, Fn14 KO mice ( $n = 4$  mice per time point). Data are shown as mean  $\pm$  SD ( $^aP < 0.05$ ); **B:** Fold increase of IL-33 mRNA in ECs and LPCs on day 5 after injection of 5-FU ( $n = 3$  mice per experiment). Data are shown as mean  $\pm$  SD; **C:** Sections were prepared from samples collected on days 0 (naïve), 5, and 8 after injection of 5-FU from WT and Fn14 KO mice and probed with anti-IL-33 antibody (green) ( $n = 3$  mice per experimental condition). Red: Nuclear staining. Scale bar = 100  $\mu$ m. Representative images are provided; **D:** LPCs from naïve WT or Fn14 KO ileum were stimulated with recombinant IL-33 *in vitro* and the number of IL-13-producing cells per mouse was measured using flow cytometry. Results of repeated experiments ( $n = 3$ ) are summarized.

WT and Fn14 KO ileum homogenate induced IL-13 $\alpha$ 2 expression in WT MEFs; however, this induction was not observed when MEFs were treated with IL-33-depleted homogenate (Figure 7B and C). These data

suggest that IL-33 was responsible for the induction. Overall, these results indicate that IL-33 expression, which is enhanced in the absence of Fn14, could upregulate IL-13 $\alpha$ 2 expression, neutralize IL-13



**Figure 6 Upregulation of IL-13R $\alpha$ 2 expression in 5-FU treated Fn14 KO mice.** A: Fold increase of IL-13R $\alpha$ 2 and IL-13R $\alpha$ 1 mRNA in total ileum from WT or Fn14 KO mice after 5-FU treatment ( $n$  = about 3-5 mice per group). Data are shown as mean  $\pm$  SD; B: Representative images from immunohistochemistry of IL-13R $\alpha$ 2 in ileum from WT and Fn14 KO mice on days 0 (naïve), 5, and 8 after injection of 5-FU. Green, IL-13R $\alpha$ 2; red, nuclei. Scale bar = 100  $\mu$ m; C: Quantification of IL-13R $\alpha$ 2 expression. The IL-13R $\alpha$ 2-positive area of images from the experiment described in (B) were quantified following normalization by mucosal length ( $n$  = 4 mice per group). <sup>a</sup> $P$  < 0.05; D: Recombinant IL-13 (400  $\mu$ g) was injected into naïve WT or Fn14 KO mice. After 3 d, ileum samples were collected, and sections were probed with anti-IL-13R $\alpha$ 2 antibody. Green, IL-13R $\alpha$ 2; red, nuclei. Scale bar = 100  $\mu$ m; E: Quantification of IL-13R $\alpha$ 2 expression normalized to mucosal length. The IL-13R $\alpha$ 2-positive area of images from the experiment described in (D) were quantified ( $n$  = 4 mice per group). There was no statistically significant difference between WT and Fn14 KO mice.

signal, and ameliorate 5-FU-induced diarrhea.

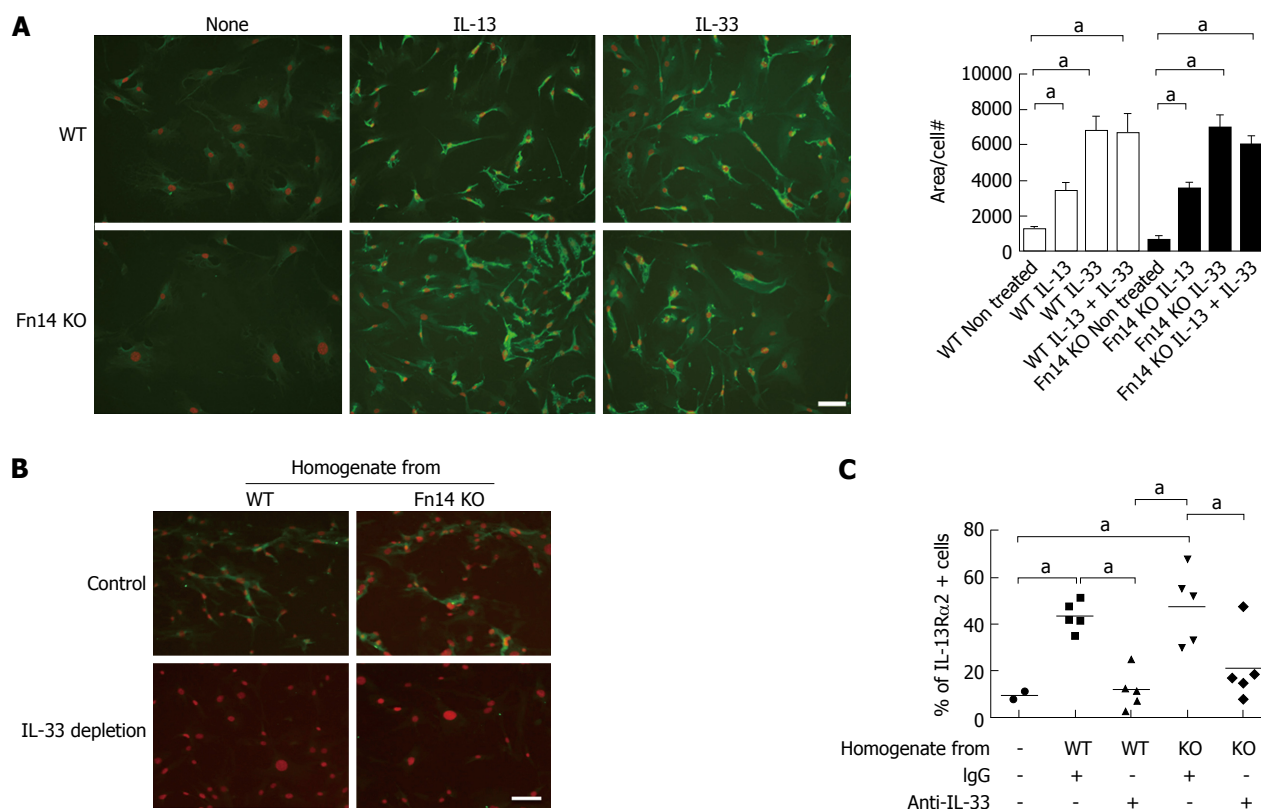
## DISCUSSION

Blocking the TWEAK/Fn14 pathway suppressed 5-FU-induced diarrhea, but did not affect tumor growth in the CT26 tumor-bearing model or the antitumor effects of 5-FU. In our mechanistic studies, we found that 5-FU-induced diarrhea was associated with IL-13,

and its expression was Fn14-dependent. IL-13R $\alpha$ 2, the IL-13 neutralizing receptor, was induced by IL-13, which was upregulated in the absence of Fn14. Thus, an Fn14 deficit limited both IL-13 expression and activity. The TWEAK/Fn14 pathway coordinates these factors, thereby contributing to 5-FU-induced diarrhea.

Fn14 is often upregulated in malignant tumors, and high Fn14 levels are related to higher tumor grades and poor prognosis in esophageal<sup>[18]</sup> and gastric





**Figure 7 Recombinant IL-33 and IL-33 from 5-FU-treated ileum induced IL-13R $\alpha$ 2 expression in mouse embryonic fibroblasts.** A: Mouse embryonic fibroblasts (MEFs) obtained from WT and Fn14 KO mice were cultured with IL-13 or IL-33 for 3 d and probed with anti-IL-13R $\alpha$ 2 antibody. Green, IL-13R $\alpha$ 2; red, nuclei. Scale bar = 200  $\mu$ m. IL-13R $\alpha$ 2-positive area was normalized by number of nuclei in the image and quantified (right). Data are shown as mean  $\pm$  SD of four images from four independent cultures.  $^{\circ}P < 0.05$ ; B: WT MEFs were cultured with the homogenate of ileum obtained from WT or Fn14 KO mice 2 d after 5-FU treatment. IL-33 was depleted using immunoprecipitation with anti-IL-33 antibody or control IgG. After 3 d of culture with homogenate, cells were probed with anti-IL-13R $\alpha$ 2 antibody. Green, IL-13R $\alpha$ 2; red, nuclei. Scale bar = 100  $\mu$ m; C: The percent of IL-13R $\alpha$ 2-positive cells was quantified from the images collected during the experiment described in (B). Each dot represents an individual culture. Bars indicate means.  $^{\circ}P < 0.05$ . KO indicates Fn14 KO mice.

cancers<sup>[19]</sup>. Previous studies reported that TWEAK/Fn14 signaling promotes invasion, migration, and survival in many types of cancer cells, including glioblastoma, neuroblastoma, and breast cancer cells<sup>[20-22]</sup>. One study demonstrated therapeutic benefits when using a TWEAK-blocking antibody for cancer therapy<sup>[23]</sup>. These reports indicated that TWEAK/Fn14 signaling relates to tumor progression and blocking the TWEAK/Fn14 pathway could be beneficial in cancer treatment. Another recent report showed that anti-Fn14 antibody treatment prevented cancer-induced cachexia and prolonged survival<sup>[24]</sup>. By contrast, some reports show that TWEAK/Fn14 signaling has an antitumorigenic function<sup>[25]</sup>. Therefore, role of TWEAK/Fn14 signaling in cancer seems to be cell-type dependent. Careful investigation of the effects of blocking TWEAK/Fn14 signaling on each cancer cell type is needed.

In Fn14 KO mice, IL-13, TNF- $\alpha$ , IFN- $\gamma$ , and IL-17A levels were low after 5-FU administration compared with WT mice. All these cytokines are involved in intestinal epithelial cell damage and diarrhea. Specifically, IL-13 is an important mediator of allergen-induced diarrhea<sup>[26]</sup>. This study demonstrated the critical role that IL-13 plays in 5-FU-induced diarrhea using IL-13R $\alpha$ 1 KO mice. IL-13 disrupts tight junctions,

which affects intestinal barrier function<sup>[27,28]</sup>. In this study, ZO-1 expression levels in 5-FU-treated WT mice appeared to coincide with diarrhea score. Our previous study showed that IL-13 combined with TNF- $\alpha$  induced damage of tight junctions and intestinal EC death in an Fn14-dependent manner<sup>[4]</sup>. All these results support our hypothesis that 5-FU-induced diarrhea is primarily mediated by the IL-13 pathway, which affects the barrier function of ECs.

We hypothesized that IL-33 was responsible for induction of IL-13 in 5-FU-induced diarrhea. IL-13-producing cells were induced when WT LPCs were stimulated with IL-33. By contrast, IL-33 failed to induce IL-13 expression in Fn14 KO LPCs both *in vitro* and *in vivo* despite exposure to sustained high levels of IL-33. This observation indicates that IL-33-stimulated IL-13 expression is Fn14 dependent; however, LPCs did not express Fn14. This result suggests that the expression of IL-13 by LPCs in response to IL-33 requires some indirect priming, likely through soluble factors, by Fn14-expressing cells, such as ECs. In this situation, IL-33 functions as an alarmin in WT mice to induce IL-13 expression and diarrhea. In an Fn14-deficient microenvironment, LPCs could not induce IL-13 expression in response to IL-33. This result



could be explained by IL-33 having a different function depending on the presence or absence of Fn14.

Previous studies reported that IL-33 is synthesized as a full-length protein that is localized in the nucleus of cells in epithelial barrier tissue<sup>[8,29]</sup>. Full-length IL-33 is not fully bioactive with respect to IL-33 receptor binding and NF- $\kappa$ B activation potential until it undergoes cleavage by caspase-1<sup>[14]</sup>. Full-length IL-33 can also be cleaved by inflammatory proteases from neutrophils, such as cathepsin and elastase. This cleavage results in IL-13 expression or IL-6 secretion<sup>[30,31]</sup>. The nuclear localization sequence is located in the N-terminus of IL-33 and is removed following cleavage. Therefore, IL-33 leaves the nucleus after cleavage. In this study, IL-33 localized to the cytosol on day 5 of 5-FU treatment in WT ECs, whereas IL-33 was highly expressed and localized to both the nucleus and cytosol in Fn14 KO ECs on day 5. This observation could indicate that in WT mice, IL-33 was fully cleaved and bioactive (*i.e.*, capable of inducing IL-13 expression in LPCs) but in Fn14 KO mice, the nuclear-localized IL-33 was in a different molecular form that served some other function with respect to IL-13 expression. Further studies are needed to investigate IL-33 processing, cellular localization, and function.

In addition to reducing IL-13 secretion, we found that upregulation of IL-33 in Fn14 KO mice actively protected ECs from damage. We demonstrated for the first time that IL-33 potently induced IL-13R $\alpha$ 2, an IL-13 neutralizing receptor. IL-13R $\alpha$ 2 plays an important protective role in cases of mucosal damage, such as oxazolone-induced colitis<sup>[32]</sup>, and EC damage after  $\gamma$ -irradiation<sup>[17]</sup>. In these cases, IL-13R $\alpha$ 2 expression or IL-13 neutralization prevents damage in the intestine. Recently, IL-13R $\alpha$ 2 was reported to bind chitinase-like protein chitinase 3-like 1 and regulate oxidant injury, apoptosis, pyroptosis, inflammasome activation, and antibacterial responses<sup>[33]</sup>. This mechanism may also contribute to the protective effect of the IL-33-IL-13R $\alpha$ 2 axis in 5-FU-induced damage of the epithelial barrier. In contrast to IL-13 expression, recombinant IL-33-induced IL-13R $\alpha$ 2 expression seems to be similar between WT and Fn14 KO mice. Therefore, sustained exposure to high levels of IL-33 could explain the enhanced induction of IL-13R $\alpha$ 2 expression in Fn14 KO mice. Collectively, these results indicate that induction of IL-13R $\alpha$ 2 expression by IL-33 is an important mechanism to ameliorate 5-FU-induced diarrhea and intestinal damage in Fn14 KO mice.

In our study, blocking the TWEAK/Fn14 pathway did not affect tumor growth in the CT26 tumor-bearing model or the antitumor effects of 5-FU. Our results demonstrated that disrupting TWEAK/Fn14 signaling alters multiple processes in various cell types, including expression of IL-33 and tight junction proteins in ECs, induction of IL-13 expression in LPCs, and the induction of IL-13R $\alpha$ 2 expression in mesenchymal cells. These changes affected the sensitivity of normal intestinal cells to chemotherapeutic agents and ameliorated

5-FU-induced diarrhea. Therefore, we believe that disrupting the TWEAK/Fn14 pathway could reduce the side effects of 5-FU, thereby making chemotherapy more effective.

## COMMENTS

### Background

The chemotherapeutic antimetabolite agent 5-fluorouracil (5-FU) is frequently used to treat a wide range of cancer types. However, chemotherapy with 5-FU is often associated with severe gastrointestinal side effects, such as diarrhea and anorexia. The tumor necrosis factor-like weak inducer of apoptosis (TWEAK) and its receptor Fn14 form a signaling pathway that is involved in the repair of injured tissue. The TWEAK/Fn14 pathway affects inflammation and epithelial cell death, especially in the intestine. In this study, we investigated the effect of blocking TWEAK/Fn14 signaling on chemotherapy-induced diarrhea.

### Research frontiers

The TWEAK/Fn14 pathway has been shown to play major roles in various pathological situations, including inflammatory bowel diseases, nephritis, myositis, and cancer. Developing new diagnostic methods and therapies targeting TWEAK/Fn14 signaling could improve outcomes of numerous conditions.

### Innovations and breakthroughs

This is the first study reporting the prevention of 5-FU side effects using anti-TWEAK antibody injection. In mechanistic studies, we found that IL-33 induced IL-13 receptor  $\alpha$ 2 expression and its expression was enhanced in the absence of Fn14. This effect protected mice from severe diarrhea induced by the IL-13-receptor  $\alpha$ 1 pathway. These results also potentially identified a new tissue-protective function of IL-33 that was regulated by the presence of Fn14.

### Applications

Because an anti-TWEAK antibody has already been developed for humans, it could be used to treat the severe side effects of 5-FU in the near future. This treatment could be used to improve the efficacy of anticancer drugs.

### Peer-review

The manuscript can be evaluated as an excellent (A) work concerning about an innovative molecular study: this study could improve significantly the common use of 5FU into clinical practise with the possibility of reducing adverse effects that may decrease patient's compliance to therapy. The study reflects a great "equipe" work with a solid scientific contribution.

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