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The protective effect of IL-12/23 neutralizing antibody in sarcopenia associated with dextran sulfate sodium-induced experimental colitis

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Abstract

Background The improvement of colitis symptoms by treatment with IL-12/23 p40 neutralizing antibody should increase the muscle mass and the function of the sarcopenia phenotype.

Methods An experimental colitis model was induced by oral administration of 2% dextran sulfate sodium (DSS) for 7 days. During induction of colitis, IL-12/23 p40 neutralizing antibody was injected twice on Days 3 and 5. The total body mass index was measured by dual-energy X-ray absorptiometry. The muscle function was measured by forelimb grip strength and fatigue running distance. The muscle fibre cross-sectional area (CSA) was calculated after the transverse section and haematoxylin and eosin staining, and gene expression was confirmed by RT-qPCR. Differentiated C2C12 cells were used as in vitro models and treated with recombinant IL12/23 proteins to mimic the enhanced cytokines in colitis.

Results The symptoms of colitis were alleviated by injection of IL-12/23 p40 neutralizing antibody compared with phosphate-buffered saline (PBS), and the disease activity index score was significantly lower on Day 8 (0.0 ± 0.00 of cont. vs. 11.3 ± 0.9 of DSS + PBS, $P < 0.0001$; DSS + PBS vs. 7.7 ± 1.25 of DSS + p40Ab, $P < 0.0001$). The CSA of the gastrocnemius and tibialis anterior muscle fibres decreased in mice with DSS-induced colitis (gastrocnemius, 1258.2 μm^2 ± 176.45 of cont. vs. 640.1 μm^2 ± 59.83 of DSS + PBS, $P < 0.0001$; tibialis anterior, 1251.8 μm^2 ± 331.48 of cont. vs. 678.9 μm^2 ± 67.59 of DSS + PBS, $P < 0.0001$), and the treatment of IL-12/23 p40 neutralizing antibody partially restored CSA of the gastrocnemius (640.1 μm^2 ± 59.83 of DSS + PBS vs. 1062.0 μm^2 ± 83.41 of DSS + p40Ab, $P < 0.0001$) and tibialis anterior (678.9 μm^2 ± 67.59 of DSS + PBS vs. 1105.3 μm^2 ± 143.15 of DSS + p40Ab, $P = 0.0003$).vs. 640.1 μm^2 ± 59.83 of DSS + PBS, $P < 0.0001$) and tibialis anterior (1251.8 μm^2 ± 331.48 of cont. vs. 678.9 μm^2 ± 67.59 of DSS + PBS, $P < 0.0001$), and the treatment of IL-12/23 p40 neutralizing antibody partially restored CSA of the gastrocnemius (640.1 μm^2 ± 59.83 of DSS + PBS vs. 1062.0 μm^2 ± 83.41 of DSS + p40Ab, $P < 0.0001$) and tibialis anterior (678.9 μm^2 ± 67.59 of DSS + PBS vs. 1105.3 μm^2 ± 143.15 of DSS + p40Ab, $P = 0.0003$). In the evaluation of muscle function, grip strength and fatigue distance decreased by colitis were partially restored (grip strength: 139.9 g ± 5.38 of cont. vs. 83.9 g ± 5.48 of DSS + PBS, $P < 0.0001$; DSS + PBS vs. 118.6 g ± 4.05 of DSS + p40Ab, $P < 0.0001$; fatigue distance: 872.5 m ± 104.01 of cont. vs. 58.2 m ± 107.72 of DSS + PBS, $P < 0.0001$; DSS + PBS vs. 328.0 m ± 109.71 of DSS + p40Ab, $P = 0.0015$) by injection of IL-12/23 p40 neutralizing antibody.

Conclusions Our study demonstrates that Il-12/23 acts directly on muscle to induce atrophy, and the IL-12/23 p40 neutralizing antibody is effective not only in suppressing colitis but also in maintaining muscle mass and improving muscle function in an experimental colitis model.

Keywords IL1; IL23; inflammatory bowel disease; neutralizing antibody; sarcopenia

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Introduction

Inflammatory bowel disease (IBD) is a chronic disorder of the gastrointestinal (GI) tract and includes ulcerative colitis (UC) and Crohn's disease (CD), which are subgroups of IBD.[1] UC is a GI condition characterized by inflammation of the large intestine or colon, which targets the colonic mucosa.[1] In contrast, CD is a transmural disease and can affect any portion of the GI tract with progressive inflammation, but the large and small intestines are the most typically affected.[2] Even though the incidence of IBD is steadily reducing in high-incidence areas, it is increasing in low-incidence areas such as Asia, southern Europe and most of the developing world.[3,4]

Muscle loss is a common pathophysiological feature of IBD as a GI disease. Sarcopenia affects 42% of IBD patients, according to recent research.[5] In addition, as a result of persistent inflammation, sarcopenia frequently coexists with malnutrition.[6] According to the study by Bryant et al., patients with IBD have a decreased lean body mass composition and reduced muscle mass by up to 60% when compared with healthy people.[7] This evidence showed that accompanying sarcopenia and IBD can lead to more serious consequences than when each occurs. Although there were several studies about the association between sarcopenia and IBD, studies on effective treatment methods in patients with sarcopenia and IBD are insufficient.

Recently, inflammatory cytokines targeted antibodies including anti-interleukin 12/23,[8] anti-interleukin 6,[9] anti-interleukin 17[10] and anti-TNFα[11] have been developed and some of them show clinical efficacy. In addition, studies on inflammatory cytokines related to sarcopenia are also underway.[12] However, there have been few studies that identify the effects and mechanisms of such IL antibody in models derived from sarcopenia animal models derived from IBD or sarcopenia cell models. Therefore, the purpose of this study was to determine the improvement of colitis symptoms in the dextran sulfate sodium (DSS) mouse model and mouse myoblast cell model by treatment with IL-12/23 p40 neutralizing antibody by examining the increase of muscle mass and the function of sarcopenia phenotype.

Materials and methods

Construction of dextran sulfate sodium-induced colitis mouse model and Il-12/23 antibody injection

Male C57BL/6 mice at 7 weeks old were purchased from Core Tech Co., Ltd. (Korea) and used in all experiments. Mice were allowed to acclimate to the environment for 7 days in a light and dark cycle with a temperature of 24 ± 2°C, relative humidity of 40–60%, an illuminance of 150 to 300 lux and an interval of 12 h. Mice were given unrestricted access to standard mouse chow and tap water. The experimental colitis model was prepared by inducing 8-week-old male mice with 2% (wt/vol) DSS (MW, 36 000 to 50 000, MP Biomedicals, France) dissolved in tap water for 8 days through oral administration. Control mice (n = 7) received only tap water. Il-12/23 p40 neutralizing antibody (n = 10) or normal IgG as control (n = 9) was used for intraperitoneal injection twice on Days 3 and 6 after induction of colitis. Mice were sacrificed on Day 8. Disease activity index (DAI) was measured based on parameters of body weight change, stool consistency and stool blood presence during DSS administration, as described previously.[13] Colon length was measured from the distal rectum to the proximal cecum. All animal experiments were approved by the Animal Experimental Ethics Committee of Gyeongsang National University (GNU-180823-M0044). Animals were handled in compliance with the ethical process for animal experiments by guidelines of the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC International).

Dual-energy X-ray absorptiometry

Body composition was assessed for all mice using a dual-energy X-ray absorptiometry (DXA) scanner (OsteoSys, Seoul, Korea). After anaesthesia, the mouse was placed on the scanner bed in a prone position with its limbs and tail extended away from the body. High-resolution images were obtained from a flat panel detector using a con beam X-ray source that could generate low energy (60 kV/0.8 mA) and high energy (80 kV/0.8 mA). All DXA images and quantitative results were taken using Insight software (Version 1.0.6; OsteoSys).

Evaluation of muscle function

For both control and DSS-induced colitis mice, muscle strength was measured based on forelimb grip strength using a grip strength metre (BIOSEB, Boulogne, France) before sacrifice on Day 8. To measure forelimb grip strength, the experimenter gently gripped the back and the base of the tail with both hands so that each mouse could grab a metal bar with its forelimbs. As soon as the mouse grabbed the metal bar, the maximum pulling force (grams) was recorded by pulling the animal back by its tail until the mouse lost grip. The forelimb grip strength was measured three times at 5-min intervals to obtain the average value of each mouse. As another method to evaluate muscle function, a fatigue test was performed using a treadmill (Harvard Apparatus, MA, USA). Each mouse was acclimatized to the treadmill for 10 min at a low speed (10 m/min) for 3 consecutive days before treadmill testing. For the fatigue test, mice ran at

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10 m/min for 5 min and 15 m/min for 5 min. After the initial warm-up, the exercise intensity was increased by 1.8 m/min every 3 min until mice were unable to continue running even with moderate electric stimulation (0.1 mA). The distance was recorded when running was stopped.

Histology

Colon and muscle tissues from normal controls and colitis mice were fixed with 10% formalin for 1 day, embedded in paraffin and sectioned at a thickness of 5 μm using a microtome. Histological score evaluation of the colon was performed after haematoxylin and eosin (H&E) staining. The degree of damage was analysed as described previously.[14] All histological quantifications were performed by two investigators for all H&E-stained tissue slides blinded. These sections were quantified for three independent parameters: severity of inflammation (score of 0–3), depth of injury (score of 0–3) and crypt damage (score of 0–4). The score for each parameter was multiplied by a factor reflecting the overall tissue invasion rate (×1: <25%, ×2: 26–50%, ×3: 51–75%, ×4: >75%). Values were summed to obtain the total histological score. Histologic analysis of each muscle tissue was also performed after H&E staining of transversely sectioned gastrocnemius (GN) and tibialis anterior (TA) muscles. The cross-sectional area (CSA) of each muscle fibre was measured using a Nikon microscope and software (Nikon, Japan).

C2C12 cell differentiation and Giemsa staining

C2C12 cells, a mouse myoblast cell line, were used for in vitro experiments. C2C12 cells were purchased from the American Type Culture Collection (Manassas, VA, USA) and cultured in Dulbecco's modified Eagle's medium-high glucose medium (DMEM-h; #11960044, Gibco, Carlsbad, CA, USA) supplemented with 10% (v/v) foetal bovine serum (FBS; #16000, Gibco), 100 U/mL penicillin and 100 μg/mL streptomycin (P/S; #15140, Gibco). Cells were subcultured at intervals of 3–4 days and used for the experiment. For myoblast differentiation, cells were cultured in a differentiation medium supplemented with 2% horse serum (Gibco) and 1% glutamine (Gibco) for 5–7 days. May–Grünwald and Giemsa staining were performed for quantitative measurement of myotubes. Differentiated C2C12 cells were washed with cold phosphate-buffered saline (PBS) and fixed with 100% methanol. Subsequently, the May–Grünwald staining solution (Sigma) diluted with sodium phosphate buffer (pH 6.0) was added to the cells for 5 min. Cells were then washed with distilled water and incubated for 10 min in Giemsa staining solution. After washing twice with distilled water, the diameter of myotubes was quantified using an inverted microscope (Nikon, Japan).

RNA interference

The transfection of siRNA for *Il12rb1* to C2C12 cells was performed in six-well plates. The scrambled siRNA as control and *Il12rb1* siRNA (Santa Cruz Biotechnology, CA, USA) were transfected when C2C12 cells were 80–90% confluence with Lipofectamine 3000 (ThermoFisher Scientific, Waltham, MA, USA). After 24 h, differentiation was induced and subsequent experiments were performed as described above.

Western blot

Cell lysates from cultured C2C12 cells were extracted using radioimmunoprecipitation assay (RIPA) buffer supplemented with protease and phosphatase inhibitors (Roche Applied Science, Mannheim, Germany). Equal amounts of total cell lysates were separated by 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membrane (Millipore Corporation, Billerica, MA, USA). The membranes were blocked using 5% non-fat dry milk in Tris-buffered saline with Tween-20 buffer (TBS-T; 20-mM Tris, 100-mM NaCl, pH 7.5, and 0.1% Tween-20) for 1 h at room temperature and incubated with primary antibodies for phospho-Erk, Erk (Cell Signaling Technology, Danvers, MA, USA) and β-actin (Sigma-Aldrich, St. Louis, MO, USA) for 1 h. After incubation with a horseradish peroxidase (HRP)-conjugated secondary antibody (Bio-Rad, Hercules, CA, USA), the signals were detected using an enhanced chemiluminescence solution (GE Healthcare, Buckinghamshire, UK).

RT-qPCR

Total RNAs were extracted from tissues and cultured cells with Trizol (Invitrogen, USA) according to the manufacturer's protocol. Extracted total RNA (1 μg) was used for reverse transcription to obtain the complementary DNAs (cDNAs) as templates using random hexamer and oligo(T) primers. RT-qPCR was performed using the SYBR green master mix on the ViiA7 machine (Applied Biosystems, Waltham, MA, USA). The expression of target genes was normalized to *Gapdh*. The sequences of primers used for RT-qPCR are listed in *Table 1*.

Results

Reduction of weight loss and disease activity by Il-12/23 p40 neutralizing antibody in dextran sulfate sodium-induced colitis model

DSS-induced colitis mouse model showed phenotypes similar to human acute and chronic UC that progressed to severe

Table 1 Primer sequences used for RT-qPCR analysis

Gene	NCBI acc. no.	Sequence 5′ to 3′
Il12rb1	NM_008353	Forward: TGCCGCTACTTCTCCTCAG Reverse: ACTTCATGGTTCGGTTCCCAA
Il12rb2	NM_008354	Forward: TGCCCCTGTGATTCCTCTTG Reverse: ACTGGGATAATGTGAACAGCCT
Il23r	NM_144548	Forward: AACAACAGCTCGGATTTGGTAT Reverse: ATGACCAGGACATTCAGCAGT
Myod1	NM_010866	Forward: TCCTCATAGCACAGGGGTGA Reverse: GCAAGCTGTGGGGAAAAGTG
Myog	NM_031189	Forward: CAGCCCAGCGAGGGAATTTA Reverse: AGAAGCTCCTGAGTTTGCCC
Mef2c	NM_001170537	Forward: ACGGGGACTATGGGGAGAAA Reverse: AATCTCACAGTCGCACAGCA
Cdkn1a	NM_007669	Forward: GGAACATCTCAGGGCCGAAA Reverse: CTGACCCACAGCAGAAGAGG
MuRF-1	NM_001039048	Forward: GTGTGAGGTGCCTACTTGCTC Reverse: GCTCAGTCTTCTGTCCTTGGA
Atrogin-1	NM_026346	Forward: CAGCTTCGTGAGCGACCTC Reverse: GGCAGTCGAGAAGTCCAGTC
Myh7	NM_080728	Forward: CTCCTGCTGTTTCCTTACTTGCT Reverse: AGCCTTGGATTCTCAAACGTGTC
Myh2	NM_001039545	Forward: AAGAGCCGGGAGGTTCACA Reverse: GGCGCATGACCAAAGGTTT
Myh4	NM_010855	Forward: GTAGAAGCTGTGAATTCCAAATGC Reverse: CGTTAGACCTCTCCACGTCAATC
Myh1	NM_030679	Forward: CGGTCGAAGTTGCATCCCTA Reverse: TTCTGAGCCTCGATTCGCTC

chronic inflammation, loss of body weight, loose faeces and faecal blood. UC and IBD are known to be strongly associated with fatigue, weakness and musculoskeletal manifestation.[15] In this study, the effects of Il-12/23 p40 neutralizing antibody in a DSS-induced experimental mouse colitis model were investigated. DAI score was decreased by Il-12/23 p40 neutralizing antibody injection. The first injection of Il-12/23 p40 neutralizing antibody was performed on the third day after DSS treatment. The DAI score was decreased from 2 days after the first injection (0.0 ± 0.00 of cont. vs. 5.9 ± 0.6 of DSS + PBS, $P < 0.0001$; DSS + PBS vs. 4.8 ± 0.79 of DSS + p40Ab, $P = 0.0489$). It was significantly decreased at 2 days after the second injection (0.0 ± 0.00 of cont. vs. 11.3 ± 0.9 of DSS + PBS, $P < 0.0001$; DSS + PBS vs. 7.7 ± 1.25 of DSS + p40Ab, $P < 0.0001$). Twice injection of Il-12/23 p40 neutralizing antibody also alleviated body weight loss from Day 4 and significantly on Day 8 (1.1 g ± 0.46 of cont. vs. −7.8 g ± 1.33 of DSS + PBS, $P < 0.0001$; DSS + PBS vs. −5.8 g ± 1.19 of DSS + p40Ab, $P = 0.0076$) (*Figure 1A*). Histologic changes of the bowel caused by DSS-induced colitis were significantly improved by Il-12/23 p40 neutralizing antibody injection (0.0 ± 0.00 of cont. vs. 23.4 ± 7.81 of DSS + PBS, $P < 0.0001$; DSS + PBS vs. 13.7 ± 5.59 of DSS + p40Ab, $P = 0.0111$) (*Figure 1B,C*). However, there was no significant change in bowel length for each group after treatment (6.4 cm ± 0.53 of cont. vs. 3.6 cm ± 0.73 of DSS + PBS, $P < 0.0001$; DSS + PBS vs. 4.0 cm ± 0.53 of DSS + p40Ab, P = not significant [ns]) (*Figure 1D*).

Inhibition of muscle atrophy and functional reduction by IL-12/23 p40 neutralizing antibody in a dextran sulfate sodium-induced colitis mouse model

The cross-sectional morphology of GN and TA muscles from normal control and DSS-induced colitis mouse twice treated with normal IgG or IL-12/23 p40 neutralizing antibody was assessed using H&E staining (*Figure 2A*). Muscle fibre CSAs of both muscles were significantly lower in the DSS-induced colitis mice than in normal control mice. After twice injection of IL-12/23 p40 neutralizing antibody, the reduction in muscle fibre CSA was remarkably recovered for both muscles of the GN (1258.2 μm^2 ± 176.45 of cont. vs. 640.1 μm^2 ± 59.83 of DSS + PBS, $P < 0.0001$; DSS + PBS vs. 1062.0 μm^2 ± 83.41 of DSS + p40Ab, $P < 0.0001$) and TA (1251.8 μm^2 ± 331.48 of cont. vs. 678.9 μm^2 ± 67.59 of DSS + PBS, $P < 0.0001$; DSS + PBS vs. 1105.3 μm^2 ± 143.15 of DSS + p40Ab, $P = 0.0003$) (*Figure 2B*). In the expression of the muscle atrophy-related genes, *MuRF-1* and *Atrogin-1* in the two muscles, the expression of *MuRF-1* and *Atrogin-1* was significantly increased in the normal IgG-injected group after colitis induction compared with the normal control group. But the IL-12/23 p40 neutralizing antibody-injected group showed similar expression of *MuRF-1* and *Atrogin-1* to the normal control group (*Figure 2C*). In various species, myosin is the major contractile protein of skeletal muscle, and the major fibre types are named according to the four different isoforms of the myosin heavy chain (MHC I, IIa, IIb and IIx), reflecting differences

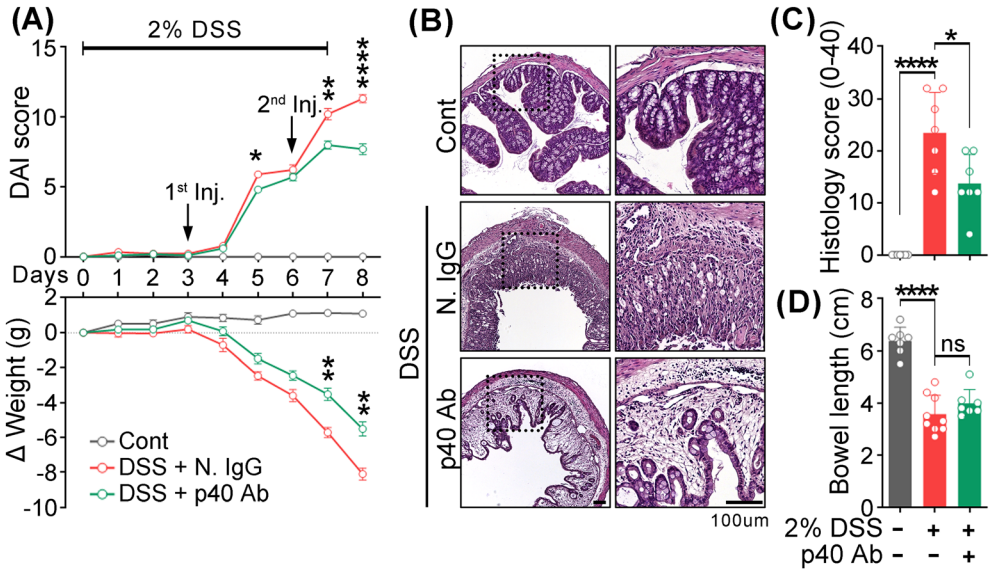

Figure 1 IL-12/23 p40 neutralizing antibody ameliorates the severity of dextran sulfate sodium (DSS)-induced colitis. (A) The severity and weight changes of DSS-induced colitis treated with phosphate-buffered saline or IL-12/23 p40 neutralizing antibody compared with normal mice. (B) Haematoxylin and eosin staining of bowel cross-section is shown. (C) Measurement of the histologic score of the bowel after haematoxylin and eosin staining. (D) Measurement of bowel length. Each symbol represents an individual mouse; bars show the mean ± SD. *$P < 0.05$, **$P < 0.005$, ***$P < 0.001$, ****$P < 0.0001$, by Bonferroni's post hoc test after one-way analysis of variance. DAI, disease activity index; ns, not significant.

in fibre properties. So, the major fibre types of muscles were evaluated according to the expression of myosin heavy chain genes *Myh7*, *Myh2*, *Myh4* and *Myh1*. The GN contains ~50% slow twitch fibres (type I fibre). In GN, the expression of the type I fibre-related gene, *Myh7* (MHC I), was not changed in colitis mouse, but the expression of type II fibre-related genes *Myh2* (MHC IIa), *Myh4* (MHC IIb) and *Myh1* (MHC IIx) was decreased, and *Myh2* and *Myh1* were confirmed to be partially recovered by injection of the IL12/23 p40 neutralizing antibody. However, the expression of Myh7 was significantly increased in the TA (type II fibre dominant muscle) of normal IgG-injected mice and recovered in the IL12/23 p40 neutralizing antibody-injected mice. Conversely, the expression of Myh2, a type II fibre gene, decreased in colitis mice injected with normal IgG and increased after injection of IL12/23 p40 neutralizing antibody, showing a similar level to control mice (*Figure 2D*). Next, changes in muscle function caused by the injection of IL-12/23 neutralizing antibody were evaluated by measuring forelimb grip strength and performing a fatigue test using a treadmill. Similar to the results of muscle fibre CSA evaluation, the decrease of forelimb grip strength in DSS-induced colitis mice was restored similarly to normal after twice injection of IL-12/23 neutralizing antibody (139.9 g ± 5.38 of cont. vs. 83.9 g ± 5.48 of DSS + PBS, $P < 0.0001$; DSS + PBS vs. 118.6 g ± 4.05 of DSS + p40Ab, $P < 0.0001$). In fatigue running distance measurement, normal IgG-injected colitis mice almost did not run, whereas mice injected with IL-12/23 neutralizing antibody recovered muscle function to a level of ~40% of normal mice in the fatigue test (872.5 m ± 104.01 of cont. vs. 58.2 m ± 107.72 of DSS + PBS, $P < 0.0001$; DSS + PBS vs. 328.0 m ± 109.71 of DSS + p40Ab, $P = 0.0015$) (*Figure 2E*).

Muscle mass, but not fat, was slightly recovered after injecting IL-12/23 neutralizing antibody to dextran sulfate sodium-induced colitis mice

Body composition was analysed with a DXA scanner on Day 8, the last day of the study. Bone mineral density (BMD), fat content and muscle content were also measured. DXA results confirmed that decreases of BMD (0.08 g/cm^2 ± 0.002 of cont. vs. 0.09 g/cm^2 ± 0.002 of DSS + PBS, P = ns; DSS + PBS vs. 0.08 g/cm^2 ± 0.002 of DSS + p40Ab, P = ns) and fat content (2.31 g ± 0.40 of cont. vs. 0.74 g ± 0.10 of DSS + PBS, $P < 0.0001$; DSS + PBS vs. 1.06 g ± 0.27 of DSS + p40Ab, P = ns) in DSS-induced colitis mice were not recovered, but muscle content (22.17 g ± 0.65 of cont. vs. 15.62 g ± 0.92 of DSS + PBS, $P < 0.0001$; DSS + PBS vs. 16.85 g ± 1.04 of DSS + p40Ab, $P = 0.0327$) in DSS-induced colitis mice is slightly recovered by injection of IL-12/23 neutralizing antibody (*Figure 3A,B*).

Induction of Il12/Il23 receptor expression by IL23 and inhibition of myoblast differentiation via activation of Erk

In mice and humans, IL-12 consists of IL-12/23 p40 and IL-12 p35 subunits. It signals through IL-12Rβ1 and IL-12Rβ2

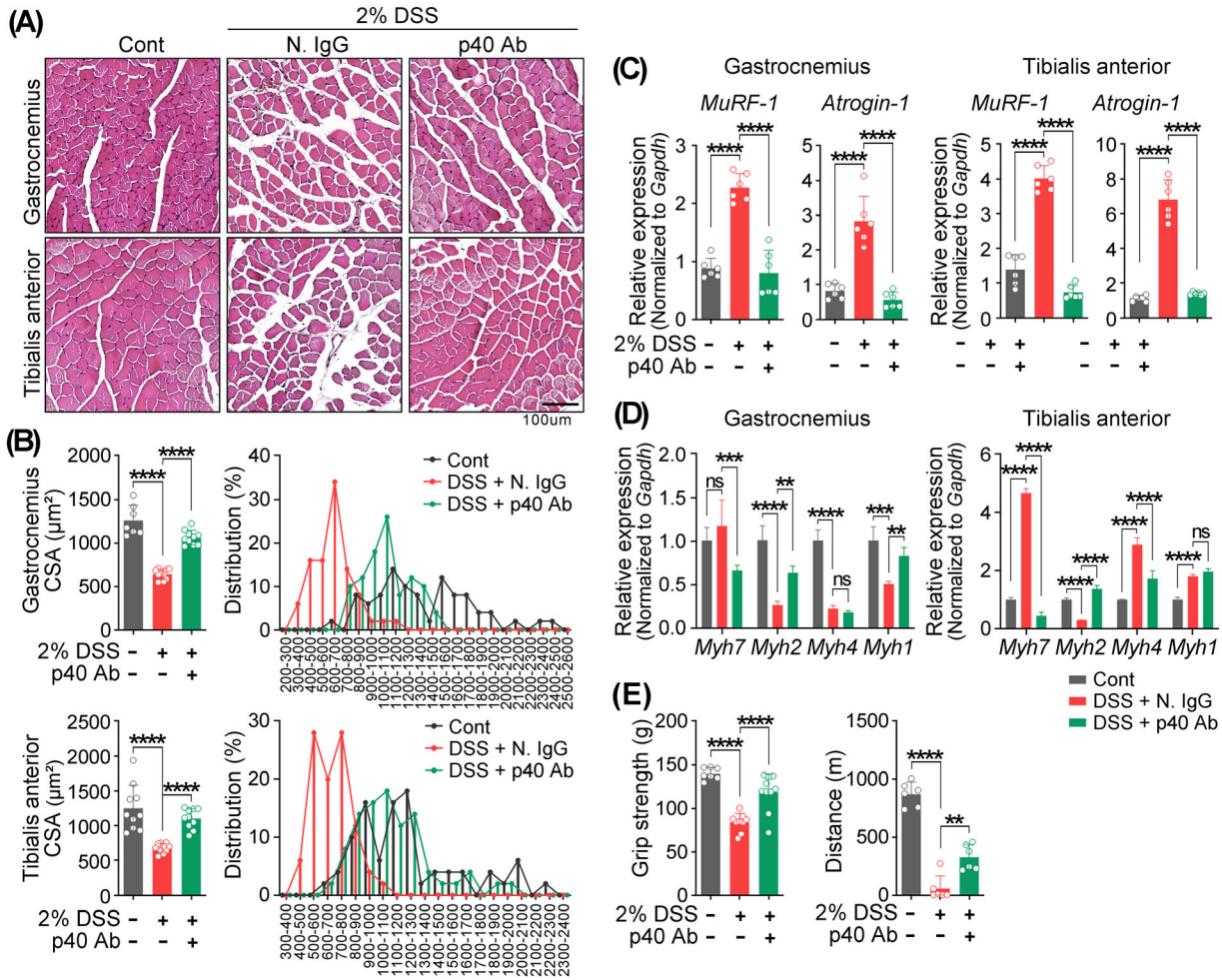

Figure 2 Muscle cross-sections of a control mouse and 2% dextran sulfate sodium (DSS)-induced colitis mouse model after injection of phosphate-buffered saline or IL-12/23 p40 neutralizing antibody. (A) Representative image of haematoxylin and eosin staining of the gastrocnemius and tibialis anterior from normal control and 2% DSS-induced colitis model. (B) Measurement of cross-sectional area (CSA) and area distribution of gastrocnemius and tibialis anterior after haematoxylin and eosin staining under light microscopy. (C) Muscle atrophy-related genes, *MuRF-1*, and *Atrogin-1* expression in gastrocnemius and tibialis anterior from normal control and DSS-induced colitis mice treated with phosphate-buffered saline or IL-12/23 p40 neutralizing antibody. (D) Muscle fibre type-specific myosin heavy chain gene expression in gastrocnemius and tibialis anterior. (E) The evaluation of muscle function by grip strength (grams) and fatigue distance (metres). Each symbol represents an individual mouse; bars show the mean ± SD. *$P < 0.05$, **$P < 0.005$, ***$P < 0.001$, ****$P < 0.0001$, by Bonferroni's post hoc test after one-way analysis of variance. ns, not significant.

subunit. IL-23 comprises IL-23 p19 and IL-12/23 p40. It signals through IL-12Rβ1 and IL-23R. IL-12 stimulation of JAK2 and TYK2 activity can induce the phosphorylation of STAT4 and other STAT molecules. IL-23 can also activate the JAK–STAT pathway, although it primarily acts on STAT3.[16] Because IL-12 and IL-23 have subunit p40 in common, simultaneous blockade of IL-12 and IL-23 through p40 targeting can effectively inhibit inflammatory colitis.[17] Therefore, the IL-12/23 p40 pathway is one of the most important pathological mechanisms of inflammatory colitis. It directly or indirectly affects muscle loss in inflammatory colitis patients. To confirm the direct role of the IL-12/23 pathway in sarcopenia, we first determined expression levels of IL12 and IL23 receptors in muscles of osteosarcopenia patients by RT-qPCR. Expression levels of both *IL12Rb1* and *IL12Rb2* were increased in the muscles of sarcopenia patients, although only *ILRb2* showed a statistically significant increase in its expression. *IL23R* mRNA level was not detected by RT-qPCR (*Figure 4A*). It was found that only *Il12rb1* was significantly increased in the muscles of DSS-induced colitis mice (*Figure 4B*). The putative role of IL12 and IL23 on C2C12 myocyte differentiation was investigated by administration of recombinant protein of IL12 or IL23 during differentiation into myotubes. The induction of differentiation decreased the expression of receptors, *Il12rb1* and *Il12rb2*, but only significantly in *Il12rb1*. The expression of Il23r was not changed by induction of differen-

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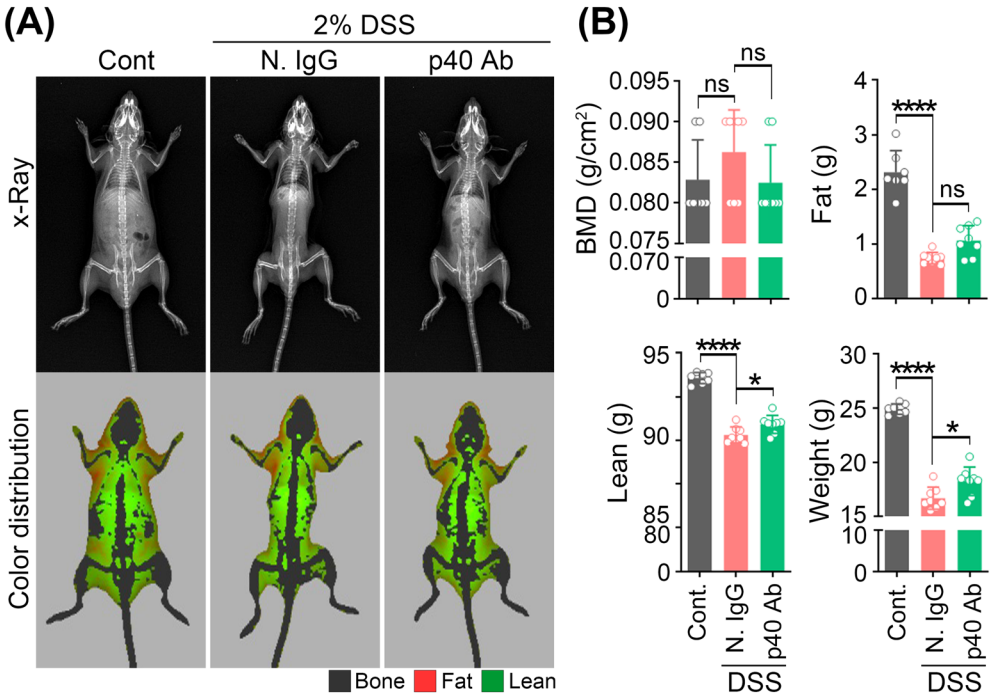

Figure 3 Analysis of body composition of a control mouse and 2% dextran sulfate sodium (DSS)-induced colitis mouse model. (A) Representative dual-energy X-ray absorptiometry images of normal control and experimental colitis model after injection of phosphate-buffered saline or p40 neutralizing antibody. (B) Average bone mineral density (BMD), fat tissue mass, lean tissue mass and body weight of each group. Each symbol represents an individual mouse; bars show the mean ± SD. $^{*}P < 0.05$, $^{**}P < 0.005$, $^{***}P < 0.001$, $^{****}P < 0.0001$, by Bonferroni's post hoc test after one-way analysis of variance. ns, not significant.

tiation. In the administration of recombinant protein of IL12 or IL23, the expression of receptors was increased mainly by treatment with IL23 recombinant protein (*Figure 4C*). Administration of the recombinant Il23 protein suppressed the expression of differentiation markers in C2C12 cells, and the expression of *Myod*, *Myog*, *Mef2c* and *Cdkn1a* was reduced by treatment with the recombinant IL23 protein at a concentration of 10 ng/mL (*Figure 4D*). The recombinant IL23 protein was found to inhibit the formation of myotubes. IL23 also decreased the average myotube diameter (17.63 μm ± 5.51 of a vehicle vs. 11.61 μm ± 3.13 of rIL23, $P < 0.0001$; *Figure 4E*), in agreement with the down-regulation of differentiation marker genes. To address the downstream signalling pathways of IL23 stimulation, we checked several signal molecules involving the pathway of myogenesis including mTOR, AMPK and STAT3, but not detected any activated signals (data not shown) except activation of Erk (extracellular signal-regulated MAPK). The activation of Erk signalling is an important pathway controlling cellular processes in muscle atrophy and was detected in the wasted muscle tissue of in vivo cachexia model and in vitro myofiber atrophy models.[18] The phosphorylation of Erk by IL23 increased until 30 min after treatment of recombinant IL23 protein in differentiated C2C12 cells and then decreased (*Figure 4F*). In order to confirm IL23 signal transduction by Erk, myoblast differentiation was confirmed by treatment with PD98059, an Erk inhibitor. The expression of differentiation markers decreased by the IL23 recombinant protein was increased by treatment with the Erk inhibitor, whereas the expression of the IL12/23 receptors, which were increased by the IL23, was decreased by the Erk inhibitor (*Figure 4G*).

Knockdown of Il12rb1 ameliorates the inhibition of C2C12 differentiation by IL23

To confirm the role of IL23 in myoblast differentiation, siRNA for *Il12rb1* was employed during C2C12 differentiation. After transfection of si-control or si-*Il12rb1*, C2C12 cells induced myotube formation with or without recombinant IL23 protein. As shown above, administration of recombinant IL23 protein resulted in a significant reduction in tube formation in si-control-transfected cells but ameliorated in the cells transfected si-*Il12rb1* in the presence of recombinant IL23 protein (*Figure 5A*). The average diameter of myotubes and distribution as size also did not decrease in si-*Il12rb1*-transfected and recombinant IL23-treated cells (*Figure 5B*). The gene expression of *Il12rb1* was ~35% of the si-control group by knockdown. The myogenic gene

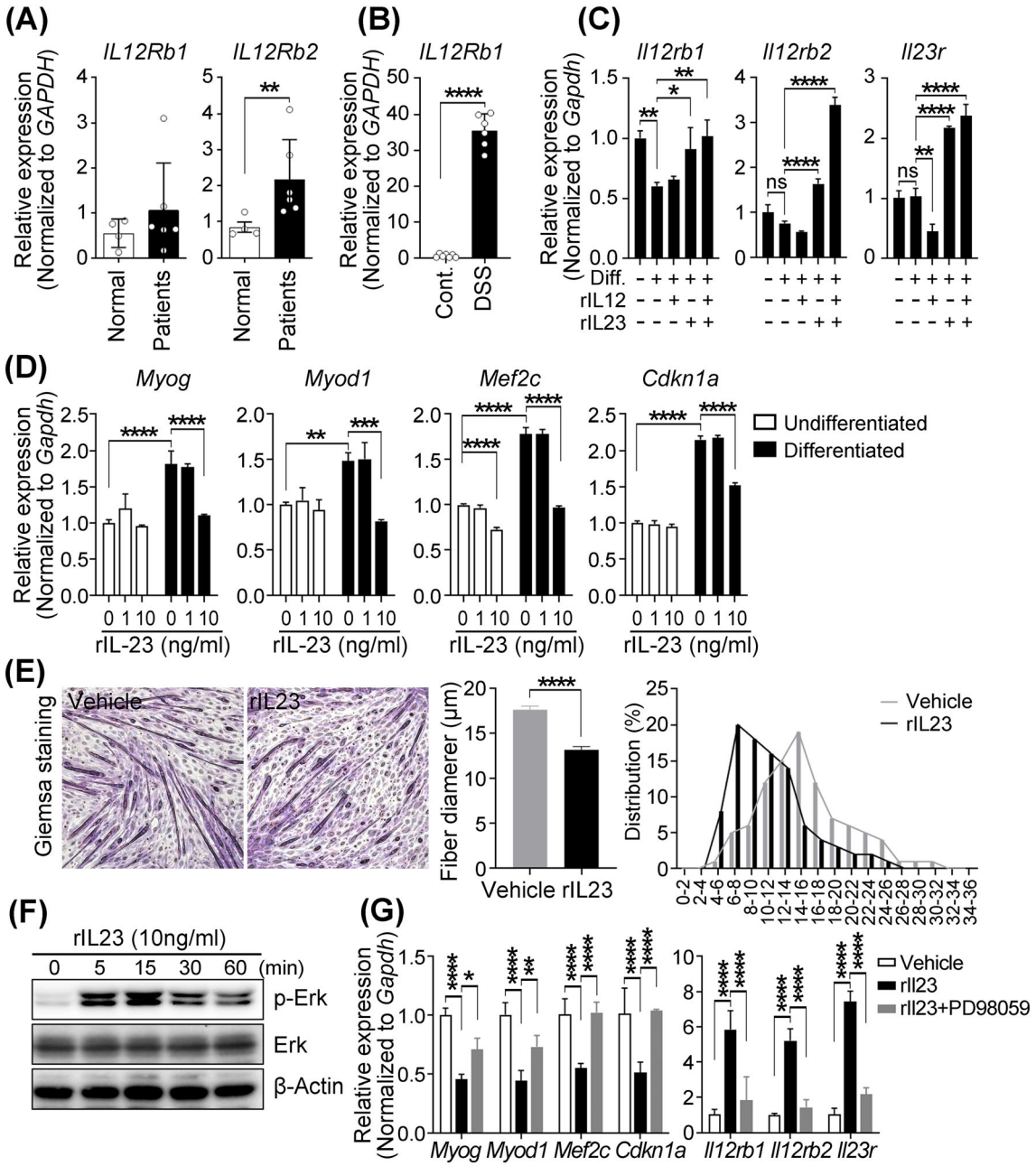

Figure 4 The change of muscle differentiation-related gene expression by treatment of recombinant Il-23 during differentiation of C2C12 myoblast cell line. (A) The expression of *IL12Rb1* and *IL12Rb2* in normal and sarcopenia patients. (B) The expression of *Il12rb1* in the tibialis anterior of control and dextran sulfate sodium (DSS)-induced colitis mice. (C) The expression of Il12 and Il23 receptors in undifferentiated or differentiated C2C12 cells treated with recombinant IL12 or IL23 protein. (D) Regulation of gene expression related to myoblast differentiation by recombinant IL-23 protein in C2C12 cell differentiation. (E) Representative images of Giemsa staining of myotube-forming differentiated C2C12 cells (left panels). C2C12 cells were differentiated with or without treatment of recombinant IL23 protein (10 ng/mL). The mean diameter of myotube (middle panel) and diameter distribution as size (right panel) of differentiated C2C12 after Giemsa staining. (F) Phosphorylation of Erk according to IL23 recombinant protein treatment time. (G) Regulation of gene expression related to myoblast differentiation by treatment of recombinant IL-23 protein and ERK inhibitor, PD98059 in C2C12 cell differentiation. Each symbol represents an individual mouse; bars show the mean ± SD. $*P < 0.05$, $**P < 0.005$, $***P < 0.001$, $****P < 0.0001$, by Bonferroni's post hoc test after one-way analysis of variance.

expression of *Myod*, *Myog*, *Mef2c* and *Cdkn1a* reduced by treatment with the recombinant IL23 protein was rescued by knockdown of Il12rb1 gene expression (*Figure 5C*). In addition, the phosphorylation of Erk increased by IL23 was also not increased by Il12rb1 knockdown (*Figure 5D*).

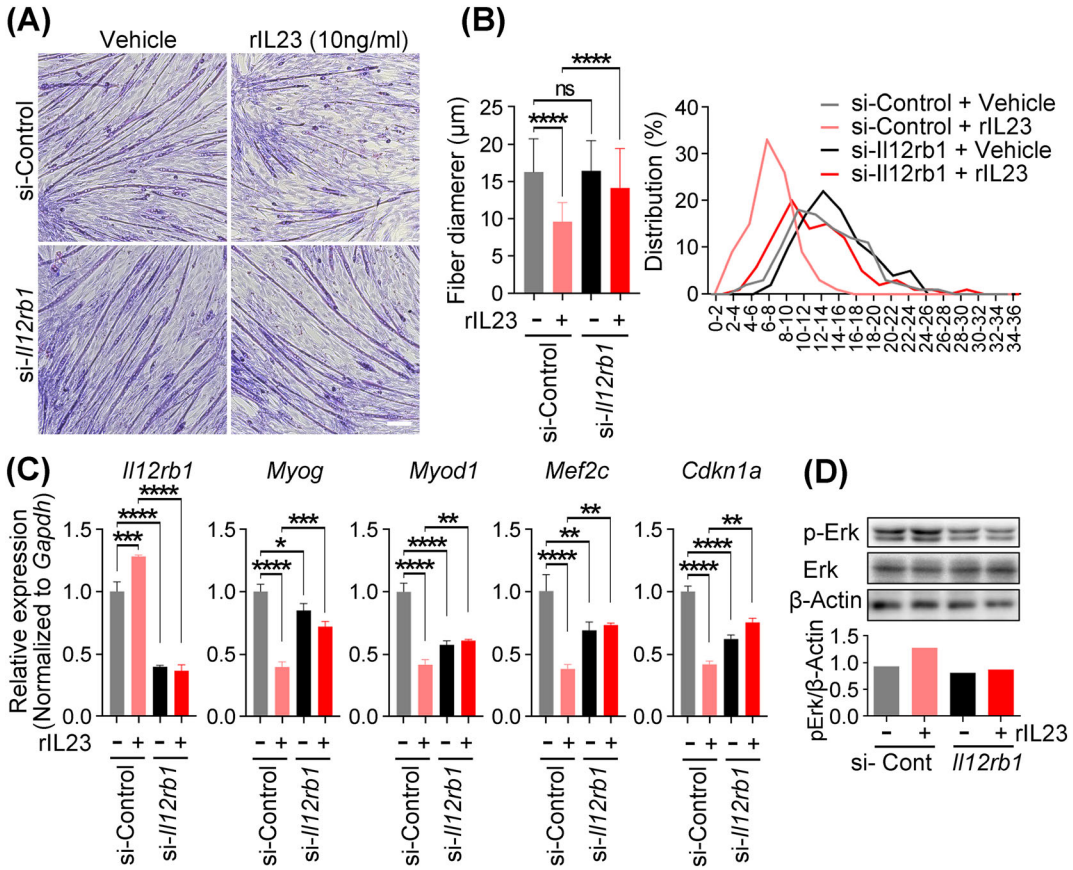

Figure 5 Effect of *Il12rb1* knockdown on the inhibited differentiation of C2C12 cells by IL23. (A) Representative Giemsa staining images of C2C12 cell myotube formation transfected with Il12rb1 siRNAs (si-Il12rb1) or scrambled siRNA (si-control) and treated with rIL23 (10 ng/mL). (B) The mean diameter of myotube and diameter distribution as size of differentiated C2C12 after Giemsa staining. (C) The expression of *Il12rb1* and myogenic genes after knockdown of *Il12rb1* and treatment of rIL23. (D) Phosphorylation of Erk after Il12rb1 knockdown and rIL23 treatment. Bars show the mean ± SD. $^{*}P < 0.05$, $^{**}P < 0.005$, $^{***}P < 0.001$, $^{****}P < 0.0001$, by Bonferroni's post hoc test after one-way analysis of variance. ns, not significant.

Discussion

To advance our knowledge of the pathophysiology of sarcopenia caused by inflammatory colitis, we investigated the molecular mechanisms of the role of IL-12/23 p40 in muscle and confirmed that p40 neutralizing antibodies can reduce sarcopenia.

As 42% of IBD patients show muscle damage, sarcopenia represents a common pathological feature of common chronic GI diseases, including IBD.[19] Additionally, most sarcopenia patients are accompanied by malnutrition as a result of chronic inflammation.[6] Malabsorption, which refers to food not properly absorbed through the small intestine, is the most common disease in IBD. It is directly related to the development of sarcopenia.[12] Intestinal inflammation can reduce the contact time between nutrients and the mucosal surface of the intestine, causing low amino acid absorption.[20] Sarcopenia is caused by a reduction in amino acid absorption, which is a primary anabolic signal in muscle.[12] In particular, a deficiency of leucine among all amino acids has a serious effect on muscle loss. The importance of leucine in sarcopenia has also been reported in a randomized controlled trial.[21]

Chronic inflammation is thought to be a major factor in IBD-related sarcopenia. Inflammatory cytokines such as interferon-gamma (IFNγ), IL-1 and IL-6 are associated with protein catabolism, which obstructs muscle protein synthesis.[22,23] Particularly, tumour necrosis factor-alpha (TNFα) can induce muscle protein degradation by inhibiting the anabolic mammalian target of the rapamycin complex 1 (mTORC1) pathway and stimulating atrogenes such as muscle atrophy box (MAFBx) and muscle ring finger-1 (MURF-1) that can increase muscle protein breakdown (MPB).[24,25] TNFα can also increase reactive oxidative stress and activate the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathway, which decreases myogenesis and causes further downstream inflammation.[26,27]

Drug interventions in individuals with IBD and sarcopenia have been reported in various studies. Several studies have shown the benefits of vitamin D supplementation in IBD

patients.[S1] The study by Das et al. has shown that vitamin D can improve muscle strength in postmenopausal women and healthy people between the ages of 18 and 40.[28] The effect of vitamin D on IBD-related sarcopenia in postmenopausal women and paediatric patients with IBD has been demonstrated.[29] However, data on such effects in elder people with IBD are insufficient. Biologic treatments such as anti-TNF drugs and newer anti-interleukin and anti-integrin agents such as briakinumab (IgG1 anti-p40 antibody), risankizumab (IgG1 anti-p19 antibody), brazikumab (IgG2 anti-p19 antibody), mirikizumab (IgG4 anti-p19 antibody) and guselkumab (IgG1λ anti-p19 antibody) have made significant advances in medical treatment for IBD.[S2–S6] K. Subramaniam et al. have shown that infliximab can reduce sarcopenia in CD patients.[S7] According to their study, muscle mass and strength were improved dramatically, whereas IL-6 levels were decreased significantly after 24 weeks of infliximab treatment.[S7] The major function of anti-TNF medicines is that they can reduce inflammation by inhibiting TNF, which can decrease catabolic signalling, apoptosis and cytotoxicity.[30] These medicines can reduce inflammation and minimize catabolic effects on skeletal muscle, which may facilitate the recovery of sarcopenia.[1]

The present study demonstrates that IL-12/23 p40 neutralizing antibody has a protective role in experimental colitis-induced muscle atrophy and muscle function. Several studies have suggested that IL-12 and IL-23 released from antigen-presenting cells can dysregulate T-cell activity causing CD.[S8,S9] Inhibition of IL-12/23 p40 might result in apoptosis of T cells in the gut mucosa, which can improve the disease.[S10] Therefore, the present findings imply that IL-12/23 p40 neutralizing antibodies might have a therapeutic effect in the treatment of IBD. Muscle fibres, the basic units of muscle, are classified according to the MHC isoforms as type I (slow-twitch, slow oxidation), type IIa (fast-twitch, fast oxidation), type IIx (fast-twitch, intermediate) and type IIb (rapid spasm, rapid glycolysis).[31] Recent studies have shown that muscle fibre type composition can be modulated in response to various external stimuli in a fibre type-specific fashion. Erk signalling is one of the fast-to-slow twitch transition stimuli.[32] In our study, Erk was activated by treatment of recombinant IL23 protein and increased myh7 gene expression in type I fibre dominant TA of colitis mice. Although we did not show direct evidence, it is possible that IL23 increased in colitis induces the activation of Erk in muscle, leading to a fast-to-slow twitch fibre transition of TA.

In conclusion, our study demonstrates that IL-12/23 p40 neutralizing antibody has a protective role against experimental colitis-induced muscle atrophy to improve muscle function.

Acknowledgements

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Conflict of interest

The authors declare no conflict of interest.

Online supplementary material

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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