



# Monoclonal Antibodies Against Mature Interleukin-18 Ameliorate Colitis and Repair Goblet Cell Function

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## Abstract

**Background** Numerous biological interventions and small molecules are used to treat Crohn's disease; however, the effectiveness of these treatments varies largely. Non-responsiveness to biological therapies is associated with interleukin (IL)-18 gene polymorphisms and high IL-18 expression has been implicated in the pathogenesis of Crohn's disease.

**Aims** The aim of this study was to elucidate the expression of precursor and mature IL-18 in patients with Crohn's disease who exhibited varied responses to cytokine-targeted treatments and determine whether selective inhibition of mature IL-18 offers a novel therapeutic avenue.

**Methods** We generated a monoclonal antibody that specifically recognizes the neopeptide of caspase-cleaved mature IL-18. Expression of precursor and mature IL-18 was analyzed in patients with Crohn's disease. Anti-mature IL-18 monoclonal antibodies were intraperitoneally administered in an acute colitis mouse model, and the disease activity index, body weight loss, tissue pathology, proinflammatory cytokine expression, goblet cell function, and microbiota composition were assessed.

**Results** Precursor and mature IL-18 expression was upregulated and goblet cell function was impaired in patients with Crohn's disease who were unresponsive to biological therapies. Administration of anti-mature IL-18 antibodies ameliorated induced colitis by repairing goblet cell function and restoring the mucus layer.

**Conclusions** The newly developed monoclonal antibody holds promise as a therapeutic alternative for Crohn's disease.

**Keywords** Crohn's disease · Interleukin-18 · Antibodies · Goblet cells

## Introduction

Crohn's disease (CD) is an etiologically complex inflammatory disorder involving inherited susceptibility, environmental factors, and altered mucosal immune responses [1]. Based on the recognition that immune dysregulation in CD involves the inappropriate production of proinflammatory cytokines, several recently developed cytokine-targeted therapies have revolutionized the treatment of CD [2], including monoclonal antibodies (mAbs) targeting tumor necrosis factor-alpha (TNF- $\alpha$ ), the p40 subunit of interleukin (IL)-12 and IL-23, and the p19 subunit of IL-23. Cytokine-targeted therapies primarily induce remission and prevent relapse by alleviating intestinal inflammation. Effectively controlling inflammation is known to reduce the risk of complications [3]. However, individual differences and variability in the efficiency of cytokine-targeted therapies have been reported, with approximately half of patients with CD showing poor

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responses to existing treatments [4, 5]. Despite numerous efforts to address this problem, treating patients with CD remains challenging.

Increased susceptibility and poor responses to TNF- $\alpha$ -targeting monoclonal antibodies have been reported to be associated with IL-18 gene polymorphisms in patients with inflammatory bowel disease (IBD) [6, 7]. IL-18 plays a significant role in modulating immune responses and inflammatory processes that maintain intestinal homeostasis in physiological conditions. Mature IL-18 is generated through proteolytic cleavage of its precursor by caspase-1 and -4, enzymes involved in the inflammasome-mediated processing of proinflammatory cytokines. IL-18 is a proinflammatory cytokine that potentially induces interferon-gamma (IFN- $\gamma$ ) production by enhancing the cytotoxicity of natural killer cells and promoting T-cell differentiation into Th1 cells [8]. IL-18 also induces the production of other proinflammatory cytokines such as TNF- $\alpha$ , IL-1 $\beta$ , and IL-6 by various immune cells, including macrophages and dendritic cells [9].

In patients with CD, serum and mucosal biopsy studies have shown elevated IL-18 levels in the active phase, in which mature IL-18 is specifically detected in the digestive tissues [10]. Excessive IL-18 activation inhibits mucus production by dysregulating goblet cell function [11]. Moreover, administering polyclonal antibodies against IL-18 or IL-18 binding proteins inhibits colitis in several mouse models [12–14]. Therefore, specifically neutralizing IL-18 might effectively control intestinal inflammation in patients with CD, especially in those who do not respond to cytokine-targeted treatments in the clinic. However, no IL-18 inhibitory treatment is currently used in clinical practice.

As IL-18 also plays a vital role in maintaining intestinal homeostasis in the steady state [15], we hypothesized that inhibiting mature IL-18, which is elevated in the intestinal tissues of patients with CD, would have an inhibitory effect on colitis without interfering with steady-state IL-18 function under homeostatic conditions. Therefore, the aim of this study was to demonstrate the expression of precursor and mature IL-18 in patients with CD who were refractory to TNF- $\alpha$  and IL-12/23 antibodies to determine whether inhibiting mature IL-18 can ameliorate colitis and to elucidate the underlying mechanism.

## Methods

### Ethical Considerations

Animal experiments were approved by the Animal Care and Use Committee of Nagoya University Graduate School of Medicine (approval number: M230086) and performed in compliance with the regulations and guidelines of animal care and use of Nagoya University. All the experiments were

performed and reported in accordance with ARRIVE guidelines. The human study was conducted following the Declaration of Helsinki for Human Research, and protocols were approved by the Research Ethics Committee of Nagoya University (approval number: 2018–0316). All participants provided written informed consent to participate in the study.

### Human Samples

Serum, colonic biopsy, and surgical samples were obtained from 31 patients with active CD (CD activity index [CDAI] scores between 150 and 450) who were resistant to anti-TNF- $\alpha$  antibodies and treated with ustekinumab at Nagoya University Hospital in Japan from October 2017 to September 2020. The patients were divided into two groups (responders and non-responders), according to their response to ustekinumab treatment. Responders were those with a CDAI score that decreased by  $\geq 100$  from baseline or those with a CDAI score  $< 150$  at 24 weeks after ustekinumab induction.

### Measurement of Human TNF- $\alpha$ and Full-Length and Mature IL-18 Serum Concentrations

Serum concentrations of full-length and mature human IL-18 and TNF- $\alpha$  were measured using an enzyme-linked immunosorbent assay (ELISA) kit (#7620; MBL, Nagoya, Japan; #E-I-002 mAbProtein, Shimane, Japan, #DTA00D; R&D, Minneapolis). Full-length and mature IL-18 and TNF- $\alpha$  concentrations were calculated using standard curves.

### Immunohistochemistry

For immunohistochemical studies, formaldehyde-fixed, paraffin-embedded biopsy and surgical specimens were deparaffinized, and antigen retrieval was performed in a target retrieval solution (Agilent Technologies, Santa Clara, CA, USA). After cooling to 20 °C, tissue sections were washed with phosphate-buffered saline (PBS), blocked with normal goat serum (#MP7451, Vector Laboratories, Burlingame, CA, USA), and incubated with anti-full-length and anti-mature IL-18 antibodies (mAbProtein) diluted 1:100 in PBS. The sections were treated with a 3% hydrogen peroxide/ethanol solution and incubated at 20 °C for 60 min with anti-rabbit IgG secondary antibody (#MP7451, Vector Laboratories), followed by signal detection using diaminobenzidine solution. The software ImageJ was used to quantify the stained areas (National Institute of Health, USA).

### Immunofluorescence Staining

To retrieve antigen, the samples that were fixed with formaldehyde were deparaffinized and boiled in a target retrieval

solution (Agilent Technologies) at a pH of 6. For mouse samples, after washing with PBS, samples were permeabilized in 0.05% Triton X-100 (Sigma-Aldrich, St. Louis, MO, USA)/PBS solution for 20 min at 20 °C. Samples were blocked with normal goat serum and incubated with primary antibody (anti-MUC-2, 1:100; Invitrogen, Waltham, MA, USA) at 4 °C for 16 h. Samples from patients with CD were blocked and permeabilized with 0.1% Triton X-100 diluted in 5% bovine serum albumin for 1 h, and then incubated with primary antibody (anti-Muc2, sc-515032, 1:100, Santa Cruz Biotechnology, Dallas, TX, USA) at 4 °C for 16 h. Both mouse and human slides were incubated with Alexa Fluor 488-conjugated secondary antibodies (#4412; Cell Signaling Technology) for 1 h. After washing, the slides were mounted in 4',6-diamidino-2-phenylindole Fluoromount-G (#010020, Southern Biotech, Birmingham, AL, USA) and then coverslips added. The stained areas were quantified using ImageJ software (National Institute of Health, USA).

Multiple immunofluorescence staining was conducted using an Opal assay kit (NEL810001KT, Akoya Biosciences, Marlborough, MA, USA) according to the manufacturer’s recommendations. Briefly, anti-human CD68 antibody (PG-M1, 1:100, DAKO) and anti-full-length and anti-mature IL-18 antibodies (mAbProtein) were incubated in 4 °C for 16 h and recognized by fluorescence of Opal570 and Opal520, respectively.

**Alcian Blue and Periodic Acid-Schiff Staining**

To perform Alcian Blue-periodic acid-Schiff (PAS) staining, a PAS and Alcian Blue staining kit (#40,582, Muto Pure

Chemicals, Tokyo, Japan) was used. Images were obtained under a universal fluorescence microscope (BZ-9000, Keyence). The number of goblet cells was determined after hematoxylin and eosin staining by counting five high-power fields (400×) in crypts.

**Mouse Model of Acute Colitis**

Male C57BL/6 J mice, 7–9 weeks of age, were purchased from CLEA Japan (Tokyo, Japan). The mice were maintained under specific pathogen-free conditions with a 12-h day/night cycle and controlled humidity and temperature. Temperature was maintained at 18–23 °C with 40–60% humidity. Each experimental group comprised seven mice. For the 2,4,6-trinitrobenzene sulfonic acid (TNBS) acute colitis model, we first conducted a preliminary study, which indicated low survival rates at high TNBS concentrations. Because exploring the efficiency of drug administration is difficult with early deaths, mice in the IL-18 mAb and isotype IgG groups were intrarectally administered TNBS (Wako Chemicals, Osaka, Japan) dissolved in 50% ethanol at a dose of 1 mg on day 1.

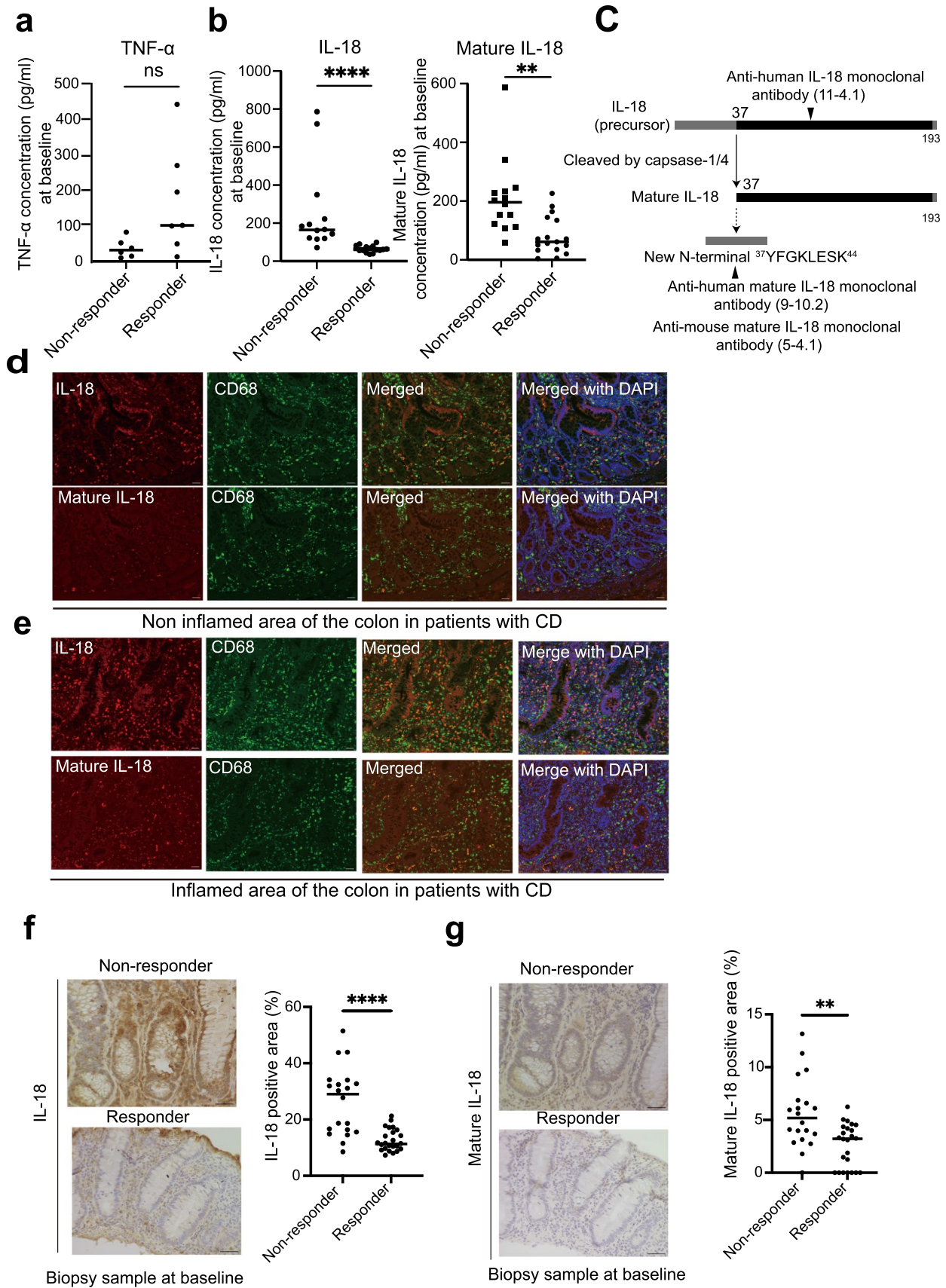
Mice in the control group were administered intrarectally without TNBS on day 1 without intervention.

Mice in the isotype IgG and IL-18 mAb groups, which modeled TNBS-induced colitis, were treated intraperitoneally with IgG (200 µg) (Bio X Cell, Lebanon, NH, USA) or anti-mature IL-18 mAb (5-4.1) (200 µg) daily for 5 d. All mice were euthanized using carbon dioxide on day 5, and colon tissues were collected. The severity of colitis was evaluated based on changes in body weight, disease activity

**Table 1** Ustekinumab-treated responder and non-responder baseline patient information

|                                 | Non-responder (N=14) | Responder (N=17) | P value   |
|---------------------------------|----------------------|------------------|-----------|
| Age (mean ± SD)                 | 44 ± 13.29           | 40.53 ± 8.73     | 0.4122    |
| Gender                          |                      |                  |           |
| Male (%)                        | 12 (85.71%)          | 13 (76.47%)      | > 0.9999  |
| Female (%)                      | 2 (14.29)            | 4(23.53%)        | > 0.9999  |
| Body Weight (mean ± SD)         | 55.09 ± 9.51         | 56.34 ± 12.8     | 0.7633    |
| BMI (mean ± SD)                 | 19.4 ± 2.6           | 20.2 ± 3.8       | 0.5226    |
| Location                        |                      |                  |           |
| Ileal (%)                       | 3 (21.43%)           | 2 (11.76%)       | 0.6358    |
| Colonic (%)                     | 2 (14.29%)           | 0 (0%)           | 0.4815    |
| Ileocolonic (%)                 | 9 (64.28%)           | 15 (88.24%)      | 0.1975    |
| Duration of disease (mean ± SD) | 14.5 ± 9.61          | 14.88 ± 8.56     | 0.9075    |
| With history of Operation (%)   | 10 (71.43%)          | 14 (82.35%)      | > 0.9999  |
| Hb (mean ± SD)                  | 12.16 ± 2.40         | 10.95 ± 2.09     | 0.4782    |
| Hct (mean ± SD)                 | 38.44 ± 5.86         | 33.54 ± 5.46     | 0.0228*   |
| CRP (mean ± SD)                 | 1.32 ± 2.08          | 0.59 ± 0.50      | 0.1687    |
| CDAI at baseline (mean ± SD)    | 217.93 ± 46.33       | 310.29 ± 77.12   | 0.0005*** |
| CDAI at 24 weeks (mean ± SD)    | 179.21 ± 61.53       | 142.59 ± 46.17   | 0.0683    |

\*P < 0.05, \*\*\*P < 0.001





**Fig. 1** IL-18 and mature IL-18 expression increased in patients with CD who were refractory to anti-IL-12/23 and anti-TNF- $\alpha$  antibodies. **a** TNF- $\alpha$  expression was measured by ELISA on serum of non-responders ( $n=14$ ) and responders ( $n=17$ ). **b** IL-18 and mature IL-18 expression in the serum of non-responders ( $n=14$ ) and responders ( $n=17$ ). **c** Schematic diagram showing the binding sites of mature anti-human IL-18 mAb. **d, e** Multiple immunofluorescence staining of CD68 and full-length or mature IL-18 in colonic surgical tissues from normal and inflamed lesions of patients with CD. **f** Anti-IL-18 mAb (11-4.1) recognition of precursor and mature IL-18 in endoscopic biopsy samples and quantification of IL-18-positive areas. **g** Immunohistochemistry with anti-IL-18 mAb (9-10.2) showing mature IL-18 in endoscopic biopsy samples and quantification of the mature IL-18-positive areas. Scale bar: 40  $\mu\text{m}$ . Data are presented as the means  $\pm$  SD. Statistical comparisons were performed using an unpaired  $t$ -test. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , \*\*\*\* $P < 0.0001$

score, colon length, and histological score. Histological evaluation was performed blinded, as described previously.

### Quantitative PCR

Total RNA was extracted using the acid guanidinium thiocyanate-phenol-chloroform extraction method, and purified RNA samples were reverse-transcribed into cDNA using ReverTra Ace (Toyobo, Tokyo, Japan). Quantitative PCR (qPCR) was performed using an Mx3000P thermal cycler (Agilent Technologies). TaqMan probes and primers for mouse CXCL2 (Mm00436450\_m1), mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Mm99999915\_g1), and mouse IL-6 (Mm00446190\_m1) were purchased from Life Technologies (Carlsbad, CA, USA). Data were analyzed using the comparative threshold cycle (CT) method and normalized to GAPDH levels.

### Measurement of Tissue Cytokine Concentrations

Mouse CXCL2 and IL-6 levels in colon tissues were measured using an ELISA. Distal colons were dissected, opened longitudinally, and washed twice with PBS. Colon homogenates were prepared using tissue homogenizers in a lysis buffer containing a protease inhibitor cocktail (#5871, Cell Signaling Technology, Danvers, MA, USA), and protein concentrations were measured. The samples were stored at  $-80^\circ\text{C}$  until assayed. Cytokine concentrations were determined using a Quantikine ELISA kit for mice (#M6000B-1; #MM200; R&D Systems, Inc., Minneapolis, MN, USA).

### Microbiome Analysis

Fecal samples were collected, and DNA was extracted using a DNeasy PowerSoil Kit (Qiagen, Hilden, Germany). Bacterial DNA was amplified using universal primers targeting the V3–4 region of the 16S rRNA gene (F: 5'-TCGTCGGCAGCGTCAGATGTGTATAAG

AGACAGCCTACGGGNGGCWGCAG-3', R: 5'GTC TCGTGGGCTCGGAGATGTGTATAAGAGACAGGA CTACHVGGGTATCTAATCC-3') with KAPA HiFi Hot-Start ReadyMix (KAPA Biosystems, Boston, MA, USA). PCR products were pooled to create a library and then sequenced on an Illumina MiSeq sequencer (Illumina, San Diego, CA, USA). Paired-end reads were generated using a MiSeq Reagent Kit v3 with  $2 \times 300$  reads and 600 cycles (Illumina). For basic analysis of the 16S rRNA gene sequence data, Quantitative Insights Into Microbial Ecology (QIIME 2–2021.4 with DADA2) [16] and SILVA (version 138) were used. Linear discriminant analysis effect size (LEfSe) [17] was used to compare intestinal microbiome compositions.

### Statistical Analyses

Statistical analyses were conducted using GraphPad Prism 9 (IBM Corp., Armonk, NY, USA). Data are presented as means  $\pm$  standard deviations (SD). Statistical comparisons were conducted using the Mann–Whitney U test or analysis of variance (ANOVA) with Tukey's multiple comparison post-hoc test. Statistical significance was defined as  $P < 0.05$ .

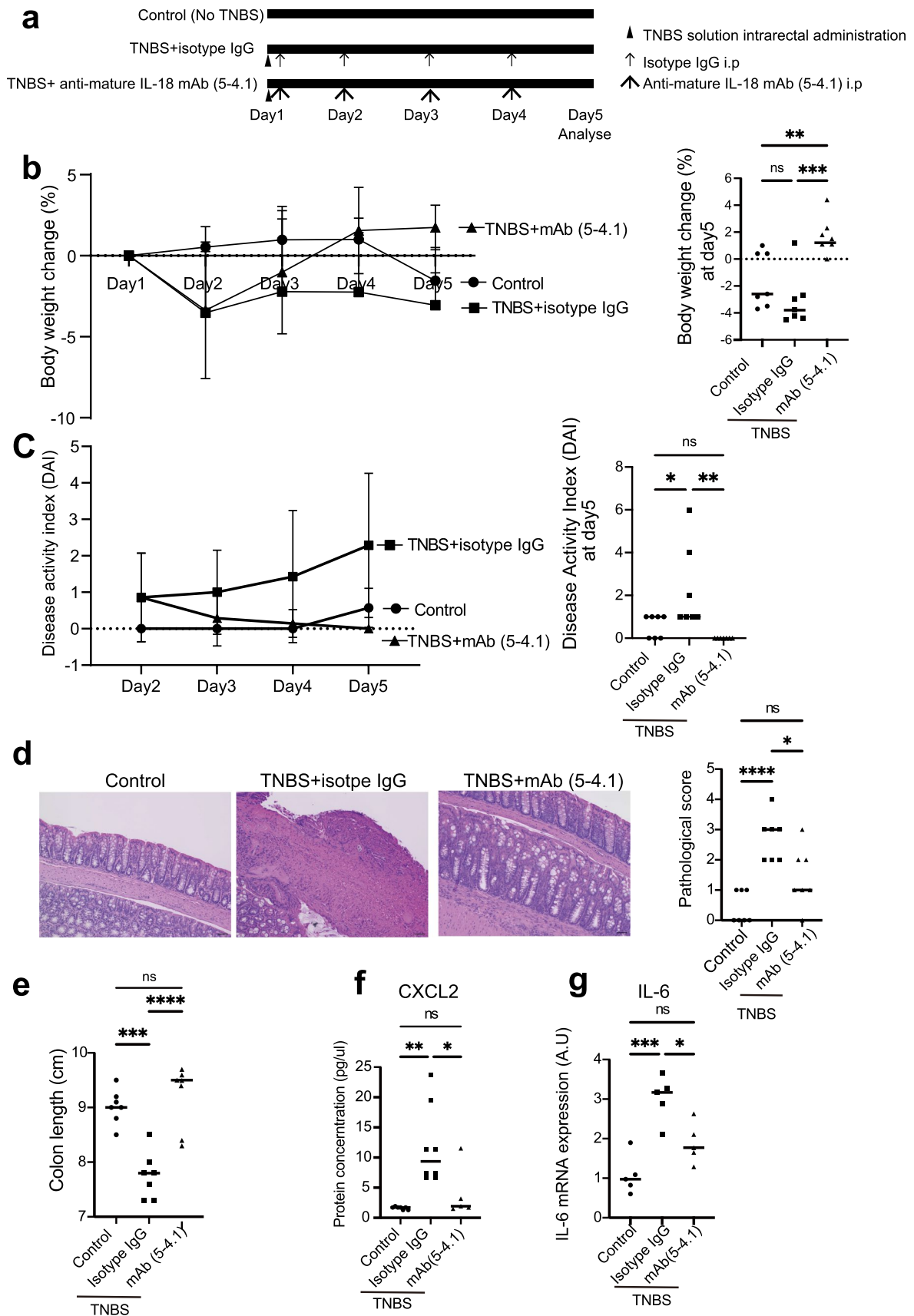
### Databases

The IBD Transcriptome and Metatranscriptome Meta-Analysis (IBD TaMMA; <https://ibd-meta-analysis.herokuapp.com>) database was used to analyze IL-18 expression based on RNA-sequence data from patients with CD. To determine the origin of IL-18 secretion cell types in patients with CD, we searched the scRNA-seq database derived from the Single Cell Portal (SCP1423, [https://singlecell.broadinstitute.org/single\\_cell](https://singlecell.broadinstitute.org/single_cell)).

### Results

#### Mature IL-18 Is Upregulated in the Serum and Colon Tissues of Patients Refractory to Anti-IL-12/23 and Anti-TNF- $\alpha$ Antibodies

To identify factors associated with resistance to treatment with multiple biologics, 31 patients with CD who were refractory to TNF- $\alpha$  antibody therapy were categorized as responders or non-responders to ustekinumab based on their CDAI scores. Baseline patient characteristics and clinical findings indicated that the CDAI scores of non-responders were significantly lower than those of responders and that mean serum hematocrit levels in



**Fig. 2** Administering anti-mature IL-18 mAb (5-4.1) ameliorates TNBS-induced colitis. **a** Schematic illustration of the experiment. Mice were intrarectally administered TNBS on day 1 and treated intraperitoneally with isotype IgG (200 µg) or mAb (5-4.1) (200 µg) daily for 5 d. **b** Body weight change and **c** disease activity index (DAI) scores in each group. **d** Representative hematoxylin and eosin staining (left panel) and histological scoring (right panel) of colon sections on day 5 after TNBS administration. **e** Length of dissected colons on day 5 after TNBS administration. **f** CXCL2 protein expression and **g** IL-6 mRNA expression in the colon on day 5 after TNBS administration.  $n=7$  per group, Scale bar: 40 µm. Data are presented as the means  $\pm$  SD; \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\*  $P < 0.001$  by multiple unpaired *t*-test or one-way analysis of variance (ANOVA), followed by Tukey's post-hoc test

non-responders were higher than those in responders (Table 1).

We then measured TNF- $\alpha$  and IL-18 expression levels at baseline in serum to investigate the different expression patterns of proinflammatory cytokines in non-responders and responders. TNF- $\alpha$  levels were not significantly different between the two groups (Fig. 1a). However, despite the lower CDAI in non-responders than in responders at baseline, IL-18 and mature IL-18 concentrations in serum at baseline were significantly higher in non-responders than responders (Fig. 1b).

To detect precursor and mature human IL-18 expression, we generated two types of mAbs, including anti-human IL-18 antibody (11-4.1), which recognizes the inactive precursor of human IL-18, and mature human IL-18 and anti-human IL-18 neopeptide antibodies (9-10.2), which recognize the new N-terminal<sup>37</sup> YFGKLESK<sup>44</sup> of IL-18 cleaved by caspase-1 and 4 [18] (Fig. 1c). Immunodeficiency, specifically insufficiency of macrophages and macrophage-derived IL-18, has been reported to be culprits in CD pathogenesis [19, 20]. Analysis of bulk RNA-seq and scRNA-seq databases of patients with CD indicated excessive expression of IL-18, primarily secreted by macrophages (Supplementary Fig. 1a and b). To identify the cells that express IL-18 and mature IL-18, we investigated the expression of IL-18 and the pan-macrophage marker, CD68, in patients with CD. The expression of the mature IL-18 was very rare in normal tissues from patients with CD but upregulated after inflammation was activated. Moreover, IL-18 was expressed in both normal and inflamed tissues of these patients and upregulated in inflammation lesions (Fig. 1d and e). Therefore, we explored the expression patterns of IL-18 as well as mature IL-18 in the colon tissues of patients with CD showing different responses to ustekinumab. IL-18 expression was upregulated in endoscopic biopsy samples of non-responders compared with those of responders (Fig. 1f). In addition, mature IL-18 expression in the same samples was significantly higher in patients with CD who were refractory to multiple biological agents than in

responders to therapy (Fig. 1g). In surgical intestinal tissues, IL-18 and mature IL-18 expression was upregulated in non-responders compared with that in responders (Supplementary Fig. 1c and d). Therefore, we hypothesized that increased expression of mature IL-18 is involved in non-responsiveness and exacerbates intestinal inflammation in patients with refractory CD and that inhibiting mature IL-18 can suppress intestinal inflammation without disrupting the barrier function of epithelial cells.

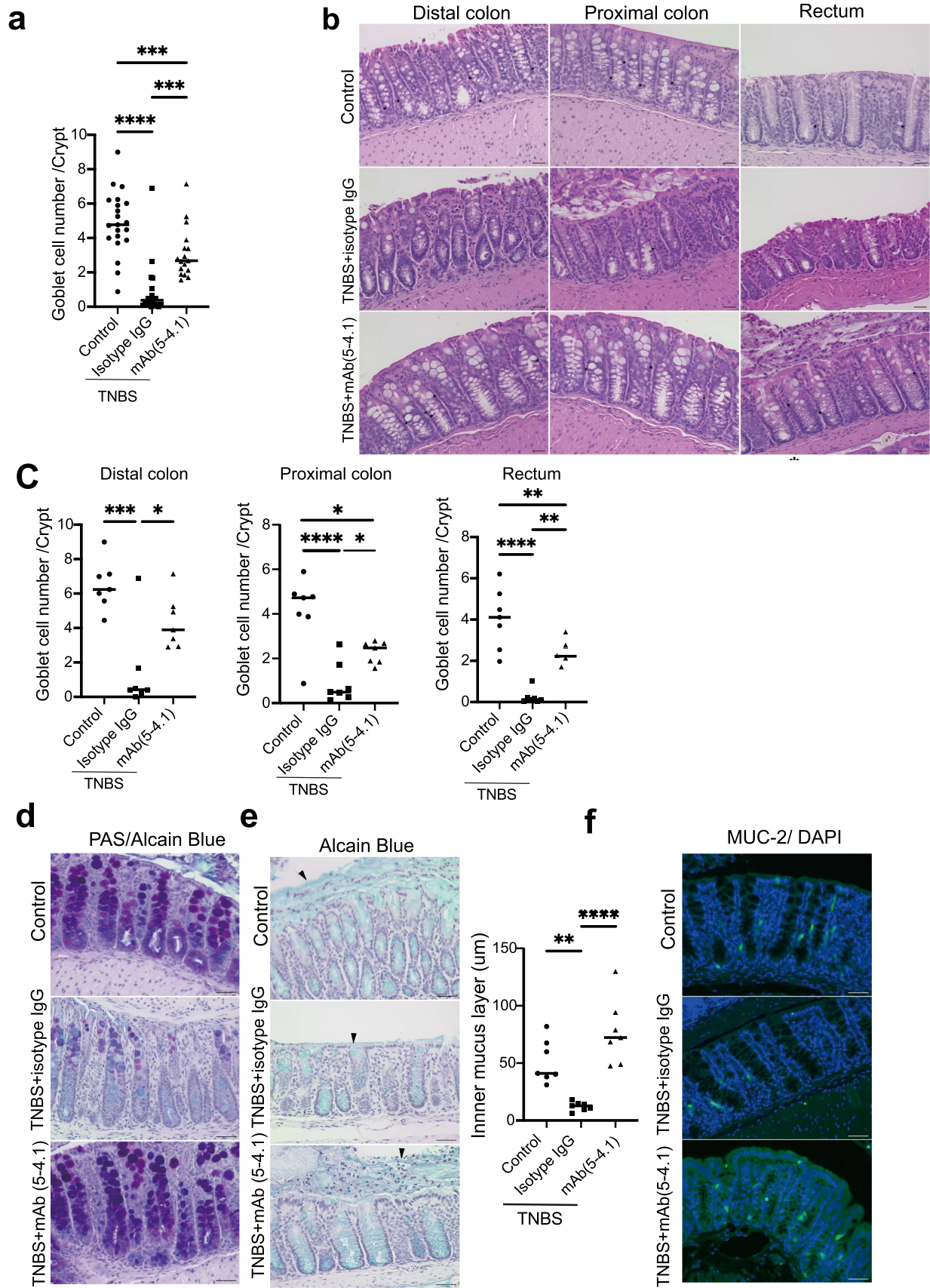
### Administering mAb (5-4.1) Has a Treatment Effect in TNBS-Induced Colitis

We developed an antibody, mAb (5-4.1), that specifically recognizes the neopeptide of mouse IL-18 cleaved by caspase-1 and caspase-4 (Fig. 1c) and demonstrated its specific binding to mature mouse IL-18 and dose-dependent inhibitory effect on IL-18-induced chemokines [21]. Mice in the isotype IgG and IL-18 mAb groups were intrarectally administered TNBS and treated intraperitoneally with isotype IgG or mAb (5-4.1) daily (Fig. 2a) to investigate the potential ameliorating effects of mAb (5-4.1) on acute colitis. The mAb (5-4.1)-treated group showed significantly less body weight loss than the isotype IgG-treated group (Fig. 2b). Furthermore, the mAb (5-4.1)-treated group showed lower disease activity index (DAI) scores, better histological scores, and higher colon lengths than those in the control group (Fig. 2c–e). CXCL2 and IL-6 expression was significantly downregulated in the mAb (5-4.1)-treated group compared with that in the isotype IgG-treated group (Fig. 2f and g).

### Administering mAb (5-4.1) Repairs Goblet Cell Function in TNBS-Induced Colitis

Because excessive IL-18 expression disrupts goblet cell function and mucus secretion, we investigated the effects of mAb (5-4.1) on goblet cell function. Histological analysis of tissue sections revealed reduced goblet cells after TNBS administration. In addition, colon tissue from the mAb (5-4.1)-treated group exhibited more goblet cells than those from the isotype IgG-treated group (Fig. 3a). Because TNBS-induced colitis led to intestinal epithelial damage and loss, we also quantified and compared goblet cell numbers in complete crypts without epithelial damage in each visual field. The numbers of goblet cells per crypt decreased after TNBS induction but rebounded after treatment with the mAb (5-4.1), suggesting that the restoration of goblet cells by mAb (5-4.1) contributes to alleviating intestinal inflammation (Fig. 3b and c). Regarding goblet cell function, Alcian Blue-PAS and Alcian Blue staining also showed an increased number of goblet cells and thicker







**Fig. 3** Anti-mature IL-18 mAb (5-4.1) ameliorates intestinal inflammation in TNBS-induced colitis model by repairing goblet cells. **a** Quantification of goblet cells in inflamed area. Three sections per mouse,  $n=7$  mice for each group. **b** Representative hematoxylin and eosin staining in non-inflamed area. **c** Quantification of goblet cells in different locations. **d** Representative Alcian blue-PAS double staining of colons on day 5 after TNBS administration. **e** Representative Alcian Blue staining (upper panels) and assessment of the thickness of the inner mucus layers (lower panel). **f** Immunofluorescence staining of MUC-2 on day 5 after TNBS administration. Data are presented as the means.  $n=7$  mice for each group.  $*P<0.05$ ,  $**P<0.01$ ,  $***P<0.001$  by one-way ANOVA, followed by Tukey's post-hoc test

mucus layer in the mAb (5-4.1)-treated group than in the isotype IgG-treated group (Fig. 3d and e). Immunofluorescence staining revealed that MUC-2, the main product and indicator of goblet cell expression, was reduced in the colons of mice with TNBS-induced colitis, whereas administration of mAb (5-4.1) suppressed the reduction in MUC-2 expression (Fig. 3f).

### Patients with CD Refractory to Anti-IL-12/23 and Anti-TNF- $\alpha$ Antibodies Exhibit Impaired Goblet Cell Function

We analyzed goblet cell function in patients with CD refractory to TNF- $\alpha$  antibody therapy. Alcian Blue staining revealed fewer goblet cells in non-responders than in responders (Fig. 4a); similarly, MUC-2 expression was lower in non-responders than in responders (Fig. 4b). These findings indicate a potential association between impaired goblet cell function and resistance to biologics. Notably, this finding supports the possibility of using an anti-mature-IL-18 antibody to restore goblet cell function in patients with refractory CD.

### Administering mAb (5-4.1) Changes Gut Microbiota Composition

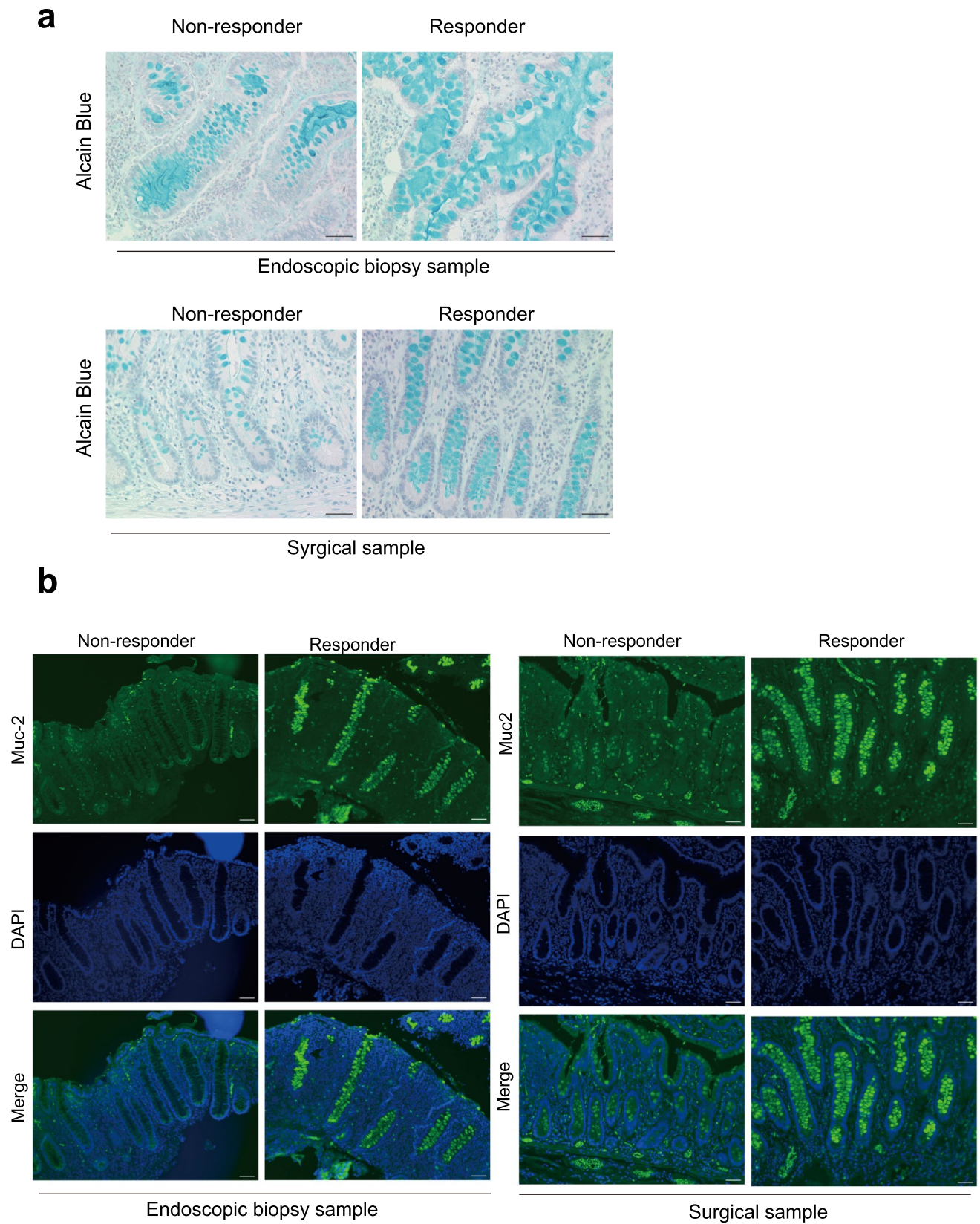
We analyzed gut microbiota composition. No differences were observed in observed species, Chao-1, and Shannon indices among the control, isotype IgG-treated group, and IL-18 mAb-treated group (Supplementary Fig. 2a and b). The relative abundance of bacteria did not differ among the three groups at the phylum level (Supplementary Fig. 2c and d). However, the abundance of *Faecalibaculum* and *Clostridium* increased with TNBS administration and decreased with mAb (5-4.1) treatment. Conversely, the abundance of *Negativibacillus* decreased with TNBS administration and increased with mAb (5-4.1) treatment (Fig. 5a–d).

## Discussion

Administering an anti-mature IL-18 antibody alleviated intestinal inflammation in a murine model of experimental colitis, primarily by rescuing goblet cell function and restoring the mucus layer at the host-microbiota interface. Consistent with our results, other studies have shown the advantages of inhibiting IL-18 with neutralizing anti-IL-18 antibodies or IL-18 binding protein in dextran sulfate sodium (DSS)- or TNBS-induced colitis models [12–14]. Furthermore, conditional deletion of IL-18 in epithelial cells protected mice from DSS-induced colitis by restoring goblet cell function. Excessive IL-18 signaling by genetic deletion of the IL-18 binding protein decreased mature mucus-producing goblet cells and severe colitis. These studies suggest that excessive IL-18 disrupts goblet cell function and maturation, impairing mucus secretion and compromising the defense against bacteria [22]. Additionally, antibiotic treatment was shown to alleviate experimental intestinal inflammation by inhibiting inflammasome and caspase-1 activation, decreasing mature IL-18 production [23]. These reports strongly support our research, which demonstrates the pathogenicity of cleaved mature IL-18 in intestinal inflammation and potential treatment effect of its inhibition. However, the role of IL-18 in intestinal homeostasis has not been elucidated.

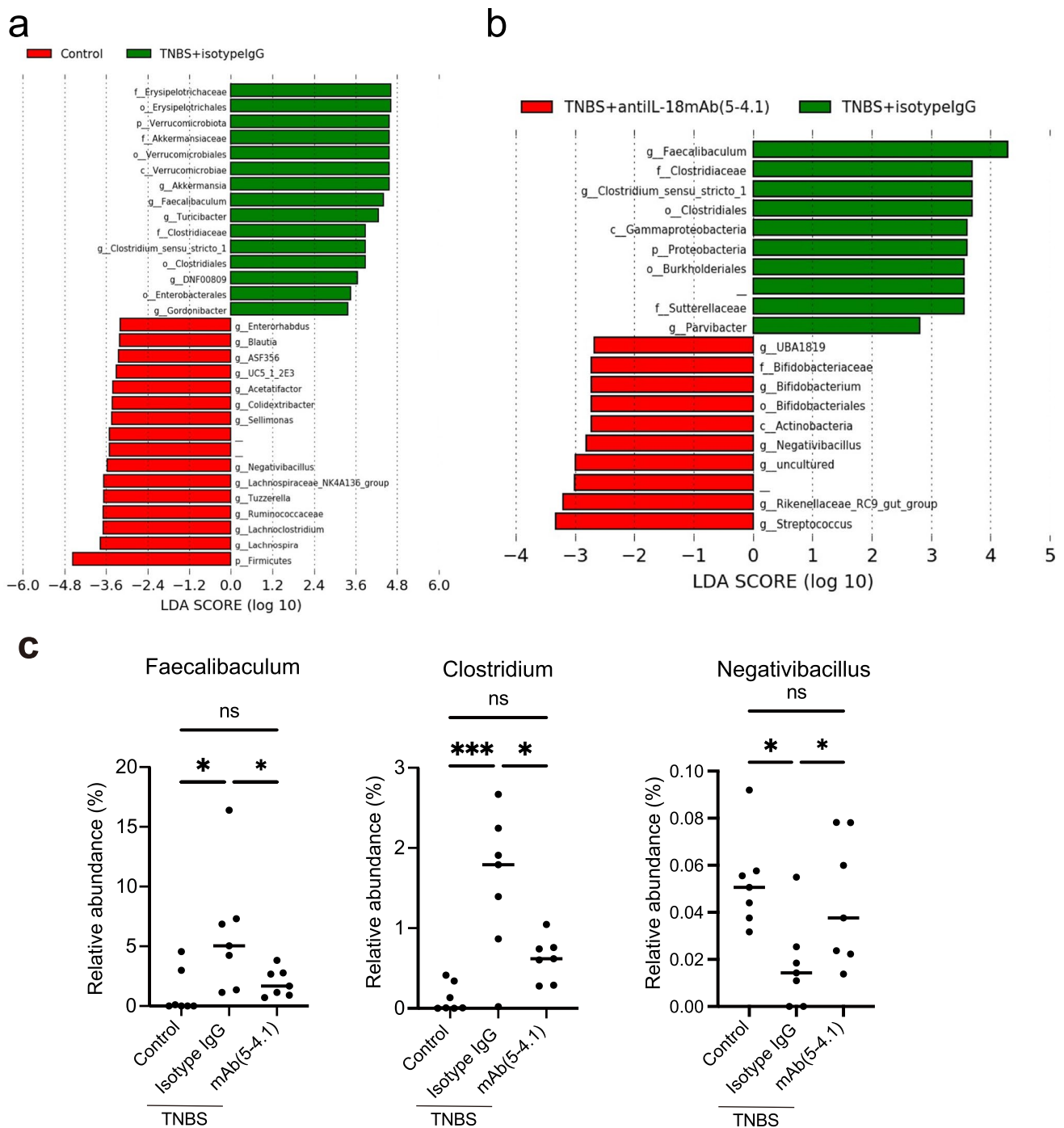
Under normal physiological conditions, IL-18 activates antimicrobial peptide secretion by Paneth cells and regulates the microbial community to prevent intestinal inflammation [15]. During intestinal infection, epithelial IL-18 facilitates the expansion of Lgr5+ stem cells for tissue repair via IL-22, promotes intestinal epithelial cell migration, induces rapid turnover of cells, and enhances intestinal host defense mechanisms [24]. Downregulated IL-18 expression in NLRP6-deficient mice leads to damaged secretion of goblet cell mucus granules, mucus layer dysfunction, an impaired host-microbial interface, and increased susceptibility to pathogen invasion [25]. Because protective and detrimental IL-18 functions in the gut have been reported, the use of an IL-18 blocking treatment in a clinic setting remains controversial. However, our results suggest that targeting only mature IL-18 and not depleting full-length IL-18 expression via antibody or even gene editing may restore goblet cell function and improve intestinal inflammation without disrupting the steady-state function of IL-18.

Macrophage-derived IL-18 has been reported to promote the depletion of goblet cells, thus playing a key role in experimental colitis [13, 19]. Consistent with these previous reports, our results indicate that the majority of IL-18-producing cells in patients with CD are macrophages, and mature IL-18 is positively correlated with inflammation flares. Therefore, our results suggest that our newly



**Fig. 4** Impaired goblet cell numbers in patients with CD who were refractory to anti-IL-12/23 and anti-TNF- $\alpha$  antibodies. **a** Representative Alcain Blue staining in non-responders and responders. **b** Repre-

sentative immunofluorescence staining of MUC-2 in non-responders and responders



**Fig. 5** Effects of anti-IL-18 mAb (5-4.1) on gut microbiota composition. **a–c** Comparison of the fecal microbiome between the two groups using linear discriminant analysis effect size (LEfSe). Bacteria indicated by red or green bars represent bacteria that increased or decreased significantly in each group. **d** The abundance of Faecalibaculum, *Clostridium*, and *Negativibacillus* increased after TNBS administration and decreased after mAb (5-4.1) administration. n=7 for each group. Data are presented as the means; \*P<0.05 by one-way ANOVA, followed by Tukey’s post-hoc test

synthesized anti-IL-18 antibody alleviated colitis by repairing goblet cell function, showing promise for application in patients with CD.

An underlying relationship between IL-18 and the regulation of gut microbiota exists. In the steady state, microbiota-derived signals are necessary for IL-18 production and antimicrobial mucus product expression; IL-18 abrogation



may induce commensal dysbiosis [18]. *Proteus mirabilis* is a pathogenic bacterium that promotes intestinal inflammation by triggering epithelial IL-18 secretion [26]. However, reports on the cytokine IL-18 and specific microbiota alterations in IBD are rare, especially under anti-IL-18 antibody treatment. Therefore, we examined alterations in the gut microbiota following the induction of experimental murine colitis and IL-18 antibody treatment. Order Clostridiales, family Clostridiaceae, and genus *Clostridium* were down-regulated following IL-18 antibody treatment. Consistent with our results, Clostridiales have been associated with active clinical CD [27]. Moreover, these bacteria are down-regulated in IL-18-deficient mice and are involved in constructing the intestinal mucus layer, leading to a significant loss of immune cells [28, 29]. Notably, IL-18 does not solely contribute to intestinal homeostasis; rather, the mature IL-18 and altered gut microbiota work together to maintain intestinal homeostasis and protect the gut microenvironment from invasion.

Nevertheless, this study had several limitations. The detailed functions of operational taxonomic units are still not clearly understood. This study also did not include investigating alterations in metabolites derived from the microbiota, which regulates immune responses and strengthens the intestinal barrier by sensing the inflammasome [30, 31].

In conclusion, administering mAb (5-4.1) alleviated intestinal inflammation in a murine experimental colitis model by rescuing goblet cell function and restoring the mucus layer at the host-microbiota interface. This antibody may serve as a novel therapeutic agent for patients who have been found refractory to biologics.

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**Author's contribution** All authors contributed to the conception and design of the study. Material preparation and data collection and analysis were performed by all authors. The first draft of the manuscript was written by Jingxi Mu, and all authors reviewed and commented on the manuscript. All authors read and approved the final manuscript.

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**Data availability** The original data and materials generated in this study are available from the corresponding author on reasonable request.

## Declarations

**Conflict of interest** TU is an employee at Shimane University and is the co-founder and current Chief Medical and Scientific Officer of mAbProtein, a biotechnology company that focuses on the development and commercial use of mAbs in inflammation research, diagnosis, and treatment. The potential conflicts of interest of TU do not alter the authors' adherence to any of the journal policies on sharing data and materials. The other authors declare no potential conflicts of interest.

**Ethical approval** Animal experiments were approved by the Animal Care and Use Committee of Nagoya University Graduate School of Medicine (approval number: M230086) and performed in compliance with the regulations and guidelines of animal care and use in Nagoya University. All the experiments were performed and reported in accordance with ARRIVE guidelines. The human study was conducted following the Declaration of Helsinki for Human Research, and protocols were approved by the Research Ethics Committee of Nagoya University (approval number: 2018-0316).

**Consent to participate** All participants provided written informed consent to participate in the study.

**Consent for publication** Not applicable.

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## References

- Graham DB, Xavier RJ. Pathway paradigms revealed from the genetics of inflammatory bowel disease. *Nature*. 2020;578:527–539.
- Friedrich M, Pohin M, Powrie F. Cytokine networks in the pathophysiology of inflammatory bowel disease. *Immunity*. 2019;50:992–1006.
- Cushing K, Higgins PDR. Management of Crohn disease: a review. *Jama*. 2021;325:69–80.
- West NR et al. Oncostatin M drives intestinal inflammation and predicts response to tumor necrosis factor-neutralizing therapy in patients with inflammatory bowel disease. *Nat Med*. 2017;23:579–589.
- Atreya R, Neurath MF. IL-23 Blockade in anti-TNF refractory IBD: from mechanisms to clinical reality. *J Crohns Colitis*. 2022;16:ii54–ii63.
- Wang Y et al. Genetic polymorphisms in the IL-18 gene and ulcerative colitis risk: a meta-analysis. *DNA Cell Biol*. 2014;33:438–447.
- Bank S et al. Genetically determined high activity of IL-12 and IL-18 in ulcerative colitis and TLR5 in Crohns disease were



- associated with non-response to anti-TNF therapy. *Pharmacogenomics J.* 2018;18:87–97.
8. Okamura H et al. Cloning of a new cytokine that induces IFN-gamma production by T cells. *Nature.* 1995;378:88–91.
  9. Yasuda K, Nakanishi K, Tsutsui H. Interleukin-18 in health and disease. *Int J Mol Sci.* 2019. <https://doi.org/10.3390/ijms20030649>.
  10. Leach ST et al. Local and systemic interleukin-18 and interleukin-18-binding protein in children with inflammatory bowel disease. *Inflamm Bowel Dis.* 2008;14:68–74.
  11. Kaplanski G. Interleukin-18: Biological properties and role in disease pathogenesis. *Immunol Rev.* 2018;281:138–153.
  12. Siegmund B et al. Neutralization of interleukin-18 reduces severity in murine colitis and intestinal IFN-gamma and TNF-alpha production. *Am J Physiol Regul Integr Comp Physiol.* 2001;281:R1264–R1273.
  13. Kanai T et al. Macrophage-derived IL-18-mediated intestinal inflammation in the murine model of Crohn's disease. *Gastroenterology* 2001;121:875–888.
  14. Ten Hove T et al. Blockade of endogenous IL-18 ameliorates TNBS-induced colitis by decreasing local TNF-alpha production in mice. *Gastroenterology.* 2001;121:1372–1379.
  15. Levy M et al. Microbiota-modulated metabolites shape the intestinal microenvironment by regulating NLRP6 inflammasome signaling. *Cell.* 2015;163:1428–1443.
  16. Bolyen E et al. Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. *Nat Biotechnol.* 2019;37:852–857.
  17. Segata N et al. Metagenomic biomarker discovery and explanation. *Genome Biol.* 2011;12:R60.
  18. Nariai Y et al. Generation and characterization of antagonistic anti-human interleukin (IL)-18 monoclonal antibodies with high affinity: Two types of monoclonal antibodies against full-length IL-18 and the neoepitope of inflammatory caspase-cleaved active IL-18. *Arch Biochem Biophys.* 2019;663:71–82.
  19. Neurath MF. Targeting immune cell circuits and trafficking in inflammatory bowel disease. *Nat Immunol.* 2019;20:970–979.
  20. Casanova JL, Abel L. Revisiting Crohn's disease as a primary immunodeficiency of macrophages. *J Exp Med.* 2009;206:1839–1843.
  21. Uchida Y et al. Generation of antagonistic monoclonal antibodies against the neoepitope of active mouse interleukin (IL)-18 cleaved by inflammatory caspases. *Arch Biochem Biophys.* 2022;727:109322.
  22. Nowarski R et al. Epithelial IL-18 equilibrium controls barrier function in colitis. *Cell.* 2015;163:1444–1456.
  23. Chakravarti D et al. Telomere dysfunction activates YAP1 to drive tissue inflammation. *Nat Commun.* 2020;11:4766.
  24. Chiang HY et al. IL-22 initiates an IL-18-dependent epithelial response circuit to enforce intestinal host defence. *Nat Commun.* 2022;13:874.
  25. Wlodarska M et al. NLRP6 inflammasome orchestrates the colonic host-microbial interface by regulating goblet cell mucus secretion. *Cell.* 2014;156:1045–1059.
  26. Zhang J et al. Elucidation of proteus mirabilis as a key bacterium in Crohn's disease inflammation. *Gastroenterology.* 2021;160:317–330.e11.
  27. Yilmaz B et al. Microbial network disturbances in relapsing refractory Crohn's disease. *Nat Med.* 2019;25:323–336.
  28. Volk JK et al. The Nlrp6 inflammasome is not required for baseline colonic inner mucus layer formation or function. *J Exp Med.* 2019;216:2602–2618.
  29. Zheng X et al. IL-18 maintains the homeostasis of mucosal immune system via inflammasome-independent but microbiota-dependent manner. *Sci Bull (Beijing).* 2021;66:2115–2123.
  30. Belkaid Y, Hand TW. Role of the microbiota in immunity and inflammation. *Cell.* 2014;157:121–141.
  31. Macia L et al. Metabolite-sensing receptors GPR43 and GPR109A facilitate dietary fibre-induced gut homeostasis through regulation of the inflammasome. *Nat Commun.* 2015;6:6734.

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