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The mTORC1 signaling modulated by intracellular C3 activation in Paneth cells promotes intestinal epithelial regeneration during acute injury☆,☆☆

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ABSTRACT

Complement activation is associated with regional inflammation during acute gastrointestinal injury (AGI). This study is designed to explore how intracellular C3 activation in Paneth cells (PCs) affects regeneration of intestinal epithelium during AGI. AGI was induced in wildtype C57BL/6 mice, with sham operation employed as control. Exogenous C3 (1 mg, I.P.) was applied at 6 h post-surgery. Intestinal crypts harvested from ileum were cultured with presence or absence of C3 (20 μg/ml), with small interfering RNA against *BST1* and complement activation inhibitor selectively applied in vitro. The intestinal integrity, percentage of PCs and intestinal stem cells (ISCs) were evaluated. Importantly, cADPR, C3 fragments, and S6-related proteins were detected in PCs to inspect the mammalian target of rapamycin complex 1 (mTORC1) signaling. AGI caused breakdown of intestinal mucosa integrity and regional inflammation. Exogenous C3 *by itself* failed to promote the growth of intestinal epithelium, but distinctly enhanced the activity of PCs via intracellular activation, which subsequently supported the expansion of ISCs inside of intestinal crypts. Inhibition of C3 activation was associated with decreased expressions of S6, S6K1 and cADPR, with blocking *BST1* found to depress cADPR only. Collectively, these data confirmed intracellular activation of C3 in PCs enhanced expansion of ISCs in response to acute injury. The mTORC1 signaling pathway in PCs contributed to this crosstalk during exogenous C3 treatment.

1. Introduction

The intestinal epithelium is an essential contributor to intestinal immunity. It is actually replaced by itself every five days to create stable barriers that protect the host, and the renewal process is cooperated with intestinal stem cells (ISCs) at the base of intestinal crypts [1,2]. ISCs, also known as crypt base columnar cells, have been recognized as responsible for quick response to catastrophic pathogenic injuries, such as irradiation, exposure to food toxins and ischemia/reperfusion [3]. The leucine-rich repeat G-protein-coupled receptor 5 (Lgr5) has been identified as a marker of ISCs through mouse engineering and cell lineage tracing studies [2]. Lgr5 is an agonist target of the *Wnt* signaling pathway, which could expand $Lgr5^{+}$ cells and accelerate intestinal recovery after irradiation [3].

Paneth cells (PCs) are granule-rich epithelial cells, which are mainly located at the base of intestinal crypts of Lieberkühn in the small intestine. These cells are universally recognized as a vital part of innate immunity for an immediate response to pathogenic invasion [4]. Dysfunction of PCs biology is associated with various severities of intestinal inflammation. A recent study progress indicates its novel role in fostering the expansion of ISCs by providing a survival niche during caloric restriction [5]. The possible mechanism of their interactions is thought to inhibiting the mammalian target of rapamycin complex 1 (mTORC1) signaling in PCs [6].

The complement system is a vital mediator of intestinal injury, particular for acute IR injury. It has been confirmed that complement activation along with circulating C3 depletion contributes to an uncontrolled intestinal injury and subsequent intestinal immunity

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depression during acute gastrointestinal injury (AGI) [7,8]. Numerous evidence have found that intracellular complement activation in intestinal epithelial cells and immune cells plays an important role in regulating local inflammation and immunity response [9–11]. More importantly, such intracellular activation has recently been proven to interact with the expansion of ISCs and organoid formation in intestinal crypts of mice [12].

Along above lines, we hypothesized that intracellular C3 activation in PCs, acting through mTORC1 signaling pathway, contributes to foster renewal of ISCs and subsequent turnover of intestinal epithelium during AGI. Herein, we performed this study to illustrate the bridging role of C3 between PCs and ISCs in response to acute injury and unravel a possible mechanism of mTORC1 interacting with such self-renewal processes.

2. Materials and methods

2.1. Mice

All mice were maintained in the wild-type C57BL/6 background and were housed in standard wired cages under a constant 12-h light and dark cycle and a specific-pathogen-free environment at controlled temperature (25 ± 1 °C). Female mice at the age of 8 to 10 weeks were allowed ad libitum access to water and foods, with a one-week rest required before the start of experiments. The study protocol was approved by the animal ethics committee at the First Affiliated Hospital of Sun Yat-Sen University.

2.2. AGI induction experiments

As we previously published, the colon ascendens stent peritonitis (CASP) operation was employed to imitate the pathogenesis of AGI [7]. In brief, mice were anesthetized intraperitoneally (0.5% pentobarbital sodium in PBS, 20 μl/g) and 5-mm-long sterile plastic stent (2.0 mm^2, Venflon; BOC, Sweden) were inserted into the anti-mesenteric wall of proximal ascending colon and fixed by suture at about 1 cm distant to the ileocecal valve. Then, a drop of stool was milked from the cecum into the stent, followed by abdominal closure with interrupted suture. As for control purpose, a sham operation with a stent fixed outside the colon at the same location was produced before abdominal closure. After those operations, all mice were brought back to the cages for resuscitation, with free access to water for next 48 h.

Evaluation of intestinal injury was performed after 24 h of surgery, as previously described [13]. Briefly, five-scaled scoring system was utilized to blindly evaluate the intestinal integrity under light microscopy. Score 0 denotes no damage, score 1 denotes sub-epithelial space at villous tip, score 2 denotes loss of mucosal lining of the villous tip, score 3 and 4 denote loss of $< 1/2$ and $\geq 1/2$ of villous structure respectively, and score 5 denotes transmural necrosis.

2.3. Exogenous C3 treatment

As our previously published works [7,8], a single dose of 1 mg complement C3 protein (MyBioSource, San Diego, CA, USA), diluted in 200 μl PBS plus 0.1% bovine serum albumin (BSA) under sterile conditions, was intraperitoneally injected in treated mice after 6 h of surgery; whereas only 200 μl PBS plus BSA was injected similarly in controlled mice at the same time point. All mice were randomly labelled as four groups as follows: (1) Sham (AGI^-), $C3^-$; (2) Sham, $C3^+$; (3) AGI^+, $C3^-$; (4) AGI^+, $C3^+$, with 10 mice in each group. As regarding for in vitro studies, additional reagents were added as follows: C3 protein (20 μg/ml, MyBioSource, San Diego, USA), anti-human C3 antibodies (1:600, ab200999, Abcam), in the presence or absence of CTSL inhibitor (ab58991, Abcam). Of note, partial crypts were transfected with 100 nM short interfering RNA (siRNA) targeting bone stromal antigen 1 (*Bst1*, Thermo Scientific, J-044021-11/12) by incubating those crypts at 37 °C for 30 min with transfection mixture (120 μl, Lipofectamine, Invitrogen) in crypt suspensions before mounting to Matrigel.

2.4. Light microscopy

After 48 h of CASP surgery, the ileum nearest the stented colon was harvested and cut into $1 \times 1 \times 2\,mm^3$ segments by a microtome and stained with hematoxylin and eosin once tissues were fixed with paraformaldehyde and embedded in paraffin. The intestinal integrity after acute injury was evaluated blindly with the following scoring system: score 0, no damage; score 1, sub-epithelial space at villous tip; score 2, loss of mucosal lining of the villous tip; score 3, loss of less than half of villous structure; score 4, loss of more than half of villus structure; and score 5, transmural necrosis. The ratio crypts: villi: heights in each section was calculated using stereological image software (ImageJ 1.51j8, Wayne Rasband, USA).

2.5. Immunohistochemistry (IHC) and fluorescence (IF)

Intestinal tissues were fixed in 10% formalin, paraffin embedded and sectioned. Immunochemistry analysis was performed as previously described [8]. Antigen retrieval was performed with Borg Decloaker RTU solution (Biocare Medical) in a pressurized Decloaking Chamber (Biocare Medical) for 3 min multiple 5 μm serial sections were incubated with affinity-purified rabbit anti-Lgr5 (1:500, ab71225, Abcam) for 1 h at room temperature. On the following day, those sections were washed three times with PBS for 10 min each, coated with anti-fade mounting medium (Molecular probes). At last, coverslips were applied with DPX mountant (CV5000, Leica Instruments). For immunofluorescence, rat anti-Occludin (1:200; bs-10011R, Bioss), rat anti-Laminin (1:200; ab11575, Abcam), anti-rabbit IgG FITC-Cy3 (CW0114S & 0159S, CWBIO) along with 4′,6-diamidino-2-phenylindole (DAPI, KGA215-50, Nanjing KeyGEN Biotech) were used to stain frozen sections of harvest intestinal sections. A fluorescence microscope (742BR1154, BIORAD) was used to observe immunofluorescent staining.

2.6. Isolation and flow cytometric analyses (FCM) of PCs and ISCs

After 24 h of surgery, mice were sacrificed through cervical dislocation under anesthesia, followed by harvest of intestinal crypts from the terminal ileum as described previously [14]. Briefly, the ileum within 1 cm close to the cecum was opened longitudinally and washed with ice-cold PBS. Afterward, the villi were scraped off with a cover slip, and the intestine was divided into pieces. After repeated PBS washing, those pieces were incubated in 2 mM EDTA-PBS solution and gently rocked at 4 °C for 30 min. After that, the EDTA solution was removed and the tissue fragments were vigorously suspended in PBS and filtered through a 70 μm mesh filter at least three times. Then, the crypt-rich fractions were centrifuged at 300*g* for 5 min at 4 °C. The pellets were suspended in the Matrigel (Bioscience) at the concentration of 10 crypts/μL. At last, the suspensions were marked with antibody cocktails including CD45-PE, EPCAM-APC (eBioscience, San Diego, CA, USA), CD31-PE, Ter119-PE, CD31-PE, Ter119-PE and CD24-Pacific Blue (BioLegend, San Diego, CA, USA), and re-suspended with SEME/7-aminoactinomycin D (7-AAD) solution (1:500 dilution), followed by flow cytometry selection and analysis using a forward and side scatter gating strategy (Novocyte™; ACEA Bioscience, San Diego, CA, USA). The flow-count fluorospheres (Beckman Coulter) was employed to calculate absolute counts, which setting a flowing event as 20,000 cells by default. PCs should be isolated as $CD24^{hi}Epcam^+CD31^-Ter119^-CD45^-$ $7\text{-}AAD^-$ cells, with ISCs isolated as Lgr5-$EGFP^{hi}CD45^-7\text{-}ADD^-$ cells. After that, those cells were deeded into the culture media in the following described.

2.7. Enzyme-linked immunoassay (ELISA) analysis

PCs from culture media under various conditions were collected, with cell lysates stored in radioimmunoprecipitation assay buffer (Thermo Fisher Scientific). The mouse cADPR ELISA Kit (ml332780, Shanghai, China), ribosomal protein S6 (ab204128, Abcam, USA) and pS6K1 ELISA Kit (ab176651, Abcam, USA) were used to detect the concentrations of such proteins in harvested PCs. According to the manufacture's protocol, the levels of sensitivity were 1 ng/ml for cADPR, 5 μg/ml for RP S6, and 1 ng/ml for pS6K1, respectively.

2.8. Western blotting (WB)

Proteins were extracted from in vitro cultured crypts or PCs were denatured by the addition of radioimmunoprecipitation assay (RIPA) buffer. Protein concentrations were determined using the bicinchoninic acid assay (BCA) method (Pierce Biochemicals, Rockford, Ill, USA), normalized for all samples and separated on NuPAGE Novex 4–12% Bis-Tris gels (Pierce Biochemicals, Rockford). Subsequently, proteins were transferred to polyvinylidene difluoride membranes (BioRad, Richmond, CA), blocked with 5% skimmed milk in tris-buffered saline Tween (T8060, Solarbio), and incubated overnight with rabbit anti-S6 fragments antibody (1:3000, ab32359, Abcam), rabbit anti-P-S6 antibody (1:4000, ab109393, Abcam), rabbit anti-Bst1 antibody (eBioscience, 14-1579-82, San Diego, CA), rabbit anti-Lgr5 antibody (Thermo Fisher Scientific) at a dilution 1:1000, goat anti-C3/C3 fragments antibody (1:1000, EMD Millipore) and complement receptor 1 (CR1; CD35, 1:500, Santa Cruz Biotechnology), and mouse anti-β-actin antibody (1:5000, Sigma-Aldrich) in TBST containing 5% skimmed milk. After three washes with TBST, the membranes were incubated with appropriate HRP-conjugated secondary antibodies (Santa Cruz Biotechnology). Protein bands were visualized using a chemiluminescent ECL™ detection kit (GE Healthcare) and captured with a digital camera (Cannon Inc., Japan).

2.9. Real-time PCR (RT PCR)

Total RNA was extracted from isolated intestinal crypts with the use of Ultrapure RNA mini kit (CW0581M, CWBIO, China) and Trizon reagent (CW0580S, CWBIO, China). Reverse transcription was performed using RNA to cDNA HiFiScript (CWBIO, China). The resulting cDNA was amplified by CFX Connect ™ Real-Time PCR Detection System (CFX96, Bio-Rad) with running for 40 cycles at 95 °C for 20 s and 60 °C for 1 min. The abundance of each target mRNA was normalized by that of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA and presented as 2[(Ct/GAPDH - Ct/gene of interest)]. The primer sequences used to detect the genotypes of interest were as follows: *Bst1* F: AAGTTGGCGATTTCTTGAGC, R: AGTCCACGGCGTTGTTTTC; *CD38* F: CCTGCTGGGCTACATTGCT, R: AGGGTTGTTGGGACAGTTTT; *GAPDH* F: AAGAAGGTGGTGAAGCAGG, R: GAAGGTGGAAGAGTGGGAGT. The expression of *CD38* was detected to reflect the activity and synthesis of cADPR, as previously described [15].

2.10. Transmission electron microscopy (TEM)

Immediately after removal from mice, 1.0–2.0 mm sections of ileum were placed into Karnovsky's KII solution (2.5% glutaraldehyde, 2.0% paraformaldehyde, 0.025% calcium chloride, in a 0.1 M sodium cacodylate buffer, pH 7.4), fixed overnight at 4 °C, and stored in cold buffer. Next, they were post-fixed in 2.0% osmium tetroxide, stained en-bloc with uranyl acetate, dehydrated in graded ethanol solutions, infiltrated with propylene oxide/Epon mixtures, flat embedded in pure Epon, and polymerized overnight at 60 °C. At last, 1 mm sections were cut, stained with epoxy resin 618, and examined by light microscopy. Representative areas were chosen for electron microscopic study and the Epon blocks were trimmed accordingly. Thin sections were cut with an LKB 8801 ultra-microtome and diamond knife, stained with uranyl acetate and lead citrate, and examined in a transmission electron microscope (FEI Quanta 250, FEI©, USA). All images were captured by an Advanced Microscopy Techniques digital CCD camera (Sony Alpha 7 II, Japan). For morphometric analyses, vertical grid lines were drawn perpendicular to the most apical strand, and intersections of strands and grid lines helped to define the number of horizontally oriented strands in the tight junction (TJ) meshwork [16]. The distance between the most apical and contra-apical tight junction strand was defined as depth of TJ. For quantification of strand discontinuities, strand breaks larger than 20 nm were counted and given per 1 μm of horizontally oriented TJ strand length.

2.11. Crypt culture media

Isolated crypts were counted and embedded in matrigel (BD Bioscience 356231 with reduced growth factor) that contained 1 μM jagged (AnaSpec Bio) at 5–10 crypts/μl and cultured in a modified form of medium as described previously [14]. In brief, DMEM/F12 (Gibco) was added to the culture media, with the absence or the presence of exogenous C3 and CTSL inhibitor (ab58991, Abcam). 30–50 μl drops of matrigel with crypts were plated onto a flat bottom 48-well plate (Corning 3548) and allowed to solidify for 30 min in a 37 °C incubator. The matrigel should be changed every 48 h in fully humidified chambers filling 6% CO_2.

2.12. Statistical analysis

Unless otherwise indicated, data were presented as means ± SEM. Statistical significance of differences was determined with Student's *t*-test for comparison between two groups or by two-way analysis of variance with *Bonferroni* correction for multiple comparisons. All analyses were performed by GraphPad Prism Software (Ver. 7.0, San Diego, California, USA). A two-tailed $P < 0.05$ was considered statistically significant.

3. Results

3.1. Exogenous C3 reverses AGI-induced collapse of intestinal structural integrity

To evaluate the impact of exogenous C3 treatment on intestinal barrier during acute injury, we supplemented C3 protein at 6 h after AGI surgery and harvested ileum at 48 h post-operation from survived mice in each group. Consistent with our previous reports [7,17], AGI induced by a CASP surgery could lead to around 75.4% mortality rate within 48 h of acute injury. However, exogenous C3 treatment could markedly reduce the mortality rate by 62%, as compared with AGI+, C3– group (Fig. S1, $P < 0.001$). By further intestinal integrity evaluation, AGI was associated with a significant breakdown of intestinal barrier, which was characterized by considerable villus detachment and destruction, increased injury score, decreased villus/crypt size ratio, reduced occludin percentage, increased depth of TJ and reduced amount of TJ, accordingly (Fig. 1). In sham-operated mice, harvested intestine was morphologically normal, with no changes observed when additional C3 treatment applied. The local inflammation due to AGI, such as edema of intestinal villi and infiltration of neutrophils, was not amplified by additional C3 supplementation. By contrast, exogenous C3 treatment in early stage of AGI appeared to reduce such regional reactions at crypts, increase the number of crypts and connecting proteins between intestinal epithelial cells, and foster the turnover of intestinal tissue (Fig. 1).

3.2. Exogenous C3 increases ISCs and PCs numbers

To investigate the relationship between C3 activation and repair of

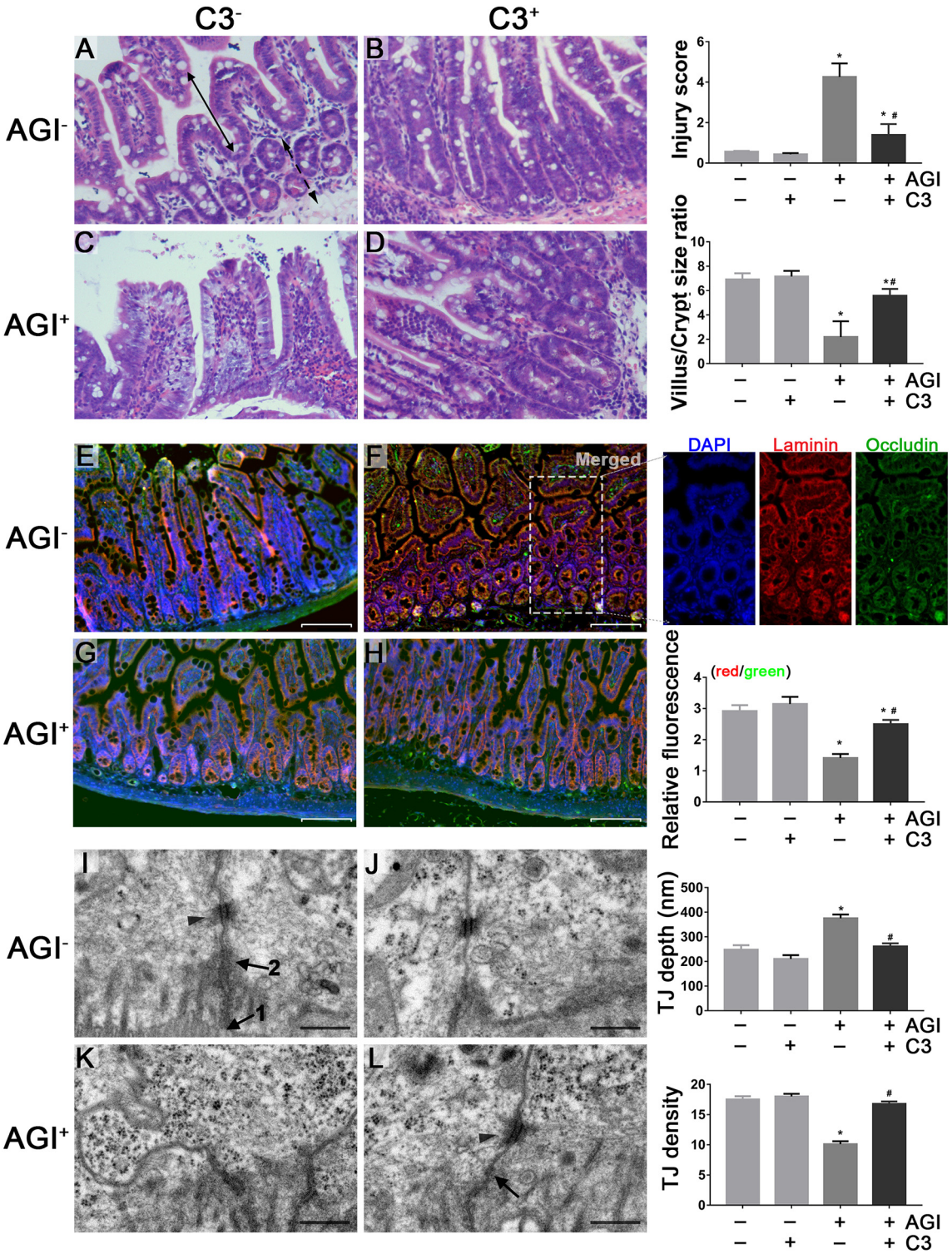

Fig. 1. The evaluation of intestinal epithelial integrity during AGI.

All ileum sections were harvested from a live mouse at 48 h post-surgery. HE staining (A–D), IF staining (E–H) and TEM imaging (I–L) were employed to assess the integrity of intestinal epithelium during AGI. This acute epithelial injury can be characterized with detachment of microvillus from mucosal layer of ileum plus extensive infiltration of neutrophils (C), numerous necrosis of enteral epithelial cells along with reduced occludins around the submucosa (G), and collapse of tight junction between epithelial cells (K). The exogenous C3 application at 6 h after AGI in vivo could attenuate such injury by reducing regional inflammation (D) and promoting turnover of epithelium (H) and new tight junction (L). Images (A, E, I) from sham-operated mice (AGI-) were used as control, with pair images (B, F, J) plus C3 as additional references. Representative images of n = 5 independent experiments. Scale bars, 100 and 0.5 μm for E–H and I-L images, respectively. HE staining images zoomed by magnification ×200; Black solid and dotted arrows with double arrowheads standing for size of villus and crypt, respectively; Black triangles indicating desmosomes between epithelial cells, whereas black arrows with single arrowhead indicating tight junction nearest the lumen (arrow 1 to arrow 2). All error bars in histograms (right panel) denote SEM. The average height of crypt and villus was calculated by Image Pro Plus ™, as well as Relative fluorescence (Laminin/Occludin). $^{*}P < 0.05$ vs. AGI− groups, $^{\#}P < 0.05$ vs. C3− group.

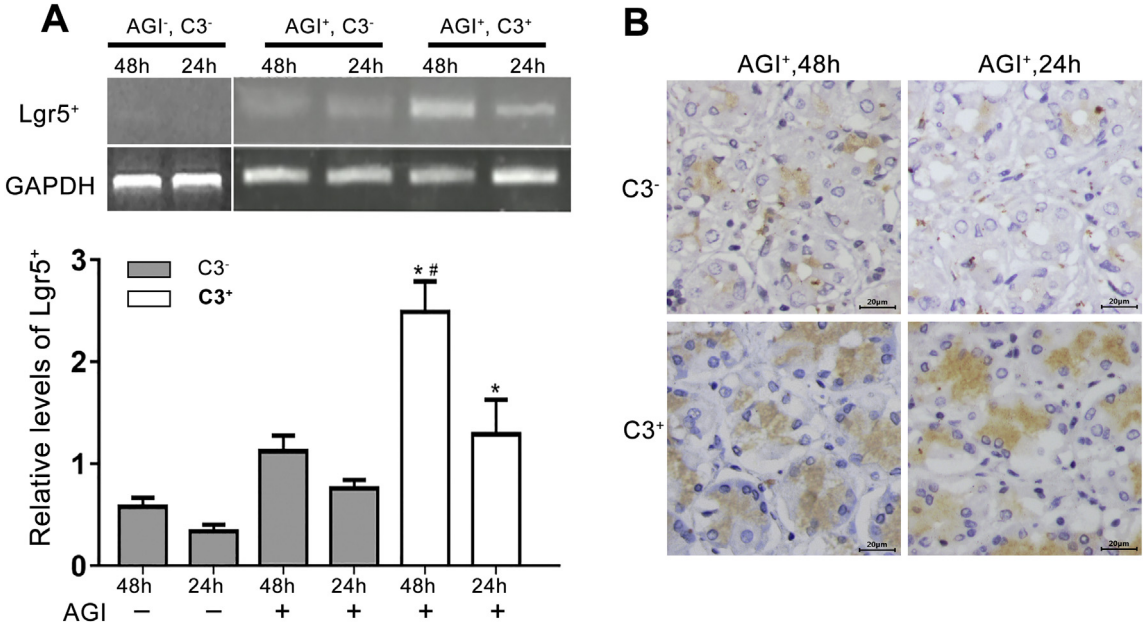

Fig. 2. The proliferation of Lgr5$^+$ISCs can be enhanced with exogenous C3 treatment during AGI.
The proliferation of ISCs under crypts of ileum was assessed at 24 h and 48 h post-surgery by qRT-PCR (A) and IHC (B) analyses, respectively. The levels of Lgr5+ expressed by ISCs were significantly increased in C3+ groups compared to C3– groups. Importantly, the number of Lgr5$^+$ ISCs at 48 h post-surgery was markedly increased compared with that at 24 h post-surgery. Brown marked areas indicate Lgr5$^+$ ISCs, with scale bars at 20 μm. All error bars in histograms denote SEM. Representative histograms of n = 5 independent experiments. *P < 0.05 vs. C3– groups at the same timepoint, #P < 0.05 vs. C3+ group at 24 h after AGI. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

intestinal barrier during acute injury, crypts were harvested from AGI-induced mice at 24 h and 48 h after surgery, respectively. The function of ISCs was slightly increased in response to AGI; however, the mRNA expression of Lgr5 in ISCs was not significantly different between two time points without the administration of C3 (Fig. 2A). By contrast, exogenous C3 treatment at early stage of AGI could significantly enhance the mRNA expression of Lgr5, especially at 48 h post-operation (P < 0.05, Fig. 2A). In addition, by using IHC method, the amount of Lgr5$^+$ ISCs was also markedly increased under exogenous C3 treatment, especially at 48 h after surgery (Fig. 2B).

To address how C3 treatment influenced the frequency of Lgr5$^+$ ISCs, we further detected the relative amount of PCs and ISCs isolated from crypts using Flow cytometry analysis (Fig. 3). It indicated that C3 supplementation led to a 36.3% increase in isolated PCs in harvested crypts compared with those without C3 treatment (60.8% vs. 24.5%, P < 0.01; Fig. 3A). Meanwhile, the secretory function of PCs in response to AGI-induced infection was distinctly reinforced with C3 treatment (Fig. S2). Knockdown of *Bst1* mRNA was realized with specific siRNA (Fig. S3). After that, it was found that the expansion of PCs in C3-treated mice was significantly depressed compared to those without silencing *Bst1* (41.3% vs. 60.8%, P < 0.05). Besides, it was also found that the CTSL inhibitor could achieved the similar depression effect on PCs, but more severe than those with *Bst1* siRNA (24.7% vs. 41.3%, P < 0.05). Similarly, the number of ISCs could be enhanced with C3 treatment, but also be suppressed with additional silence of Bst1 or CTSL (Fig. 3B). Nevertheless, blockage of *Bst1* mRNA appeared to apparently depress the proliferation of ISCs in comparison with inhibition of C3 intracellular activation in PCs (2.9% vs. 6.1%, P < 0.01).

3.3. Exogenous C3 strengthens the mTORC1 signal pathway in PCs

To further confirm the role of exogenous C3 treatment in promoting the function of PCs, additional studies in vitro were appended. As mentioned in the Materials and methods section, isolated PCs from AGI-produced mice were seeded into essential matrigel medium to receive the stimulation from C3 and other vital proteins. Exogenous C3 could promote the mRNA expression of *Bst1* in PCs directly and subsequently enhanced the mRNA expression of downstream *CD38* by RT-PCR analyses (Fig. 4). Additionally, the mTORC1 signaling pathway could be distinctly depressed by silencing the Bst1 mRNA in PCs, which was characterized by decreased mRNA expression of both *Bst1* and *CD38*. However, a simplex inhibition of intracellular C3 activation with CTSL inhibitor could not shut down this signaling pathway as muting the *Bst1* did. It suggested that partial intracellular C3 activation still worked inside the PCs to initiate the mTORC1 signaling.

To further confirm that, we performed immunoassay and western blotting to detect the levels of mTORC1-related proteins, with levels of C3 fragments also explored meanwhile. First, the production of cADPR could be obviously reduced with the knockdown of *Bst1* mRNA, but partially affected by CTSL inhibitor. The findings here indicated that CTSL inhibitor could reduce the production of C3a in PCs but fail to abrogate the depletion of C3 in PCs (Fig. 5B). Besides, it was found that the production of CR1 mainly located on the membrane of PCs was not correlated with exogenous C3 stimulation, as similarly as the C5a protein. More importantly, the production of S6 and S6K1 were both increased under the supplementation of exogenous C3 (Fig. 5C), but this enhanced production cannot be suppressed with silencing *Bst1* (Fig. 5D). Besides, suppressing the intravascular C3 activation with CTSL inhibitor was quite effective to down-regulate the synthesis of S6 and other effectors related to mTORC1 pathway. All taken together, it suggested that intracellular activation of C3 might be required for the initiation of mTORC1 signaling pathway inside the PCs.

4. Discussion

In this study, the interactions between PCs and ISCs were confirmed during acute intestinal injury. Our work suggested that intracellular activation of C3 in PCs enhanced the function of PCs, followed by promoting the proliferation of ISCs and renewal processes of intestinal epithelium. More importantly, the findings further indicated that the mTORC1 signaling pathway could be regulated by intracellular C3

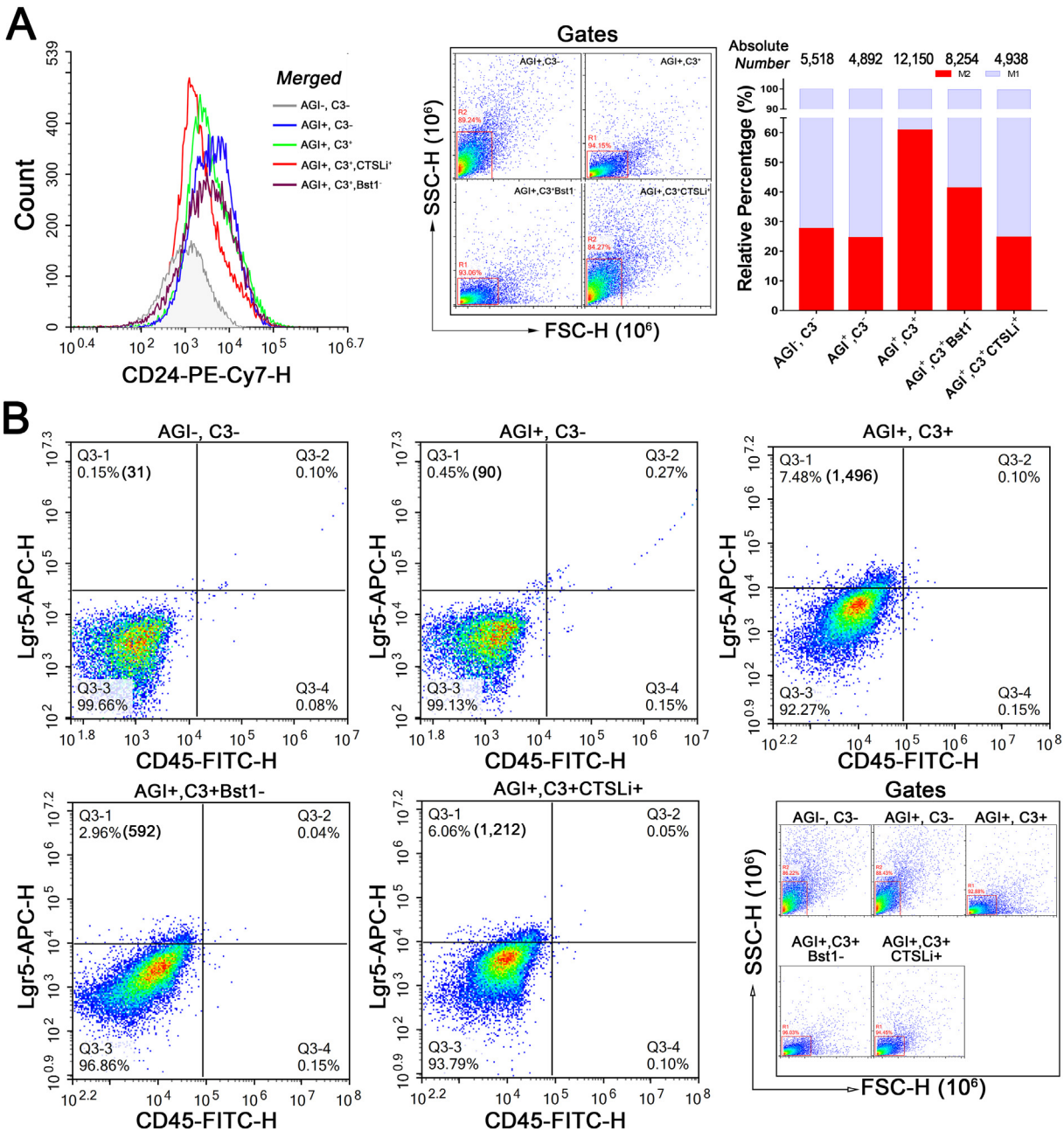

Fig. 3. The exogenous C3 treatment enhances the expansion of ISCs in need of PCs.
FCM was utilized to detect the amount of harvested PCs (A) and Lgr5-GFP^{+}ISCs (B) from ileum in each group at 48 h post-surgery. The relative percentage of low- and high-expression of CD24 for each group (20,000 cells per sample) presents as: 1. Sham (AGI−), C3−: 72.32% vs. 27.59%; 2. AGI+, C3−: 75.45% vs. 24.46%; 3. AGI+, C3+:39.14% vs. 60.75%; 4. AGI+, C3+/Bst1−: 74.88% vs. 24.69%; 5. AGI+, C3+/CTSLi+: 58.48% vs. 41.27%, respectively. The selected gates for PCs detection were attached aside except the sham-operated group. The relative percentage of CD45^{-}Lgr5^{+} ISCs in Q1 (Left-upper quadrant) of each flow dot plot for each group (20,000 cells per sample) presents as: 1. AGI−, C3−: 0.15%; 2. AGI+, C3−: 0.45%; 3. AGI+, C3+: 7.48%; 4. AGI+, C3+/Bst1−: 2.96%; 5. AGI+, C3+/CTSLi+: 6.06%. Representative histograms of n = 5 independent experiments. Absolute numbers of PCs and ISCs marked in histogram and Q1 dot plot within brackets, respectively.

activation via CTSL to release essential cytokines from PCs.

Generally, acute injury to small intestine would cause extensive loss of villous epithelium, with breakdown of intestinal barrier followed if intestinal epithelial regeneration is not promptly. In our preliminary studies, we confirmed that C3 depletion in circulation was strongly associated with that uncontrolled intestinal injury and subsequent severe sepsis [7,17]. Actually, the C3 depletion resulted from immense C3 activation during AGI could be postponed with exogenous C3 treatment, with no responsive inflammation or injury found meanwhile. Moreover, exogenous C3 administration might inhibit the local inflammation by suppressing the regulatory T cells via NF-κB signaling pathway [8]. Hence, it is applicable to supply the complement system during AGI.

There is recognized evidence that C3 activation inside functional cells could arouse certain regional injury, such as in tubular epithelial cells, intestinal epithelial cells, vascular epithelial cells and retinal cells [18–21]. Alternative activation pathway appears to contribute local injury through initiating the formation of C3 convertase on cellular surface. As a double-edged sword, it is also confirmed that C3 activation is vital for regional renewal during such injury [12,22]. Our work along

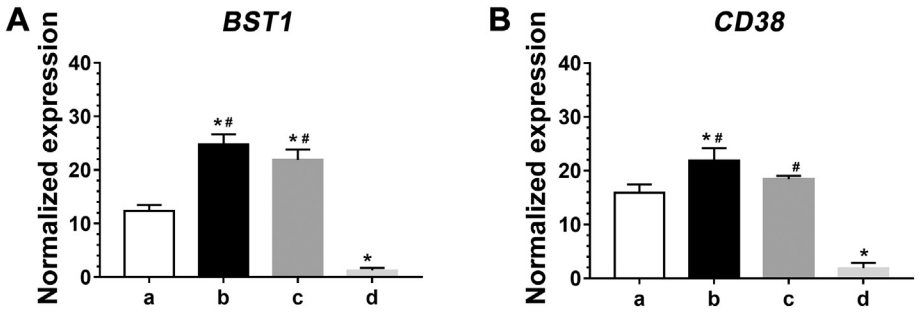

Fig. 4. The mRNA expression of *Bst1* and *CD38* in PCs during AGI.
All isolated PCs were obtained from AGI-produced mice without C3 treatment at 24 h after surgery. qRT-PCR method was utilized to detect the mRNA expression of *Bst1* (A) and *CD38* (B) in isolated PCs. The mRNA level of *CD38* was detected to explore changes of cADPR. Their expression levels were normalized to GAPDH. Groups: a:AGI+, C3−; b:AGI+, C3+ &CTSLi+; c:AGI+, C3+; d:AGI+, C3+ & Bst1−. Abbreviations: RFU, relative fluorescence units. $^{*}P < 0.05$ vs. C3– group. $^{\#}P < 0.05$ vs. C3+ Bst1− group. Error bars in histograms denote SEM. Representative images of n = 7 independent experiments.

with its preliminary studies confirms that intracellular activation of C3 in PCs plays essential role in fostering the repair of intestinal tissue, rather than deteriorating the tissue injury [13]. A recent experimental study indicates that intracellular C3a activation is required for mini-gut organoid generation, particular for intestinal crypts in quick response to AGI [12]. That study using a classical ischemia/reperfusion model to cause AGI actually induced limited inflammatory reactions by C3a in comparison to our study using a severe sepsis-mediated AGI. Therefore, the role of C3a activation inside of PCs in mediating proinflammatory activities might be more powerful than enhancing the turnover of ISCs in our study. Interestingly, our in vitro data suggested that the intracellular level of C3a was consistent with C3 in PCs (Fig. 5B).

To survive from acute injury, various cells all need energy intake to produce proteins, lipids and nucleotides while also suppressing catabolic pathways such as autophagy and apoptosis. It is believed that mTORC1 plays an indispensable role in regulating all survival processes via balancing between anabolism and catabolism in response to environmental changes [23]. Generally, mTORC1 promotes the production of vital growth factors largely through the phosphorylation of two enzymes: S6K1 and 4EBP [24]. In this study, we show that the levels of S6K1 and its related proteins in PCs with restriction of C3 co-stimulation were reduced. Those proteins inside PCs were also reduced by partially inhibiting the intracellular C3 activation with CTSL inhibitor. Besides, we observed an increased production of regenerating gene (Reg) proteins such as Reg-IIIγ and Reg-IIIα in PCs under exogenous C3 treatment [13]. All these together suggested that C3 activation in PCs were closely associated with the regulation of mTORC1 signaling. Exogenous C3 treatment in early stage of AGI could sustain a stable C3 levels inside PCs to stimulate adjacent ISCs. Beyond that, a possible uptake pathway through hydrolytic C3 recycling mechanism has been discovered in several types of immune cells, further suggesting the source and composition of intracellular C3 store [25].

The current study had several limitations. First, a lack of C3 deficiency model would unavoidably weaken the power of current findings. Second, *Bst1* siRNA was utilized to block the downstream of mTORC1 signaling pathway; however, rapamycin was not applied to mute the mTOR to identify the relationship between C3 intracellular activation and mTORC1 or mTORC2 inhibition. To our knowledge, mTORC1 is the main compound of mTOR signaling and is strongly associated with the regulation of ISCs in specific conditions [6,26,27]. Importantly, it is confirmed that Bst1 is the essential factor for producing cADPR in PCs to further foster the expansion of ISCs [28]. However, our findings cannot exclude a possibility of another signaling pathway fired by C3 activation to collaborate with mTORC1. At last, it was suggested that

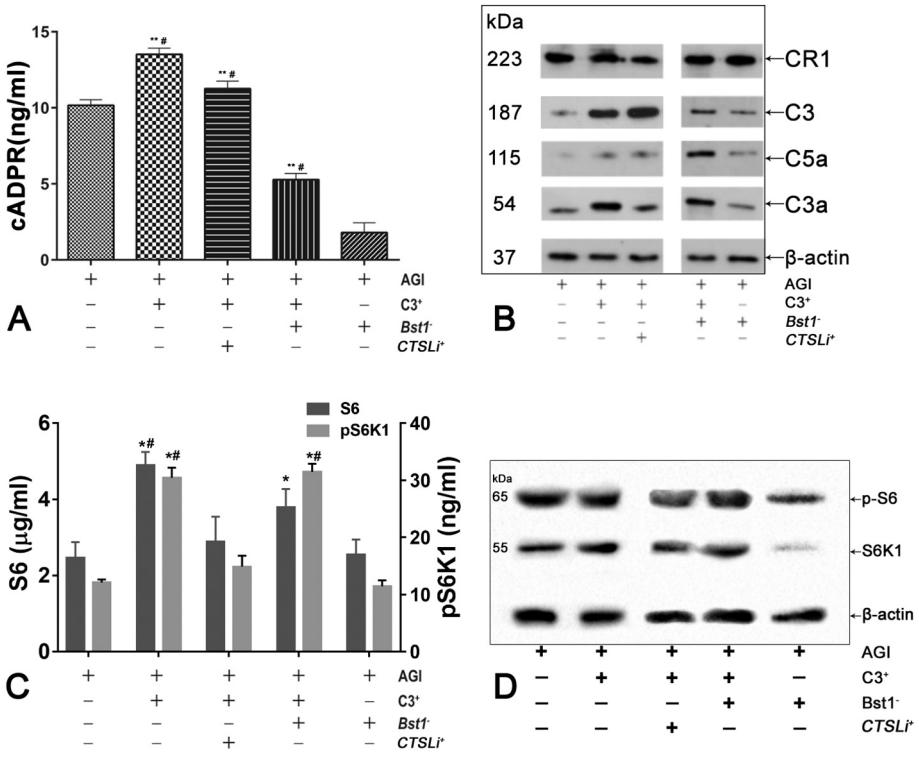

Fig. 5. The production of C3 fragments, cADPR and S6-accociated proteins in PCs during AGI.
All isolated PCs were obtained from AGI-produced mice without C3 treatment at 24 h after surgery. The production of cADPR (A) in PCs was compared in the absence or presence of C3 contained medium by using ELISA method, with C3 fragments and receptor (B) detected via WB method, S6-associated proteins via ELISA (C) and WB (D), respectively. As for WB analysis, β-actin was used as a protein loading control. Representative images of n = 5 independent experiments, error bars in histograms denote SEM. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. C3– group, $^{\#}P < 0.05$ vs. C3+/CTSLi+ group.

exogenous C3 may not directly promote the function of ISCs, but rather through their niche cells during an inflammatory process. However, additional in vitro studies to explore internal relationship were required to confirm the above point. A previous report found that ISCs were no sense of external signals except from PCs when self-renewal through mTORC1 signaling pathway [6]. Unfortunately, such finding was confirmed in specific model of caloric restriction, with further validation required in other models causing intestinal injury.

In summary, we first demonstrated that complement C3 activation inside PCs could enhance the expansion of ISCs through mTORC1 signaling pathway in response to acute injury. Exogenous C3 treatment for AGI would not lead to an uncontrolled regional inflammation, but rather promote an improved repair of intestinal epithelia. However, due to the limited results, more rigorous studies should be performed to validate the key role of C3 in enhancing the regenerative process.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.intimp.2018.12.002.

Ethical approval

All animal procedures were performed under the guidelines on the care and use of laboratory animals (Institute of Laboratory Animal Resources) and were approved by the animal ethics committee of The First Affiliated Hospital of Sun Yat-Sen University (Guangzhou, PR China).

Condense summary

1. The activated PCs by intracellular C3 activation are attributed to the proliferation of ISCs and subsequent intestinal epithelial regeneration during acute gastrointestinal injury. 2. Intracellular C3 activation regulates the mTORC1 signaling inside PCs to promote the expansion of ISCs in response to such injury.

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Conflict of interest

The authors have no conflicts of interest.

Author contributions

JY, KS and KY performed experiments, analyzed data and contributed in writing manuscript. WD, GL, WS and MT directed animal experiment. YY contributed in data analysis, designed the experiments, and supervised the work.

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