

Interleukin-22 regulates the homeostasis of the intestinal epithelium during inflammation

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Abstract. Interleukin-22 (IL-22) has both pro-inflammatory and anti-inflammatory properties in a number tissues depending on the environment. Epithelial cells usually have a rapid turnover and are fueled by tissue stem cells. However, the question of whether IL-22 regulates tissue homeostasis through the modulation of stem cells remains unanswered. In this study, we investigated the role of IL-22 in the homeostasis of intestinal epithelial cells (IECs) during inflammation through a 3D organoid culture system. qPCR was performed to detect the changes in important gene transcriptions, and immunohistochemistry and western blot analysis were carried out to determine protein expression. As a result, we found that the expression of IL-22 was synchronously altered with the damage of the intestine. IL-22 treatment promoted cell proliferation and suppressed the cell differentiation of intestinal organoids. Surprisingly, IL-22 also led to self-renewal defects of intestinal stem cells (ISCs), thereby eventually resulting in the death of organoids. In examining the underlying mechanisms, we found that IL-22 activated signal transducer and activator

of transcription 3 (Stat3) phosphorylation and suppressed the Wnt and Notch signaling pathways. Importantly, Wnt3a treatment attenuated the organoid defects caused by IL-22, which consolidated the importance of Wnt pathway at the downstream of IL-22. Collectively, the findings of this study indicate that IL-22 regulates the homeostasis of the intestinal epithelium and is critical for the regeneration of the intestine during inflammation. Thus, the data of this study may provide a potential strategy and a basis for the treatment of diseases of intestinal inflammation in clinical practice.

Introduction

The intestinal epithelium forms a physical barrier that separates the luminal contents of the gut from the internal milieu (1). Intestinal epithelial cells (IECs) also perform several other functions, such as the sampling of the intestinal microenvironment, the sensing of both beneficial and harmful microbes and the secretion of numerous factors in response to positive or negative signals, which are crucial for intestinal homeostasis (2). To fulfill these diverse functions, the intestinal epithelium has unique anatomical and cellular adaptations, and IECs comprise several specialized cell types with distinct function (2,3). The dynamic crosstalk between IECs and local immune cells represents one of the basic features of intestinal homeostasis (2,3). These interactions are not only essential for maintaining homeostasis, but are also critical for the pathogenesis of inflammatory bowel disease (IBD) and intestinal epithelium restitution following injury.

Interleukin (IL)-22 belongs to the IL-10 cytokine family and is expressed by several types of lymphocytes, including CD4⁺ T cells, Th17 cells, natural killer (NK) cells and neutrophils (4-14). IL-22 binds to a heterodimeric receptor consisting of IL-22RA1 and IL10-RB. In contrast to the wide expression pattern of IL-10RB, IL-22RA1 is expressed by non-hematopoietic cells, such as epithelial cells of the gastrointestinal tract and skin (15-17). Thus, IL-22 can function in both lymphocytes and epithelial cells. In the intestine, IL-22 has been

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shown to prevent prolonged inflammation in models of dextran sodium sulfate (DSS)-mediated colitis and graft versus host disease (13,18,19). On the contrary, IL-22 can induce inflammatory responses in the other intestine models (20). These findings indicate that IL-22 has dual functions of promoting and antagonizing inflammation, which likely depends on the specific context. To date, these complex roles of IL-22 are not yet fully understood.

It is well known that intestinal stem cells (ISCs) are located at the crypt base and specifically express markers, such as leucine-rich repeat-containing G-protein coupled receptor 5 (Lgr5) (21). They play significant roles in intestinal homeostasis and regeneration. An *ex vivo* 3D culture system of intestinal organoids (epithelial mini-guts), highly mimicking the growth *in vivo*, has been successfully established by Clever's laboratory. This was done by culturing intestinal epithelial organoids in Matrigel and supplementing epidermal growth factor (EGF), activators of the Wnt and Notch pathways, and an inhibitor of the bone morphogenetic protein (BMP) pathway (22). This powerful system has been widely used for the study of intestinal epithelium homeostasis (22-26).

In this study, we applied DSS and X-ray induced colitis mouse models, and an *ex vivo* organoid culture system to explore the role of IL-22 in regulating the homeostasis of the intestinal epithelium during inflammation. We found that IL-22 was dynamically expressed with the development of intestinal inflammation. IL-22 promoted cell proliferation, but inhibited the differentiation of intestinal organoids. It also suppressed the self-renewal capacity of ISCs and accordingly resulted in the destruction of organoids. Mechanistically, IL-22 activated signal transducer and activator of transcription 3 (Stat3) activity, but suppressed the Wnt and Notch signaling pathways, in which the change in Wnt signaling was more dominant.

Materials and methods

Ethics statement. All animal experiments conformed to the regulations drafted by the Association for Assessment and Accreditation of Laboratory Animal Care in Shanghai and were approved by the East China Normal University Center for Animal Research.

Animal experiments. For the model of IBD, a total of 35 male (n=5/group) C57BL/6 mice (8 weeks old, weighing approximately 20 g) were obtained from Shanghai SLAC Laboratory Animal Co., Ltd. (Shanghai, China) and maintained under SPF experimental animal conditions (temperature, 23±2°C; humidity, 65±5%) in a 12-h light/dark cycle and fed a standard laboratory diet and water in East China Normal University Center for Animal Research. Acute colitis was induced at day 0 by the administration of 2.5% (w/v) DSS (molecular weight, 36-50 kDa; MP Biomedicals, Santa Ana, CA, USA) in their drinking water and this was then changed to normal drinking water at day 5. The mice were sacrificed at different time points as indicated (days 0, 2, 4, 6, 8, 10 and 12) and intestinal samples were collected for RNA extraction or histological analysis.

For *in vivo* regeneration assay, a total of 20 male (n=5/group) C57BL/6 mice (8 weeks old, weighing approximately 20 g) were obtained from Shanghai SLAC Laboratory Animal Co., Ltd.

and kept under SPF experimental animal conditions (temperature, 23±2°C; humidity, 65±5%) in a 12-h light/dark cycle and fed a standard laboratory diet and water in East China Normal University Center for Animal Research. The mice were irradiated by 9.0 Gy X-ray using a Biological X-ray irradiator (RS 2000 series; Rad Source Technologies, Inc., Suwanee, GA, USA) operated at 250 kVp, 15 mA. The mice were sacrificed every other day after irradiation by cervical dislocation.

Organoid culture. Small intestinal crypt isolation and culture were performed as previously described (21,22). Briefly, the small intestine was washed in cold PBS. The tissue was then chopped into approximately 3-5-mm-thick sections, washed with cold PBS and incubated in 5 mM EDTA in PBS for 30 min on ice. The tissue fragments were suspended vigorously, and the supernatants, including most of the villi were discarded. The sediment was further resuspended with cold PBS; the yielded supernatants enriched in crypts were collected and filter through a 70 µm cell mesh (BD Biosciences, San Jose, CA, USA). The crypts were then pelleted by centrifugation (132 x g, 5 min), suspended in ADF medium (advanced DMEM F12) (Thermo Fisher Scientific, Waltham, MA, USA) containing 1% GlutaMAX (Thermo Fisher Scientific). The crypts were centrifuged at low speed (33 x g, 3 min) for 2-3 times to remove single cells. The final crypts were resuspended with growth factor reduced to Matrigel (356231; BD Biosciences) at 5-10 crypts per µl, followed by seeding on a 96-well plate (5 µl per well) or 48-well plate (30 µl per well). Culture medium was made by ADF supplemented with penicillin/streptomycin, 10 mM HEPES, 1X GlutaMAX, 1X N2, 1X B27 (all from Thermo Fisher Scientific) and 1 µM N-acetylcysteine (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) containing 50 ng/ml Murine EGF, 100 ng/ml Murine Noggin and 1 µg/ml Human R-spondin-1 (all from PeproTech, Rocky Hill, NJ, USA). The medium was changed every other day.

For single cell culture, 4-6-day cultured organoids were dissociated with TrypLE express (Thermo Fisher Scientific) including 2,000 U/ml DNase (Roche Diagnostics, Indianapolis, IN, USA) for 10 min at 37°C. Dissociated cells were passed through a 20 µm cell mesh (BD Biosciences) and embedded in Matrigel containing 1 µM Jag1 (AnaSpec, Fremont, CA, USA) at up to 5,000-10,000 cells per 30-50 µl. The culture medium was regular organoid culture medium supplemented with 10 µM ROCK inhibitor Y27632 (Sigma-Aldrich; Merck KGaA).

For rescue experiments, 100 ng/ml IL-22 (R&D Systems, Minneapolis, MN, USA), 200 ng/ml Wnt3a (R&D Systems), 1 µM Jag1 (AnaSpec), 5 µM WP1066 (Stat3 inhibitor; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), 3 µM Stattic (Stat3 inhibitor; Santa Cruz Biotechnology, Inc.) were used at different combinations in organoid culture medium: Vehicle (without IL22), Stattic, WP1066, Wnt3a, Jag1, IL-22, IL-22 plus Stattic, IL-22 plus WP1066, IL-22 plus Wnt3a, IL-22 plus Jag1.

Reverse transcription-quantitative PCR (RT-qPCR). RNA from the organoids, intestines (IECs) and immune cells [macrophages, monocytes, CD4⁺ T cells and dendritic cells (DCs)] were sorted from mouse peripheral blood by corresponding magnetic beads; Miltenyi Biotec, Bergisch Gladbach,

Germany] was obtained by tissue homogenization using TRIzol reagent (Takara Bio, Inc., Otsu, Japan), followed by RNA precipitation in isopropanol, resuspension in DEPC-treated water and verification as DNA free by PCR. cDNA was synthesized with the Superscript RNase H2 Reverse Transcriptase kit (Takara Bio, Inc.) using 2.5 mM random hexamers (Takara Bio, Inc.). Quantitative (real-time) PCR (qPCR) was performed using the SYBR-Green PCR kit (Takara Bio, Inc.) on a Mx3005P thermal cycler (Stratagene, San Diego, CA, USA). The amplification conditions included 3 processes, namely hold stage (1 cycle): 95°C for 1 min; PCR stage (40 cycles): 95°C for 5 sec, 55°C for 30 sec; melt curve stage (1 cycle): 95°C for 5 sec, 55°C for 30 sec and 95°C for 30 sec. The sequences of the main primers were as follows: *β-actin* sense, CAGCCTTCCTTCTTGGGTAT and antisense, TGATCTTGATCTTCATGGTGC; *Il-22* sense, ACATCG TCAACCGCACCTTT and antisense, CAGCCTTCTGAC ATTCTTCTGGAT; *Il-22ra1* sense, CTACGTGTGCCGAGT GAAGA and antisense, GGTAGGTTGCAGGATGGAGA; *Il-10rb* sense, TTCGGAGTGGGTCAATGTCA and antisense, GACCTCAGAGTCGTACGGAG; *Lgr5* sense, CCTACTCGA AGACTTACCCAGT and antisense, GCATTGGGGTGA ATGATAGCA; olfactomedin 4 (*Olfm4*) sense, TCTTGGGCA GAAGGTGGGACT and antisense, GGACCGTCAGGTTCA GGAGC; achaete-scute family bHLH transcription factor 2 (*Ascl2*) sense, AAGCACACCTTGACTGGTACG; and anti-sense, AAGTGGACGTTTGCACCTTCA; zinc and ring finger 3 (*Znrf3*) sense, CCAGTGGTGTATGTGAAGGGTG and antisense, AATTCTGACTACGACGTTGCT; WD repeat domain 43 (*Wdr43*) sense, GCATACAGTGGCATCAAGAC and antisense, GTGAACCTAAGCGACGAAA; Prominin 1 (*Prom1*) sense, CTGAAGATTGCCCTCTATGA and anti-sense, AGTTTCTGGGTCCCTTTGAT; Axin2 sense, TGA CTCTCCTTCCAGATCCCA and antisense, TGCCCACAC TAGGCTGACA; and EPH receptor B3 (*Ephb3*) sense, GAC CTTGCTGCCCCGAAACAT and antisense, CCCACATGA CAATCCCATAGCT; *Mus musculus* mucin 2 (*Muc2*) sense, ATGACGTCTGGTGGAAATGGT and antisense, TGTCT GACAGTTGCACGTG; *Mus musculus* SAM pointed domain containing ets transcription factor (*Spdef*) sense, TGAACA TCACAGCAGACCCT and antisense, ACTTCCAGATGT CCAGGTGG; Chga chromogranin A (*Chga*) sense, ACTTCA AGACCTGGCTCTCC and antisense, CCTAGGTCCCTC TGTGGTTG; *Mus musculus* neurogenin 3 (*Nuero3*) sense, ACACAACAACCTTTTCCCGG and antisense, CCCGAT CATTGGCCTTCTTG; *Mus musculus* caudal type homeobox 1 (*Cdx1*) sense, AAGGGTTCAGAGTGAGGTG and anti-sense, TAGGGCAGGTGAAAGTGAGG; *Mus musculus* caudal type homeobox 2 (*Cdx2*) sense, CTCTCCTCTACCCA CGAAC and antisense, TGGGGCAAAGAGGCTGATA; *Mus musculus* lysozyme 1 (*Lyz1*) sense, GCTTCTACTGCA GCCATTC and antisense, GCTGACTGACAAGGGAGACT; *Mus musculus* SRY (sex determining region Y)-box 9 (*Sox9*) sense, CAAGAACAAGCCACACGTCA and antisense, GTG GTCTTCTTGTGCTGCA; *Mus musculus* defensin, alpha, 29 (*Defa29* or *Crs1c*) sense, AAAGAAGGTTTCCGTGGTGC and antisense, TGAGGCTAAGCACAATGGGA; *Mus musculus* defensin, alpha, 5 (*Defa5*) sense, ATTTGTCCTCTCTCTG CCC and antisense, ACGCGTTCTTCTTTTGCA; *Axin2* sense, TGA CTCTCCTTCCAGATCCCA and antisense, TGC

CCACACTAGGCTGACA; cyclin D1 (*Ccnd1*) sense, AGT TCATTTCCAACCCACCC and antisense, AGACCAGCC TCTTCCTCCAC; transcriptional regulator Myc-like (*C-myc*) sense, CCCTACCCGCTCAACGACAG and antisense, TTG CCTCTTCTCCACAGACACC; Eph receptor B3 (*Ephb3*) sense, GACCTTGCTGCCCCGAAACAT and antisense, CCC ACATGACAATCCCATAGCT; Eph receptor B2 (*Ephb2*) sense, GGCTTTACCTCTTTTCGACG and antisense, CAA TCCCCTTTTCTTCC; wingless-type MMTV integration site family, member 3A (*Wnt3a*) sense, GCACCACCGTCA GCAACAGC and antisense, TCCCTGGCATCGGCAAA CTC; *Notch1* sense, AATGGAGGGAGGTGCGAAGT and antisense, CAGAGGTGTCAGGCAGAGGG; hes family bHLH transcription factor 1 (*Hes1*) sense, GGAGAAGAG GCGAAGGGCAAGA and antisense, CGTGGACAGGAA GCGGGTCA; jagged 1 (*Jag1*) sense, AATGGTTATCGC TGTATCTGTCC and antisense, AGTTCTTGCCCTCAT AGTCCTC; delta like canonical Notch ligand 1 (*Dll-1*) sense, CCGATGACCTCGCAACAGAA and antisense, CCAGGG TCGCACATCTTCTC. *β-actin* served as the control gene, all reactions were performed in triplicate. The relative expression was calculated using the $2^{-\Delta\Delta C_q}$ method (27).

Histological analysis. The intestines were dissected and flushed gently with cold PBS. They were then immediately fixed by 4% PFA in PBS or snap-frozen on dry ice. Frozen samples were stored at -80°C until cut into frozen slices using a cryostat microtome. Fixed intestines were dehydrated by gradient alcohols, embedded in paraffin, and cut into 4- μ m-thick sections which were then subjected to H&E staining or immunohistochemistry (IHC).

Organoid histological staining was carried out with the sections or whole-mount sections. For the sections, organoids were collected after Matrigel was melted by Cell Recovery Solution (BD Biosciences). The organoids were then dehydrated and embedded in paraffin. The sections were deparaffinized in xylene and rehydrated in gradient alcohols. Endogenous peroxidase activity was quenched with 3% H₂O₂ in methanol for 20 min and washed in PBS. Antigen retrieval was performed by boiling the slides in 10 mM sodium citrate buffer, pH 6.0 or 10 mM Tris-base, 1 mM EDTA solution, pH 9.0. The sections were blocked with 1% BSA and incubated with the primary antibodies as listed below overnight at 4°C. For IHC staining, following incubation with secondary antibody (HRP labeled polymer, GK500710; Shanghai Genetech Co., Shanghai, China) for 30 min, the sections were developed with DAB and counterstained with hematoxylin, then dehydrated and mounted in neutral resins. For BrdU staining, before fixture, the organoids were treated with 3 μ g/ml BrdU for 2 h. A similar procedure was performed with the addition of a 30-min treatment with 2 N HCl after antigen retrieval. For immunofluorescence (IF), the sections, following primary antibody as listed below incubation overnight at 4°C were washed and incubated with secondary Alexa Fluor 488 (1:500 dilution; cat. no. ab150077; Abcam, Cambridge, MA, USA) or 647 antibodies (1:500 dilution; cat. no. ab150115; Abcam) for 2 h at room temperature with DAPI (1:10,000 dilution; cat. no. ab104139; Abcam) counterstaining for 5 min at room temperature (Thermo Fisher Scientific) and subjected

to immunofluorescence microscopy (BX53; Olympus Corporation, Tokyo, Japan).

For the whole-mount sections, the organoids were fixed with 4% PFA in a plate without melting the Matrigel, then permeabilized with 0.2% Triton in PBS and incubated overnight with primary antibodies as listed below. Fluorescence was detected as mentioned above.

The primary antibodies were as follows: Cleaved caspase-3 (1:200; cat. no. 9661; Cell Signaling Technology, Danvers, MA, USA), Ki67 (1:2,000; cat. no. ab873; Abcam), Lysozyme (Paneth cell marker; 1:2,000; cat. no. ab108508; Abcam), Chromogranin A (endocrine cell marker; 1:2,000; cat. no. 20085; ImmunoStar, Hudson, WI, USA), Muc2 (goblet cell marker; 1:100; cat. no. sc-59859; Santa Cruz Biotechnology, Inc.), phosphor-Stat3 at Tyr705 (1:200; cat. no. 4113; Cell Signaling Technology), BrdU (1:500; cat. no. B8434; Sigma-Aldrich; Merck KGaA) and IL-22RA1 (1:400; cat. no. ab211675; Abcam).

Flow cytometry. *Lgr5-EGFP-IRES-creERT2* mice (Stock no. 008875) were obtained from The Jackson Laboratory (Bar Harbor, ME, USA) and a total of 5 male, *Lgr5-EGFP-IRES-creERT2* mice (8 weeks old, weighing approximately 20 g) were kept under SPF experiment animal conditions (temperature, 23±2°C; humidity, 65±5%) in a 12-h light/dark cycle and fed a standard laboratory diet and water in East China Normal University Center for Animal Research. Endogenous GFP (green) driven by *Lgr5* promoter indicates crypt base columnar stem cells in the small intestine. Crypts from the *Lgr5-EGFP-IRES-CreERT2* mice were isolated as described above after the mice were sacrificed and organoid culture was performed. Following treatment with 100 ng/ml recombinant mouse IL-22 (R&D Systems, Inc., Minneapolis, MN, USA), organoids were collected and dissociated with TrypLE and 2,000 U/ml DNase for 15 min at 37°C. The dead cells were then excluded by scatter characteristics and propidium iodide. *Lgr5*⁺ISCs were identified by their endogenous GFP expression.

Western blot analysis. Organoids were collected after Matrigel melting. The cells were then lysed with 0.5% NP-40 lysis buffer [50 mM Tris-base (pH 7.4), 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS and Complete Mini Protease Inhibitor mixture (Roche Diagnostics)] for western blot analysis. The protein concentration was determined using a BCA Protein Assay kit (cat. no. p0011; Beyotime, Shanghai, China). The proteins (30 µg each sample) were resolved on 12% SDS-PAGE gels, transferred onto PVDF membranes (EMD Millipore, Billerica, MA, USA). Following blocking with PBS containing 3% skim milk, the membranes were incubated overnight at 4°C with primary antibodies as follows: Anti-cleaved caspase-3 (1:1,000; cat. no. 9661), anti-Bax (1:1,000; cat. no. 2772), anti-Bcl2 (1:1,000; cat. no. 3498), anti-Bcl-xL (1:1,000; cat. no. 2762), anti-Stat3 (1:1,000; cat. no. 9139), anti-phospho-Stat3 (Tyr705; 1:1,000; cat. no. 9145) (all from Cell Signaling Technology) and anti-β-actin (1:1,000; cat. no. A1978; Sigma-Aldrich; Merck KGaA). Following incubation with peroxidase AffiniPure secondary antibodies (goat anti-mouse IgG, cat. no. 115-035-003; goat anti-rabbit IgG, cat. no. 111-035-003; Jackson ImmunoResearch Laboratories,

Inc., West Grove, PA, USA) for 1 h at room temperature, the blots were then analyzed using the ECL detection system or scanned with an Odyssey Infrared Imager (LI-COR Biosciences, Lincoln, NE, USA).

Enzyme-linked immunosorbent assay (ELISA). The colons from mice with DSS-induced colitis were obtained and completely homogenized in PBS containing proteinase inhibitors. Following centrifugation at 825 x g/min for 20 min, the supernatant was collected. The level of secreted IL-22 from IECs was measured using a mouse IL-22 ELISA kit (Abcam) following the manufacturer's instructions. The experiment was repeated 3 times.

Statistical analysis. Data were statistically analyzed by one-way ANOVA or the Student's t-test. A value of P<0.05 considered to indicate a statistically significant difference. Results are presented as the means ± standard error of the means (SEM).

Results

IL-22 is dynamically expressed during intestinal inflammation. We first detected the gene transcription of *IL-22RA1* and *IL-10RB*, heterodimeric receptors of IL-22, in both immune cells and IECs by RT-qPCR. While *IL-22RA1* was specifically expressed in epithelial cells, *IL-10RB* expression was found in both immune and IECs (Fig. 1A). The results of immunohistochemistry revealed that *IL-22RA1* was expressed in the epithelium of the colon and intestine (Fig. 1B). These data suggest IL-22 signaling functions in the intestinal epithelium.

We then examined IL-22 expression in the colon in a mouse model of DSS-induced colitis. Histological analysis revealed that damage to the intestinal epithelium gradually increased, being most severe on day 6 (1 day after DSS administration), and were recovered from day 8 to day 12 (3rd day to the 7th day after DSS administration) (Fig. 1C). Notably, IL-22 expression was consistently altered with the development of colitis. It reached maximum levels on day 6 following DSS administration and decreased during the regeneration of the intestinal epithelium (Fig. 1D and E). In another mouse model of irradiation, we observed similar results (Fig. 1F and G). Taken together, our results reveal that IL-22 expression is associated with the state of intestinal damage.

IL-22 regulates the formation of intestinal organoids. As it is well known that IL-22 promotes cell proliferation during tissue repair, we wished to explore the direct function of IL-22 in IECs. In an extra *in vitro* organoid culture system, we found that IL-22 treatment led the organoids acquiring a rounded shape and they began to degenerate after 3 days (Fig. 2A). On day 4, the buds of the organoids were significantly decreased and almost all organoids were disrupted in the IL-22-treated group (Fig. 2B-D).

Subsequently, to determine the mechanisms through which IL-22 regulates organoid formation, we assessed the proliferation and apoptosis of IECs following IL-22 treatment. IHC staining for BrdU and Ki67 revealed that the majority of the cells were in a proliferative state (Fig. 2E). By contrast, we failed to detect a marked difference in cell apoptosis between the vehicle (the organoid cultured without IL-22) and the

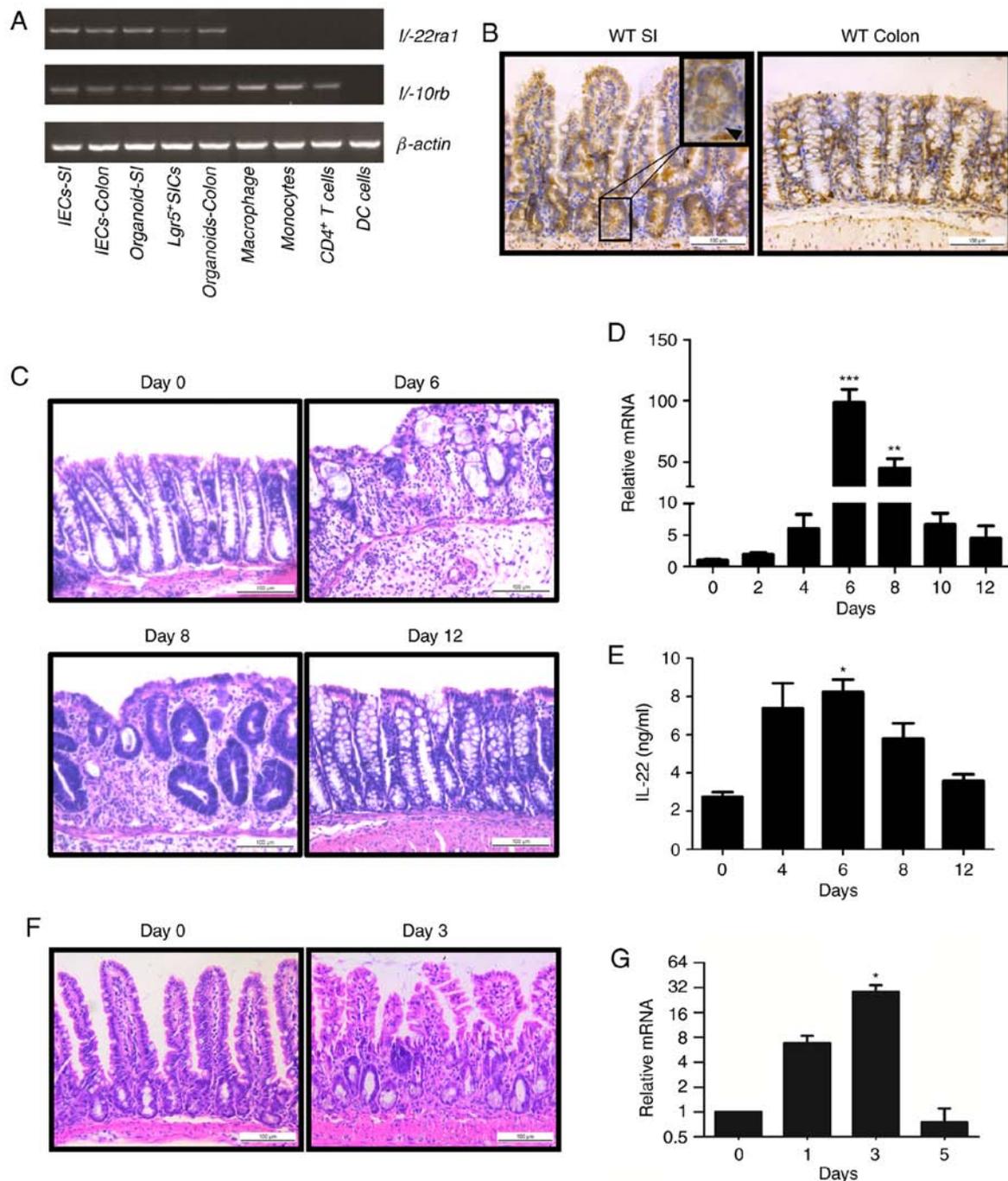


Figure 1. Interleukin (IL)-22 is dynamically expressed during intestinal inflammation. (A) The expression of IL-22RA1 and IL-10RB in different cell types was detected by RT-qPCR. IECs-SI indicates the intestinal epithelium cells from small intestine, immune cells containing macrophages, monocytes, CD4⁺ T cells and dendritic cells (DCs). (B) Immunohistochemical staining of paraffin-embedded sections of the small intestine and colon from wild-type (WT) mice by IL-22RA1 antibody (n \geq 3). Scale bar, 100 μ m. The inset is the magnification of one representative crypt and the black arrowhead points to the intestinal stem cell (ISC). (C) H&E staining of colon from WT mice at different time points during dextran sodium sulfate (DSS)-induced inflammatory bowel disease (IBD). Scale bar, 100 μ m. (D) The mRNA expression of IL-22 in colon during DSS-induced IBD in the animal model was determined by RT-qPCR. (E) The secreted IL-22 in the colon during DSS-induced IBD in the animal model was measured by ELISA. (F) H&E staining of the intestine from WT mice on days 0 and 3 after X-ray radiation. Scale bar, 100 μ m. (G) The mRNA expression of IL-22 in the intestine was examined by RT-qPCR in the X-ray model. Data are the means \pm SEM; *P<0.05, **P<0.01, and ***P<0.001 vs. day 0; analyzed by one-way ANOVA (n=5).

IL-22-treated samples, as determined by measuring the levels of apoptosis-related markers (Fig. 2E and F).

We then wished determine whether IL-22 affects the differentiation of IECs. The results of IF staining revealed that the numbers of different differentiated cell types, including Paneth cells, goblet cells and enteroendocrine

cells were significantly decreased following IL-22 treatment (Fig. 2G and H). In addition, the expression of specific markers of differentiated cells was suppressed by IL-22, as shown by RT-qPCR (Fig. 2I). Collectively, these results indicated that IL-22 regulates organoid formation through activation of cell proliferation and inhibition of IECs differentiation.

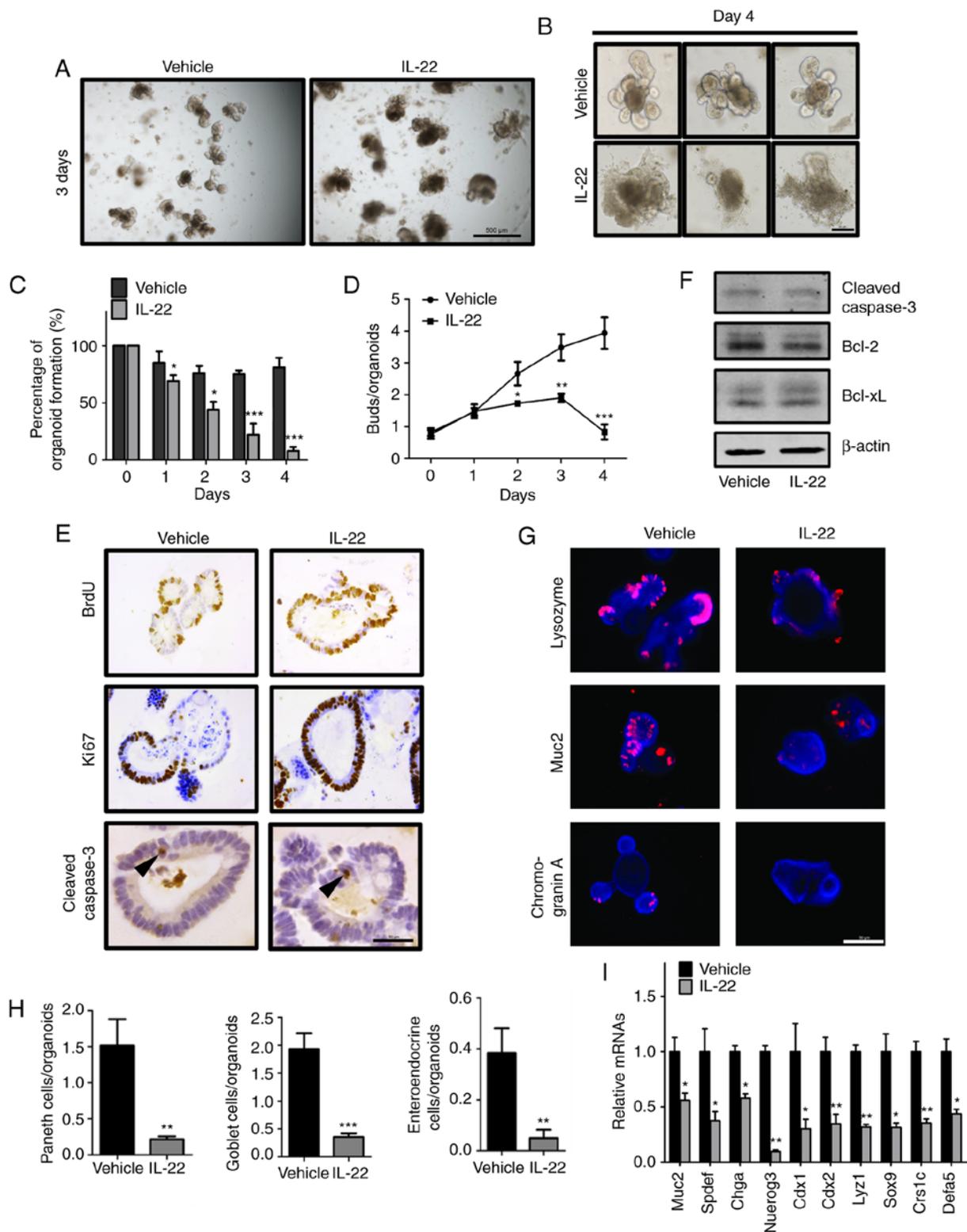


Figure 2. Interleukin (IL)-22 regulates growth of intestinal organoids. (A) Organoids were cultured for 2 days followed by treatment with the vehicle (the organoid cultured without IL-22, left panel) or IL-22 (100 ng/ml treatment group, right panel); representative images are shown on day 3 after treatment. Scale bar, 500 μ m. (B) Organoids were cultured for 2 days followed by treatment with the vehicle (upper panels) or IL-22 (100 ng/ml, bottom panels); representative 3 images are shown on day 4 after treatment. Scale bar, 50 μ m. (C and D) Organoids were cultured for 2 days followed by treatment with the vehicle or IL-22 (100 ng/ml). The percentage of (C) organoid formation and (D) the number of buds are shown at different time points after treatment. Data are the means \pm SEM; * P <0.05, ** P <0.01, and *** P <0.001 vs. the vehicle at the indicated time points; analyzed by t-test. (E) Organoids were cultured for 2 days followed by treatment with the vehicle or IL-22 (100 ng/ml). The expression of BrdU, Ki67 and cleaved caspase-3 was detected by immunohistochemical staining. Arrowheads indicate apoptotic cells. Scale bar, 50 μ m. (F) Immunoblotting of cleaved caspase-3, Bcl2 and Bcl-xL in the organoids treated with or without IL-22 for 2 days (100 ng/ml). (G) The expression of Lysozyme (Paneth cell marker), Muc2 (goblet cell marker) and chromogranin A (endocrine cell marker) was determined by immunofluorescence. Scale bar, 50 μ m. (H) Quantitative analysis of the data in (G) are presented. Data are the means \pm SEM; ** P <0.01 and *** P <0.001 vs. the vehicle; analyzed by t-test. (I) Organoids were cultured for 2 days followed by treatment with the vehicle or IL-22 (100 ng/ml). The expression of Muc2, Spdef (goblet cell marker), Chga, Nuerog3 (enteroendocrine cell marker), Cdx1, Cdx2 (columnar absorptive cell marker), Lyz1, Sox9, Crs1c and Defa5 (Paneth cell marker) was detected by RT-qPCR. Data are the means \pm SEM; * P <0.05, ** P <0.01, and *** P <0.001 vs. vehicle; analyzed by t-test.

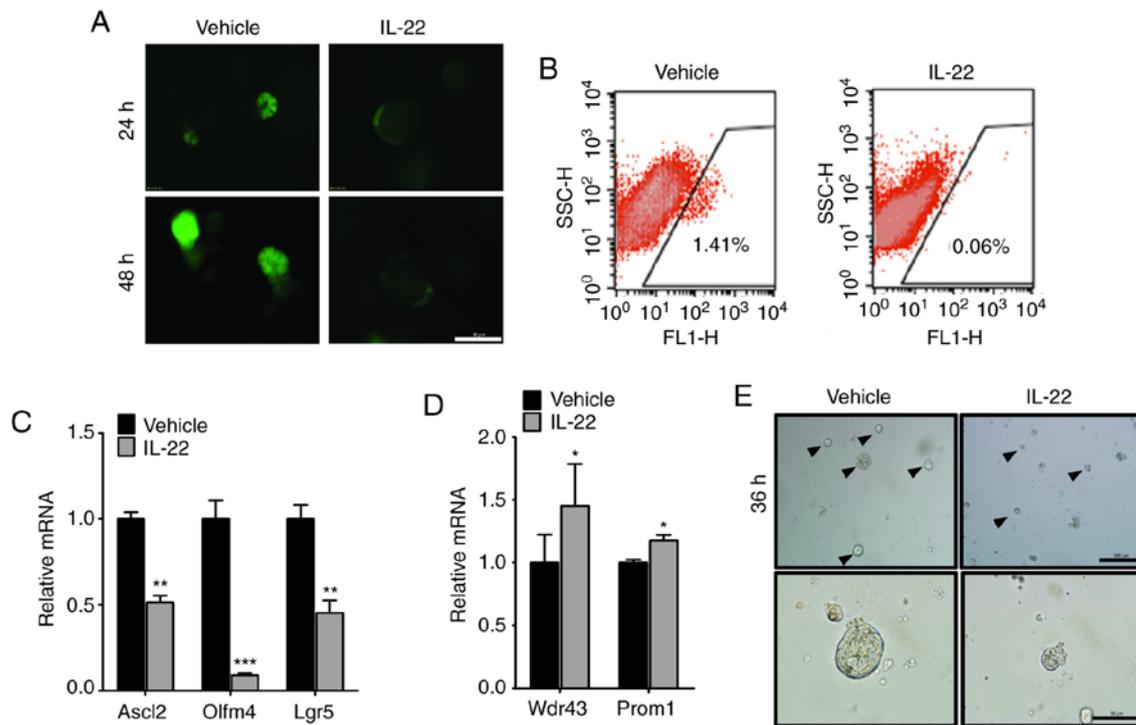


Figure 3. Interleukin (IL)-22 counteracts the self-renewal of intestinal stem cells (ISCs). (A) Organoids from *Lgr5-EGFP-IRES-creERT2* mice were treated with or without IL-22 (100 ng/ml); representative images of *Lgr5*-GFP-positive cells are shown. Scale bar, 50 μ m. (B) Organoids from *Lgr5-EGFP-IRES-creERT2* mice were treated with or without IL-22 (100 ng/ml) for 40 h, and they were then dissociated to single cells and analyzed by FACS. The percentage of *Lgr5*-GFP positive cells is shown. (C and D) RT-qPCR analysis of the expression of (C) ISC markers or (D) transit-amplifying (TA) cell markers in the organoids treated with or without IL-22 (100 ng/ml). Data are the means \pm SEM; * P <0.05, ** P <0.01 and *** P <0.001 vs. the vehicle, analyzed by t-test. (E) Single cells were dissociated from the organoids following culture for 4-6 days. The cells were then treated with or without IL-22 (100 ng/ml) for 36 h. Cysts formed from single ISCs are indicated (arrowheads). Scale bar, 500 μ m. The magnified images are shown at the bottom panels. Scale bar, 50 μ m.

IL-22 counteracts the self-renewal ability of ISCs. Paneth cells provide EGF, Notch and Wnt ligands to maintain ISC self-renewal ability, which is essential for organoid formation. Since IL-22 was found to inhibit the differentiation of Paneth cells, we wished to determine whether it also affects ISCs self-renewal. By isolating the organoids from *Lgr5-EGFP-IRES-creERT2* mice, we found that the number of ISCs, positive for *Lgr5*-EGFP, was markedly decreased by IL-22 (Fig. 3A and B). Consistently, the results of RT-qPCR revealed that the expression of stem cell markers, including *Ascl2*, *Olfm4* and *Lgr5* was significantly lower in the group treated with IL-22 (Fig. 3C). Moreover, the expression of *Wdr43* and *Prom1*, the transit-amplifying (TA) cells markers, was slightly upregulated by IL-22. This suggested that IL-22 promotes the differentiation into TA cells from *Lgr5*⁺ISCs (Fig. 3D).

To further validate the effects of IL-22 on the maintenance of ISC self-renewal, we dissociated the organoids into single cells and assessed the growth ability. We observed that the single cells could individually grow to a cyst-like structure and finally formed an organoid structure (Fig. 3E, arrowheads). On the contrary, these cells could not survive following culture for 3-4 days with the addition of IL-22 (Fig. 3E, bottom panels). Taken together, these data suggest that IL-22 counteracts the self-renewal ability of ISCs.

IL-22 promotes the phosphorylation of Stat3 and suppresses the Wnt and Notch signaling pathways. It has been reported

that IL-22 modulates the phosphorylation of Stat3 (18,28). In this regard, we assessed the changes in the levels of phosphorylated Stat3 following treatment of the organoids with IL-22. In the assay of immunofluorescence, we observed that the addition of IL-22 increased the level of phosphorylated Stat3 in the nucleus of the organoids (Fig. 4A and C). Consistently, similar results were got by immunoblotting (Fig. 4B). Moreover, in the organoids of *Lgr5-EGFP-IRES-creERT2* mice, increased levels of nuclear phosphorylated Stat3 were observed in the *Lgr5*-positive ISCs following treatment with IL-22 (Fig. 4C).

We then examined the activity of the Wnt and Notch signaling pathways, which are essential for ISC self-renewal and differentiation. It was found that both the Wnt and Notch signaling pathways were markedly suppressed by IL-22 (Fig. 4D and E). In summary, these results demonstrate that IL-22 activates the Stat3, and inhibits the Wnt and Notch signaling pathways.

IL-22 leads to defects in organoid formation mainly through the Wnt pathway. To understand the importance of signaling pathway changes caused by IL-22, we carried out rescue experiments. We applied WP1066 and Stattic as Stat3 specific inhibitors, and added *Wnt3a* and *Jag1* as respective ligands for the Wnt and Notch pathways.

In the absence of IL-22, these supplements did not affect the formation and survival of organoids (Fig. 5A and B). Notably, only the addition of *Wnt3a* recovered the defects in the organoids induced by the addition of IL-22 (43% vs.

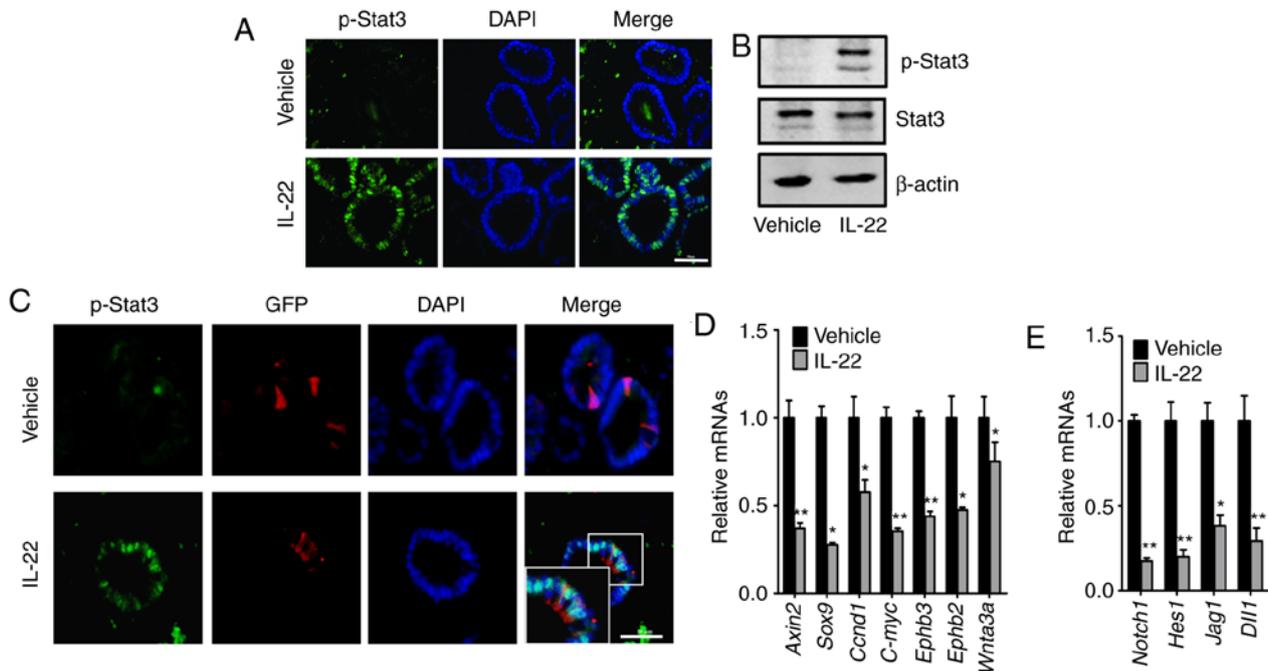


Figure 4. Interleukin (IL)-22 promotes the phosphorylation of Stat3 and suppresses the Wnt and Notch signaling pathways. (A) Organoids were cultured for 2 days followed by treatment with the vehicle or IL-22 (100 ng/ml). The expression of phospho-Stat3 at Tyr705 was detected by immunofluorescence. Scale bar, 50 μ m. (B) Western blot analysis of phosphor-Stat3 and total Stat3 in the organoids treated with either the vehicle or IL-22 for 40 h (100 ng/ml). (C) Organoids from *Lgr5-EGFP-IRES-creERT2* mice were treated with or without IL-22 (100 ng/ml) for 40 h. The expression of phosphor-Stat3 and GFP was assessed by immunofluorescence. Scale bar, 50 μ m. The inset is the magnification of representative positive staining. (D and E) The expression of target genes of (D) Wnt and (E) Notch signaling pathways was determined by RT-qPCR. Data are the means \pm SEM; * P <0.05, ** P <0.01 and vs. the vehicle; analyzed by t-test.

7%; Fig. 5A and B). Furthermore, we found that the number of Paneth cells was retrieved by Wnt3a, but not by Stattic, WP1066 and Jag1 (Fig. 5D). Notably, Stattic treatment significantly restrained IL-22-induced cell hyper-proliferation (Fig. 5C). This observation suggested that IL-22 promotes cell proliferation mainly through Stat3 activation. Consistently, the upregulation of Wnt target genes, including Axin2 and Ephb3, was confirmed by RT-qPCR following the addition of Wnt3a in IL-22-treated organoids (Fig. 5E). Moreover, the levels of ISC markers (*Lgr5*, *Olfm4* and *Znrf3*) were elevated as well. Taken together, these data suggest that IL-22 caused the deficiency of organoid formation mainly by suppressing the Wnt signaling pathway.

Taken together, in this study, we observed that the IL-22 level initially increased and gradually decreased during inflammation. Elevated IL-22 levels modulated the Stat3, Wnt and Notch pathways to accelerate the proliferation of TA cells, suppressing cell differentiation and causing the deficiency of ISC self-renewal (Fig. 6).

Discussion

It has been reported that IL-22 is a dual-natured cytokine depending on the context of inflammation. It has either pro-inflammatory or anti-inflammatory properties (13,29). In the majority of inflammatory bowel disease animal models (14,18,28,30,31), infectious disease (32-34) and graft versus host disease (GVHD) (35), IL-22 plays a protective role against inflammation. By contrast, in a Treg cell transferring animal model and during *Toxoplasma gondii*-induced

ileitis, IL-22 has been shown to play a pro-inflammatory function (20,36). In this respect, a more precise association between IL-22 and epithelial cells needs to be determined. In this study, we used an *ex vivo* organoid culture system to investigate the role of IL-22 in the homeostasis of intestinal epithelium during inflammation. The advantage of this system is that it can rule out the effects of other cells surrounding the IECs, such as mesenchyme cells and leukocytes. In this manner, we discovered a previously unknown direct association between IL-22 and the intestinal epithelium in that IL-22 promotes the proliferation but inhibits the differentiation of intestinal organoids. Moreover, it suppresses the self-renewal ability of ISCs and consequently leads to the death of organoids. These data reveal that IL-22 has more complex functions in IECs than what was previously known.

Following IL-22 treatment, we observed that the organoids became rounded without buds whose tips were enriched with ISCs and Paneth cells (21,22,37). In addition, fewer terminal differentiated cells were found in IL-22-treated organoids. It has been known that the downregulation of Wnt signaling suppresses EphB expression and thus impairs the ephrinB-EphB repulsion, which is critical for the crypt-villi axis establishment (38,39). Thus, the rounded structure caused by IL-22 treatment is likely attributed to the accelerated proliferation of IECs and the loss of migration axis, causing the cells to detach from the epithelial structures and to shed into the lumen.

In vivo, IL-22 expression was upregulated within 6-8 days of DSS-induced inflammation and on the 3rd day of X-ray radiation-induced inflammation; during this period, the intestine

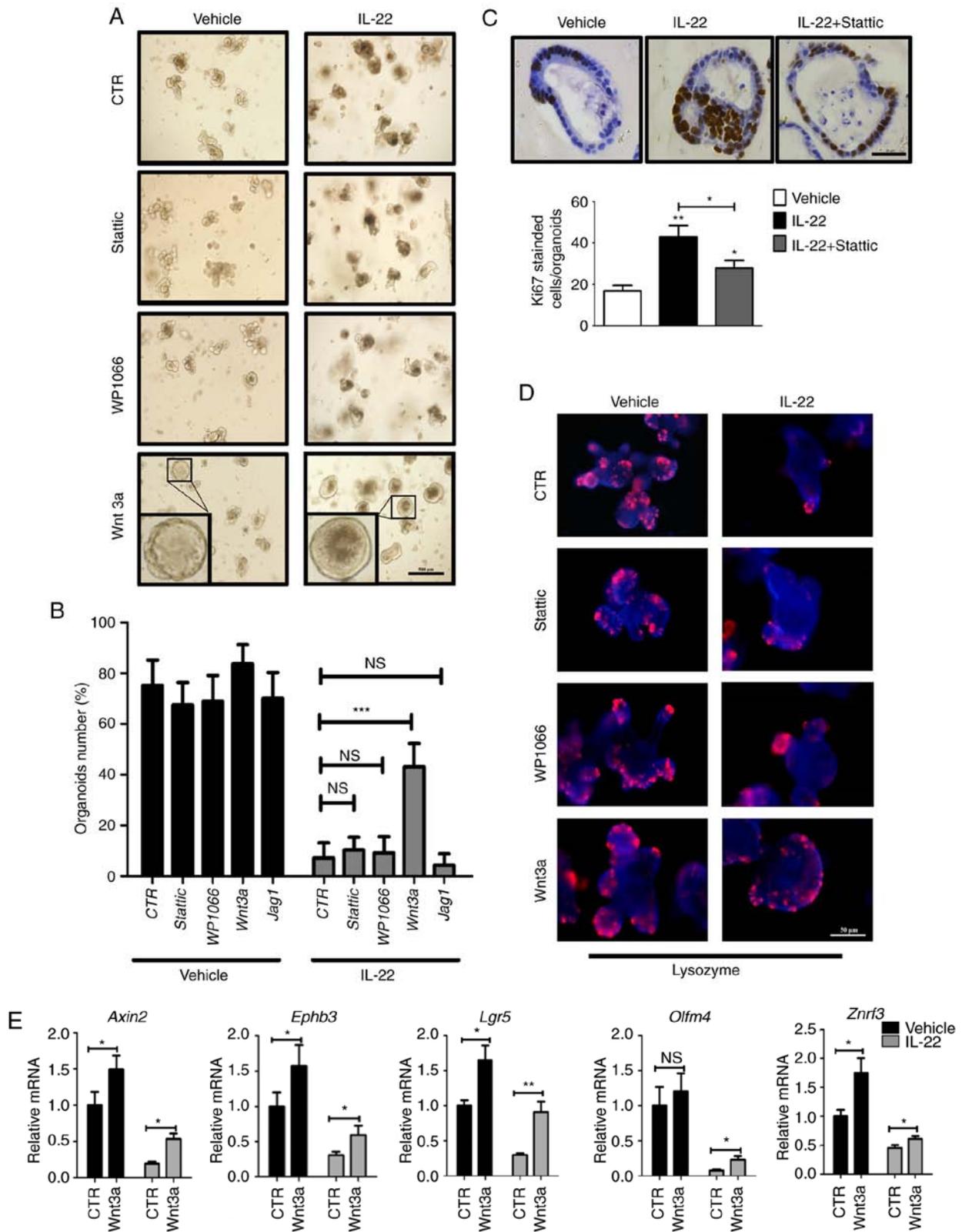


Figure 5. Interleukin (IL)-22 causes defects in organoid formation mainly through the Wnt pathway. (A) Organoids were cultured for 2 days followed by treatment with various combinations of IL-22 (100 ng/ml), Wnt3a (200 ng/ml), WP1066 (5 μ M) and Stattic (3 μ M). Scale bar, 500 μ m. The inset is the magnification of one representative organoid. (B) The number of organoids after 2 days of treatment with various combinations of IL-22, Wnt3a, WP1066, Stattic and Jag1 was counted. Data are the means \pm SEM; and *** P <0.001 vs. Control (100 ng/ml IL-22 treatment group); NS, not significant; analyzed by one-way ANOVA. (C) Organoids were cultured for 2 days followed by treatment with various combinations of IL-22 and Stattic. The expression of Ki67 was detected by immunohistochemical staining. Scale bar, 50 μ m. The quantitative analysis is presented at the bottom panel. Data are the means \pm SEM; * P <0.05, ** P <0.01 and vs. the vehicle; analyzed by one-way ANOVA. (D) Organoids were cultured for 2 days followed by treatment with various combinations of IL-22, Wnt3a, WP1066 and Stattic. The expression of Lysozyme (Paneth cell marker) was determined by immunofluorescence. Scale bar, 50 μ m. (E) Organoids were cultured for 2 days followed by treatment with various combinations of IL-22 and Wnt3a. The expression of ISC markers (Lgr5, Olfm4 and Znrf3) and Wnt target genes (Axin2 and Ephb3) was examined by RT-qPCR. Data are the means \pm SEM; * P <0.05, ** P <0.01 and vs. Control (the organoid cultured without IL-22) or Control (100 ng/ml IL-22 treatment group); analyzed by t-test.

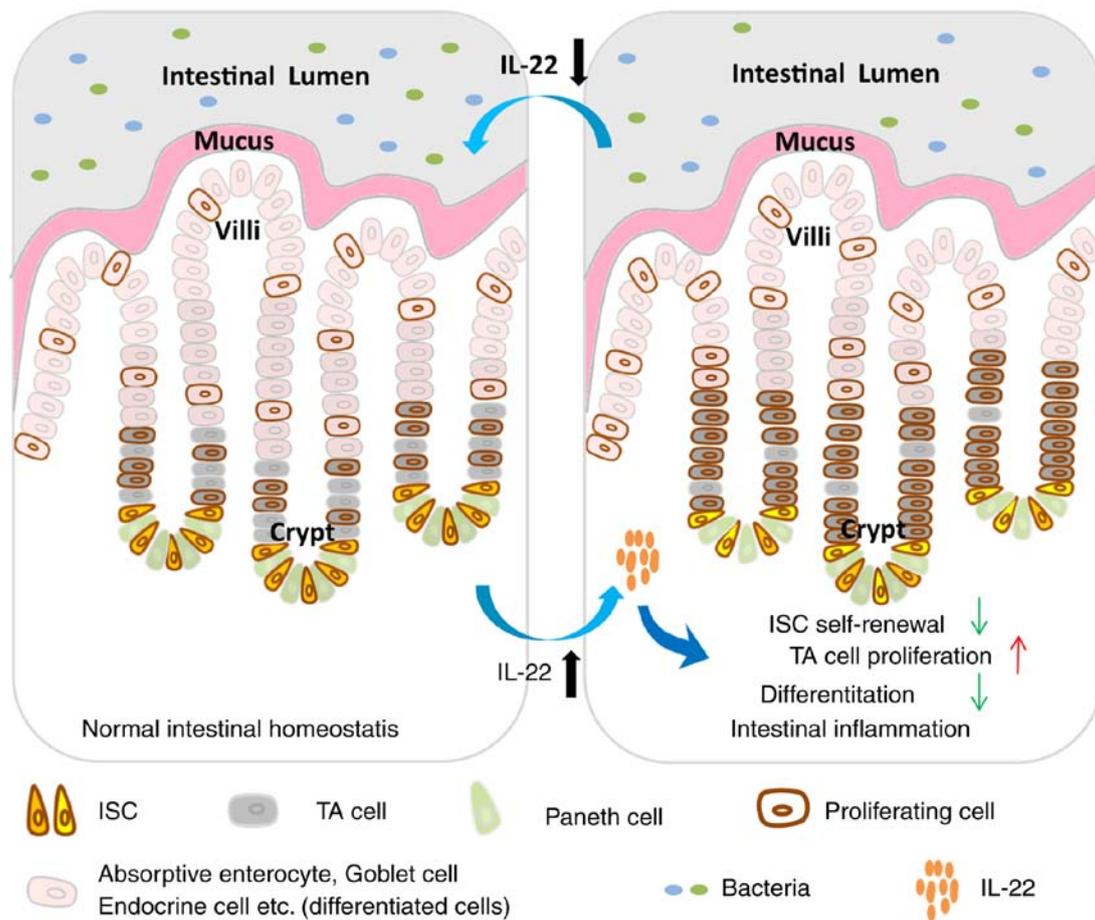


Figure 6. Interleukin (IL)-22 regulates the homeostasis of intestine epithelium during inflammation. During inflammation, elevated IL-22 promotes the proliferation of TA cells, represses cell differentiation and leads to the self-renewal defect of intestinal stem cells. Later, the level of IL-22 gradually reduces and the homeostasis of intestine epithelium is recovered.

incurs the most severe inflammatory response. We postulate that high level of IL-22 promotes anti-microbial peptide expression and alleviates the bacterial burden, which is critical for the initiation of intestinal regeneration (32,40,41). However, following bacterial clearance, the epithelium begins to recover and reconstitute; the limitation of IL-22 is important for IEC differentiation. The decreased IL-22 level is possible due to the reduced number of adaptive immune cells, such as Th17 and Th22, which are the source of IL-22, while responding the inflammatory stresses and bacterial states (33,42-44).

The stem cell niche is indispensable for the homeostasis of the intestinal epithelium. In this study, we observed that IL-22 activates Stat3 signaling to promote the hyper-proliferation of intestinal organoids. Moreover, IL-22 inhibited the Wnt and Notch signaling pathways in IECs. Notably, only Wnt3a rescued the organoid defects caused by IL-22, which suggests that the Wnt pathway is a key factor, downstream of IL-22, to regulate intestinal homeostasis. A recent study indicated that Wnt signaling synergizes with R-spondin to maintain ISC self-renewal (45). However, the mechanisms through which IL-22 influences the Wnt pathway remain unclear. One possibility is that IL-22 directly modulates important players controlling Wnt signaling. Another possibility is that IL-22 suppresses the differentiation of Paneth cells which secrete Wnt ligands for ISCs and IECs.

Our results are not consistent with those from a previous study, which demonstrated that IL-22 maintained the self-renewal ability of Lgr5-positive stem cells by using an IL-22 knockout mouse strain (35). IL-22 has been reported to have diverse functions in the intestine. For example, it can promote antibacterial peptide secretion and mucus production (18). We hypothesized that the loss of IL-22 may cause global consequences, such as impairing bacterial clearance, and therefore leads to the destruction of the intestinal barrier and structure. Inconsistently, another study recently demonstrated that IL-22 promotes the proliferation and expansion of ISCs in intestinal organoids (46). Of note, we used a much higher concentration of IL-22 (100 ng/ml vs. 5-10 ng/ml) in this study. This is more coincident with the high level of IL-22 expression during inflammation (47). We reason that a greater amount of IL-22 likely reaches a certain threshold that causes the suppression of Wnt signaling. Additionally, given that IL-22 receptors are also bound to other cytokines (10,48-50), a greater amount of IL-22 may competitively inhibit other signaling, such as IL-20 and IL-24 (48), which affect ISC self-renewal.

Given that IL-22 is well documented to regulate barrier immunity and anti-microbiota, it is considered to play a protective role in IBD (51). Agents to activate the IL-22 pathway are being developed for the treatment of patients with IBD. For

example, *Indigo naturalis* has been shown to be beneficial for ulcerative colitis in a clinical trial (52). However, a previous study demonstrated that the continuous stimulation of IL-22 potentially increases the risk of colitis-associated cancer (53). In this study, we found that the dynamic alteration of IL-22 was important for the maintenance of intestinal homeostasis during inflammation (Fig. 6), which sheds light into the role of IL-22 in regulating the homeostasis of the intestinal epithelium during inflammation. Thus, this study suggests that a prolonged administration of agents to activate the IL-22 pathway for IBD treatment may not be beneficial. Our findings further the current understanding of IL-22 and may aid in the clinical therapy of IBD.

In conclusion, in this study, we found that IL-22 treatment promoted cell proliferation, suppressed the differentiation of intestinal organoids and led to self-renewal defects of intestinal stem cells. As regards the underlying mechanisms, IL-22 activated Stat3 phosphorylation and suppressed the Wnt and Notch signaling pathways. This study reveals that IL-22 regulates the homeostasis of the intestinal epithelium and is critical for the regeneration of the intestine during inflammation. Thus, it deepens our current understanding of the regulation and function of IL-22 and provides the theoretical basis for IBD treatment.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

XiZ, JZ, YC, XuZ designed the experiments. XiZ, SL, YWa, HH, LL performed the experiments. SL DC and YWu performed the data analysis. YWu contributed materials/facilities. XiZ, JZ, YC and XuZ wrote the manuscript. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

All animal experiments conformed to the regulations drafted by the Association for Assessment and Accreditation of Laboratory Animal Care in Shanghai and were approved by the East China Normal University Center for Animal Research.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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