Repression of Mammalian Target of Rapamycin Complex 1 Inhibits Intestinal Regeneration in Acute Inflammatory Bowel Disease Models

Yuting Guan, Long Zhang, Xia Li, Xinyan Zhang, Shijie Liu, Na Gao, Liang Li, Ganglong Gao, Gaigai Wei, Zhaohua Chen, Yansen Zheng, Xueyun Ma, Stefan Siwko, Jin-Lian Chen, Mingyao Liu and Dali Li

*J Immunol* published online 29 May 2015
http://www.jimmunol.org/content/early/2015/05/29/jimmunol.1303356

**Supplementary Material**
http://www.jimmunol.org/content/suppl/2015/05/29/jimmunol.1303356.6.DCSupplemental

**Subscription**
Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

**Permissions**
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Repression of Mammalian Target of Rapamycin Complex 1 Inhibits Intestinal Regeneration in Acute Inflammatory Bowel Disease Models

Yuting Guan,*1 Long Zhang,*1† Xia Li,* Xinyan Zhang,* Shijie Liu,* Na Gao,* Liang Li,* Ganglong Gao,† Gaigai Wei,* Zhaohua Chen,* Yansen Zheng,* Xueyun Ma,* Stefan Siwko,‡ Jin-Lian Chen,† Mingyao Liu,*† and Dali Li*

The mammalian target of rapamycin (mTOR) signaling pathway integrates environmental cues to regulate cell growth and survival through various mechanisms. However, how mTORC1 responds to acute inflammatory signals to regulate bowel regeneration is still obscure. In this study, we investigated the role of mTORC1 in acute inflammatory bowel disease. Inhibition of mTORC1 activity by rapamycin treatment or haploinsufficiency of Rheb through genetic modification in mice impaired intestinal cell proliferation and induced cell apoptosis, leading to high mortality in dextran sodium sulfate- and 2,4,6-trinitrobenezene sulfonic acid–induced colitis models. Through bone marrow transplantation, we found that mTORC1 in nonhematopoietic cells played a major role in protecting mice from colitis. Reactivation of mTORC1 activity by amino acids had a positive therapeutic effect in mTORC1-deficient Rheb+/- mice. Mechanistically, mTORC1 mediated IL-6–induced Stat3 activation in intestinal epithelial cells to stimulate the expression of downstream targets essential for cell proliferation and tissue regeneration. Therefore, mTORC1 signaling critically protects against inflammatory bowel disease through modulation of inflammation-induced Stat3 activity. As mTORC1 is an important therapeutic target for multiple diseases, our findings will have important implications for the clinical usage of mTORC1 inhibitors in patients with acute inflammatory bowel disease. The Journal of Immunology, 2015, 195: 000–000.

Crohn’s disease (CD) and ulcerative colitis are two main forms of inflammatory bowel diseases (IBDs) that afflict millions of people globally (1). Patients suffering from the second most common inflammatory condition (IBDs) often need to take medications lifelong (2, 3). It is believed that environmental, immunological, and genetic factors are involved in the initiation and maintenance of gastrointestinal tract inflammation, but the exact cause of this disease has not yet been fully elucidated (4). Increasing evidence suggests that enteric pathogen-induced acute gastroenteritis, sometimes leading to mucosal damage, is a critical event to induce an inappropriately activated mucosal immune response leading to chronic IBD (5). IBDs are characterized not only by the submucosal accumulation of immune cells, but also by severe damage to the epithelial layer. Thus, it is believed that understanding how the epithelial cells respond to the inflammatory stimuli and identifying the molecular basis of epithelial cell regeneration during IBD are critical for developing therapeutics to cure these diseases.

The intestinal epithelium is a highly proliferating tissue that generates new intestinal epithelial cells (IECs) from the stem cells residing in the crypt (6, 7). Defective epithelial restitution by attenuation of multiple signaling pathways, including Hippo/Yap, Hedgehog/Gli1, DKK1, Notch, and others, impairs the maintenance of the barrier and is considered an important risk factor for IBDs (8–11). Our recent findings also suggest that Lgr4-mediated Wnt/β-catenin signaling is critical for the maintenance of intestinal homeostasis and protection against IBDs in mice (12). It suggests that signaling pathways involved in modulation of epithelial cell proliferation/survival may contribute to IBD.

The mammalian target of rapamycin (mTOR) signaling pathway plays a critical role in generating a coordinated response to the cellular environment (cell growth, proliferation, and/or survival) via modulation of protein synthesis (13). Upstream regulators, such as growth factors and amino acids, activate mTORC1 through a small GTPase Rheb that is negatively regulated by GTPase-activating protein TSC1/2 complexes (14–17). Activation of mTORC1 enhances protein synthesis through phosphorylation of ribosomal protein S6 kinase β-1 (S6K1) and eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP), which induces cell cycle progression and growth (13). Oncogenic activation of Rheb-mTORC1 signaling has been reported to induce cancer cell survival, growth, and proliferation in multiple types of malignant tumors, and inhibition of mTORC1 is a clinical therapeutic strategy for several types of cancers (18–23). As a specific inhibitor of mTOR, rapamycin (i.e., sirolimus or rapamune) is approved as a clinical drug for immunosuppression in transplant recipients.
patients and for prevention of coronary artery stent thrombosis (24). However, lung toxicity, the major adverse effect of sirolimus, has been reported in transplant recipients (25). Because the physiological role of mTORC1 in intestine homeostasis and tissue repair is not fully investigated, we hypothesize that mTORC1 plays a protective role in experimental mouse models of acute colitis.

To conclusively assess the global impact of mTORC1 during IBDs, we investigated the consequences of mTORC1 inhibition (using both pharmacological and genetic strategies) in mouse 2,4,6-trinitrobenzene sulfonic acid (TNBS)– and dextran sodium sulfate (DSS)–induced experimental colitis models. In this study, we report that mTORC1 activity was induced in mouse IECs upon DSS treatment. Suppression of mTORC1 activity by ramycycin treatment or haploinsufficiency of Rheb in mice increased the susceptibility and mortality to TNBS- and DSS-induced colitis. Inflammation-induced Stat3 activation was attenuated in the mucosa of mice with impaired mTORC1 activity, leading to increased apoptosis and decreased proliferation of IECs. Together, our findings suggest that mTORC1 is essential for protection against IBD.

**Materials and Methods**

**Mice**

Embryonic stem cells with a gene-trap insert in Rheb (BayGenomics; stock number 015808-UCD) were microinjected into C57BL/6 blastocysts. Chimeric offspring were crossed to C57BL/6 background wild-type (WT) mice to obtain gene-trapped heterozygous mice that were then backcrossed with C57BL/6 mice for at least six generations. As determined by RT-PCR and genotyping technology and used at 1:1000 dilution).

**Chemical-induced colitis**

Six- to 8-wk-old littermates were treated with DSS or TNBS to induce colitis. For DSS-induced colitis, mice were given drinking water containing 3% DSS (molecular weight, 36000–50000; MP Biomedicals) for 6 d, followed by normal drinking water until the end of the experiment. For TNBS-induced colitis, TNBS treatment was performed, as described previously (26). Briefly, mice were pretreated with 150 μl 1% TNBS (Sigma-Aldrich) in acetone and olive oil on the skin of the back on day 1, followed by rectal administration of 5 μg/kg body weight of 2.5% TNBS in 50% ethanol on day 8. For rapamycin and amino acid treatments, rapamycin (LC Laboratories) was administered by i.p. injection at 2 mg/kg, and the compound amino acid treatment (Kelun Pharmaceutical) was administered by i.p. injection at 250 mg/kg. Rapamycin and amino acids were given daily beginning on day 1 until the end of the experiment.

**Histopathology analysis**

The entire colon was excised to measure the length of the colon. Colonos were washed with PBS, fixed in 4% paraformaldehyde, and embedded in paraffin. Tissue sections were stained with H&E (Sigma-Aldrich). Colonic injury scoring was performed, as described previously (12).

**Immunohistochemistry and fluorescence**

Immunohistochemical staining was performed, as described previously (12). TUNEL was analyzed using the in situ cell death detection kit (Roche). For immunofluorescence, Alexa Fluor 488 secondary Abs (Invitrogen; 1:500) were used.

**Immunoblotting**

The entire colon was rinsed with ice-cold PBS and lysed in ice-cold 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 Diagnostic]). Protein extracts were quantified by Micro BCA Protein Assay Kit (Thermo Scientific) and resolved by SDS-PAGE.

**Bone marrow transplantation**

Bone marrow cells were collected from femurs and tibia of WT or Rheb+/− donor mice and were injected (1 × 10⁴ cells/mouse) into the tail vein of irradiated (9 Gy) recipient mice. After 7 wk, mice were given DSS, as above, to induce acute colitis. Primers targeting Rheb gene-trapped allele used to confirm successful transplantation are as follows: forward, 5′-CTTGGTATGAGGTGCGTGC-3′ and reverse, 5′-CTTGGCAATACATAACCG-3′.

**Gene expression analysis**

RNA was isolated from mouse colon tissue with TRIzol reagent (Invitrogen) and was reverse transcribed into cDNA with the Prime Script RT reagent kit (TaKaRa). Real-time PCR was performed with SYBR Green (TaKaRa). Primer pair sequences are as follows: IL-6 forward, 5′-GAAAAGATTGGCTGAAGTCG-3′ and IL-6 reverse, 5′-ATCTGCCAGAAGCACAGGA-3′; TNF-α forward, 5′-CACGCTGTCAGGAAACACCCA-3′ and TNF-α reverse, 5′-TAGCAATCGGCTAGCTG-3′; IL-1β forward, 5′-GCTCCTAAATCCTGGACG-3′ and IL-1β reverse, 5′-TCACAGAGGATGGGTTCTTC-3′; Reg3β forward, 5′-TGTCGTCCTCCGCGATGT-3′ and Reg3β reverse, 5′-ATAGAACCCATAGGCCG-3′.

**Cell and primary colon tissue culture**

Caco2 colon cancer cells were cultured in MEM with 10% FBS and maintained at 37°C in 5% CO₂. In our experiments, Caco2 cells were pretreated for 6 h with rapamycin (LC Laboratories) at 100 nM before addition of IL-6 (PeproTech) at 20 ng/ml. Full-thickness colon pieces were cultured in RPMI 1640 for 30 min or 2 h in the presence or absence of IL-6 (PeproTech); 20 ng/ml). Colon pieces were then paraffin embedded for immunofluorescence or subjected to Western blotting analysis.

**Abs**

For immunohistochemistry, primary Abs were used as follows: phospho-s6 (pS6) (Cell Signaling Technology; 1:400); Ki67 (Neomarker; 1:1000); cleaved caspase 3 (Cell Signaling Technology; 1:200); Chromogranin A (Immunostar; 1:3000); BrDU (Sigma-Aldrich; 1:500); pE BP (Cell Signaling Technology; 1:400); pSTAT3 (Cell Signaling Technology; 1:200); pSTAT3 (Cell Signaling Technology; 1:400); Cyclin D1 (Cell Signaling Technology; 1:100); and Bcl-xL (Cell Signaling Technology; 1:100). For immunofluorescence, primary Abs were as follows: β-actin (Sigma-Aldrich; 1:5000); RHEB (Abcam; 1:500); and 56K, pS6K1, S6, pS6, p4E BP, 4EBP1, STAT3, pSTAT3, Bcl-xL, and Cyclin D1 (from Cell Signaling Technology and used at 1:1000 dilution).

**Statistical analyses**

Data are expressed as means ± SEM. Means of two groups were compared using Student’s t test (unpaired, two tailed), with p < 0.05 considered to be statistically significant. Unless indicated in the figure legends, all the experiments were performed at three times with similar results.

**Results**

mTORC1 activity protects against DSS- and TNBS-induced colitis

mTORC1 plays important roles in a number of pathological conditions, but its function in acute bowel injury is still largely unknown. To explore whether mTORC1 signaling is activated during acute IBD, we employed the DSS-induced experimental colitis model. As shown in Fig. 1A, the protein level of phospho-S6K1 and phospho-S6, downstream targets of mTORC1, in mouse colonic epithelial cells before treatment was relatively low, but dramatically increased upon DSS treatment for 6 d, suggesting the potential role of mTORC1 in intestinal injury and regeneration. However, another key downstream target, phospho-4EBP1, was not activated after DSS treatment (data not shown). To test the role of mTORC1 signaling in colitis, we used rapamycin, the mTOR-specific inhibitor, to treat the mice in the DSS-induced colitis model. On day 3 of DSS treatment, no obvious histological defect was observed in the colons of mice treated with or without rapamycin, but the proliferation of the epithelial cells in...
rapamycin-treated mice was significantly reduced (Supplemental Fig. 1A–C). Surprisingly, all mice treated with rapamycin died within 11 d, whereas only 20% of mice in the control group died (Fig. 1B). Dramatic body weight loss was observed from day 4 of treatment concordant with an increased daily activity index (DAI) (Fig. 1B), indicating more severe clinical symptoms in rapamycin-treated mice. Histological analysis clearly showed that mice treated with rapamycin suffered substantial intestinal injury with significant loss of crypts and excessive immune cell invasion into the submucosa (Fig. 1C). Statistical analysis suggested that the colitis was more severe in rapamycin-treated mice than in controls (8 ± 1 versus 5.33 ± 0.57) (Fig. 1C). Moreover, we found decreased proliferation, but increased apoptosis, in colonic epithelial cells in mice treated with rapamycin compared with controls (Fig. 1D). These data suggested that rapamycin dramatically increased the susceptibility and mortality from DSS-induced IBD in mice. As expected, rapamycin treatment dramatically inhibited the phospho-S6K1 and phospho-S6 levels in colonic epithelium in DSS-induced mice (Supplemental Fig. 1D), suggesting that the activation of the mTORC1 pathway is essential for protecting mice from DSS-induced colitis. In addition, we also used the TNBS-induced colitis model to explore the protection role of mTORC1 signaling in intestinal injury. Following TNBS administration, mice treated with rapamycin displayed increased mucosal ulceration with higher histology score (Fig. 1E) and decreased cell proliferation rate (Fig. 1F). Taken together, the above findings strongly imply the protective role of mTORC1 in chemical-induced bowel injury.

**Haploinsufficiency of Rheb in mice causes hypersusceptibility to colitis**

To further confirm the physiological roles of mTORC1 signaling in DSS-induced colitis, we generated a mouse strain with a gene trap inserted into intron 1 of Rheb, the direct activator of mTORC1. Taking advantage of the β-galactosidase reporter gene that is driven by the endogenous Rheb promoter, the expression of Rheb was detected in the crypt of the colon as well as in lamina propria cells (Supplemental Fig. 2A). Consistent with a previous report, Rheb homozygous mutation led to embryonic lethality around midgestation (27). Rheb+/− mice were apparently normal and exhibited no spontaneous gut defect detectable through histological analysis (Supplemental Fig. 2B–F).

Rheb+/− mice and WT littermates were challenged with 3% DSS for 6 d, followed with regular drinking water to induce colitis. Similar to rapamycin-treated mice, all Rheb+/− mice died within 8–11 d after DSS treatment, whereas only 20% of control mice died (Fig. 2A). The average body weight of Rheb+/− mice was dramatically decreased. Specifically, at day 10 of treatment, Rheb+/− mice lost 30% of their body weight on average, which was 3-fold higher than that of the WT controls (Fig. 2A). Meanwhile, Rheb+/− mice showed elevated DAI scores relative to DSS-treated WT mice (data not shown) as well as a significant reduction of colon length (Fig. 2A). These findings were validated by histological analysis of colonic sections. Severe tissue damage with a complete loss of crypt architecture in large, continuous areas and severe transmural extensions of inflammatory cell infiltrations were consistently observed in Rheb+/− mouse colon tissues, whereas only minimal architecture erosion and ulceration were detected in the WT controls (Fig. 2B).

We confirmed that the protein level of Rheb in Rheb+/− mice was decreased in the colon tissue with or without DSS treatment (Fig. 2C). After DSS induction, the protein level of phospho-S6 was dramatically attenuated in the colons of Rheb+/− mice compared with WT controls (Fig. 2C, Supplemental Fig. 3). Although phospho-4EBP1 was not induced by DSS treatment, its level in Rheb+/− mice was dramatically reduced in DSS-induced colitis models (Fig. 2C, Supplemental Fig. 3). Furthermore, the expression of proinflammatory cytokines, such as TNF-α, IL-6, IL-1β, and IL-12, was significantly higher in the colon tissue of Rheb+/− mice than in that of WT controls. On the contrary, the expression of the anti-inflammatory cytokine IL-10 was decreased in Rheb+/− mice (Fig. 2D). To investigate the cause of the more severe colitis in Rheb-haploinsufficient mice, cell proliferation and apoptosis were examined. BrdU (Fig. 2E) and KI67 (data not shown) positive signals were significantly reduced in the crypts of Rheb+/− mice.

**FIGURE 1.** mTORC1 is required for protection against chemical-induced experimental colitis. (A) S6 and S6K1 protein and phospho-protein levels in colon tissues of WT mice treated with or without 3% DSS for 6 d were analyzed by immunostaining and Western blotting. (B) Mice treated with DMSO (n = 13) and rapamycin (n = 13) were subjected to DSS-induced colitis. Survival rate, body weight, and DAI were monitored each day. (C) H&E staining of colonic tissues from DMSO (control)- or rapamycin-treated mice after 10 d of DSS treatment. Histological damage was scored after H&E staining. (D) Ki67 immunohistochemistry and TUNEL staining of colonic tissues from mice treated with or without rapamycin on day 6 of DSS administration. Quantitation data are graphed on the right. (E) Mice treated with DMSO (n = 5) or rapamycin (n = 5) were challenged to TNBS-induced colitis. H&E staining of colonic tissues of 4 d of TNBS treatment. Histological damage was scored based on histology analysis. (F) Quantification of KI67-positive colonic epithelial cells from mice treated with or without rapamycin on day 4 of TNBS treatment. Scale bars, 100 μm. Data represent means ± SE. *p < 0.05, **p < 0.01.
Rheb signaling in nonhematopoietic cells plays a major role in protection against DSS-induced injury

Rheb haploinsufficiency in mice phenocopied the defects caused by administration of rapamycin in chemical-induced colitis in mice, suggesting that the activation of mTORC1 signaling is essential for the protection against colitis. However, Rheb is expressed in immune cells as well as in nonhematopoietic cells and regulates the mTORC1 pathway activity in both the innate and adaptive inflammatory responses (28, 29). To determine the cell populations that are critical for Rheb-mediated protection against acute colitis, four groups of bone marrow chimeras were generated. After bone marrow reconstitution, mice were subjected to DSS-induced colitis. Bone marrow cells were collected for genotyping to confirm the success of transplantation when the mice were sacrificed (Fig. 3A). Through the analysis of body weight loss and DAI, we found that Rheb−/− mice receiving Rheb+/− or WT bone marrow lost more body weight than WT mice transplanted with Rheb+/− or WT bone marrow (Fig. 3B). The DAI index also suggested that the colitis was more severe in Rheb−/− recipients than in WT recipients regardless of the genotype of transplanted bone marrow (Fig. 3B). The above clinical disease parameters were confirmed by histological analysis of colonic sections. Specifically, WT mice receiving either Rheb−/− or WT bone marrow showed well-organized crypts with few immune cells in the lamina propria and submucosa. However, extensive crypt damage with excessive leukocyte invasion into the epithelium was observed in Rheb−/− recipients (Fig. 3C). Consistent with the above observations, pS6 level was dramatically decreased in Rheb−/− recipients, suggesting that mTORC1 activity was inhibited (Fig. 3D). These results reveal that Rheb in the nonhematopoietic cells plays a major role in protection against colitis.

Activation of mTORC1 by amino acids reduces experimental colitis

As attenuation of mTORC1 activity exacerbated the experimental colitis in mice, we next explored whether activation of mTORC1 signaling by amino acids had protective effects, because amino acids activate mTORC1 in a Ragulator-Rag– and Rheb-dependent manner (30, 31). To study whether the protective role of amino acids in colitis is mainly through activation of the mTORC1 pathway, colitis was induced in Rheb−/− mice, which were then divided into three groups. One group was left with no further treatment, and the other two groups were treated with amino acids together with or without rapamycin. Severe colonic ulcers developed in DSS-treated Rheb−/− mice, but the epithelial damage was dramatically reduced by treatment with amino acids (Fig. 4A). However, when mTORC1 activity was inhibited by rapamycin in Rheb−/− mice, colonic epithelium was disrupted and the protective role of amino acids was abolished, as determined by histological analysis (Fig. 4A). Similar to previous observations, a small number of Rheb−/− colonic epithelial cells were Ki67-positive proliferating cells whose number was dramatically increased by amino acids stimulation. Rapamycin treatment further inhibited amino acids–stimulated cell proliferation (Fig. 4B). In addition, the inhibitory effect of cell apoptosis by amino acids was abolished by rapamycin (Fig. 4C).

Besides the molecular and cellular positive responses to amino acids stimulation during colitis, a significant reduction of gross symptoms was observed in DSS-challenged animals after amino acids treatment. Although amino acids treatment did not significantly affect the survival rate in WT mice, the body weight loss of amino acids–treated WT mice was reduced compared with control mice (Fig. 4D, 4E). Consistent with previous data (Fig. 2A), all Rheb−/− mice died within 11 d after DSS treatment; however, 30% of amino acids–treated Rheb−/− mice survived after exper-
mental colitis. Additionally, a significant increase in the average body weight was observed in amino acids–treated Rheb+/- mice compared with Rheb+/- controls (Fig. 4D, 4E). After amino acids treatment, the average body weight of the Rheb+/- mouse group was comparable with WT mice without amino acids treatment (Fig. 4E). Rapamycin treatment attenuated amino acids–induced positive effects in DSS-treated Rheb+/- mice. Upon rapamycin treatment, the mice died earlier, accompanied with more severe body weight loss than mice without rapamycin (Fig. 4D, 4E).

These data suggested that activation of mTORC1 by amino acids had positive effects on experimental acute colitis in both WT and mTORC1 hypoactive mice. However, rapamycin treatment abolished the therapeutic effects of amino acids, suggesting that amino acids function through the mTORC1 signaling pathway to provide protection from colitis.

Stat3 activity is inhibited in mTORC1-deficient mice during DSS-induced colitis

Stat3 has been demonstrated to play a critical role in intestinal homeostasis and inflammation-induced mucosal regeneration.
Consistent with previous observations (32), the pStat3 level is very low in mouse colonic epithelial cells under normal conditions (Fig. 5A, control). As Stat3 is an important transcription factor involved in multiple inflammatory responses, DSS-induced colitis led to high phosphorylation of Stat3 residues Ser727 and Tyr705, as expected in WT mice (Fig. 5A, WT). However, in Rheb+/- mouse colonic epithelial cells, only a very faint pStat3 signal was observed in DSS- and TNBS-treated mice (Fig. 5A, 5B). Similarly, pStat3 was downregulated in colonic epithelial cells in rapamycin-treated mice subjected to experimental colitis (Fig. 5C). Interestingly, Stat3 activity was only inhibited in the epithelial cells, but not in lamina propria cells (Fig. 5C), suggesting a cell type–specific role of rapamycin in regulation of inflammation-induced Stat3 activation. In accord with the hyperactivation of Stat3 in colonic epithelium in WT mice upon DSS treatment, we observed a significant elevation of the mRNA expression level of the Stat3 target gene Reg3γ, which is important for antimicrobial activity (33). However, Rheb+/- mice had a much lower induction of Reg3γ, which is the direct target of Stat3, suggesting that Stat3 signaling was impaired in Rheb+/- mice during colitis (Fig. 5D). In addition, Cyclin D1 and Bcl-xL, which play important roles in cell survival and inhibiting apoptosis, are also direct target genes of Stat3. In DSS-induced Rheb+/- mice, the protein level of both Cyclin D1 and Bcl-xL was reduced compared with WT mice (Fig. 5E, 5F).

The above data suggest that amino acids activate mTORC1 activity in the intestines to protect mice from experimental colitis.

**FIGURE 5.** Stat3 activity is inhibited in mTORC1-deficient mice during experimental colitis. (A) pSTAT3 (Ser727, left) and pSTAT3 (Tyr705, right) staining of the colon tissue from WT or Rheb+/- mice with or without 6 d of DSS treatment. (B) Immunofluorescence analysis of pSTAT3 (Tyr705) in colons from WT or Rheb+/- mice treated with TNBS for 4 d. (C) pSTAT3 (Tyr705) staining of the colon tissue from DSS-fed mice treated with DMSO or rapamycin. (D) Changes in the expression levels of the Stat3 target gene Reg3γ, as measured by quantitative PCR from total colon tissue. (E) Western blotting analysis of Bcl-xL and Cyclin D1 expression in colonic mucosa from mice with or without DSS treatment. (F) Bcl-xL and Cyclin D1 staining of the colon sections from mice treated with DSS. High magnification images are shown for each specimen. (G) pSTAT3 (Tyr705) staining of the colon tissue from DSS-fed Rheb+/- mice treated with or without amino acids and rapamycin (RAPA). Scale bars, 100 μm. Data represent means ± SE. *p < 0.05, **p < 0.01.

**FIGURE 6.** Inhibition of mTORC1 impairs Stat3 activation induced by IL-6. (A) Caco-2 cells were pretreated with rapamycin (100 nM) or vehicle control for 6 h, followed by treatment with IL-6 (20 ng/ml) for 30 min. Cells were subjected to Western blot (left) and immunofluorescence (right) assays to examine the pSTAT3 levels. (B) pSTAT3(Tyr705) immunofluorescence analysis of colon pieces isolated from WT and Rheb+/- mice and cultured in medium for 4 h in the presence or absence of 20 ng/ml rIL-6. Scale bars, 50 μm. (C) IL-6–stimulated colon pieces isolated from control and rapamycin-treated mice were subjected to Western blot analysis with specific Abs against pSTAT3, pS6, and pS6K1.
To examine whether Stat3 is also an important downstream target in intestinal epithelial cells, the activity status of Stat3 was determined in amino acids–stimulated mouse models. As shown in Fig. 5G, amino acids strongly induced the pStat3 level in intestinal epithelial cells in Rheb−/− mice, but rapamycin treatment of Rheb−/− mice abolished amino acids–induced activation of Stat3 protein during DSS-induced colitis.

Inhibition of mTORC1 impaired IL-6–induced Stat3 activation

To explore the upstream signals that triggered mTORC1-mediated Stat3 activation, IL-6 was selected due to its potent stimulatory effect on Stat3 and its anti-apoptotic role in intestinal epithelial cells in mice (34). In Caco-2 cell lines, IL-6 significantly induced Stat3 activation, which was partially attenuated by rapamycin treatment (Fig. 6A). These data are consistent with a previous publication that suggested that Stat3 activity is regulated by mTORC1 upon IL-6 stimulation (35). These results were further supported by primary intestinal tissue culture experiments in which Stat3 activity was determined by detecting nuclear localized pStat3 through immunofluorescence staining. pStat3 was very faint in the primary cultured colon tissue of unstimulated WT and Rheb−/− mice (Fig. 6B). IL-6 strongly stimulated pStat3 levels in cultured WT colon tissue, but in Rheb-haploinsufficient tissues both the intensity and the number of pStat3-positive signals were decreased (Fig. 6B). Both Y705 and S727 were phosphorylated upon IL-6 treatment, and the stimulation was abolished by addition of rapamycin to the tissue culture medium (Fig. 6C). These data support the possibility that mTORC1 mediated IL-6 stimulation of Stat3 activity to protect the intestinal epithelial cells from colitis.

Discussion

mTORC1 signaling has been well demonstrated to play essential roles in health and disease through distinct mechanisms (13). In this study, we found that mTORC1 signaling was activated upon intestinal epithelial damage in two chemical-induced experimental colitis mouse models. Through pharmaceutical and genetic approaches, we provide evidence that mTORC1 signaling is essential for intestinal tissue regeneration by regulating Stat3 signaling in epithelial cells. Activation of mTORC1 by amino acids treatment increases Stat3 activity and alleviates colitis in mTORC1-deficient mice. Our study will contribute to further understanding of the role of mTORC1 in intestines during inflammation.

mTOR is a key intracellular regulator of the immune system, and its specific inhibitor, rapamycin, has a strong immunosuppressive activity potentially mediated by modulating differentiation of CD4 T cells (36). A case report has suggested that treatment of a severe refractory colonic and perianal CD patient with rapamycin (sirolimus) had marked and sustained improvement in CD symptoms (36). However, in this study, we demonstrated that rapamycin treatment in DSS- or TNBS-induced acute IBD augmented IBD symptoms and increased mortality in mice. The different effects of rapamycin in human IBD compared with our experimental IBD model are mainly due to pathogenic differences between chronic colitis and acute tissue damage. Refractory CD is considered to be an autoimmune disease due to hyperactivity of the adaptive immune system. It is not surprising that inhibition of mTOR signaling benefits patients with chronic IBD. However, in this study, the experimental colitis models involved an acute cell damage that disrupted the mucosal integrity, which did not develop to an autoimmune response stage. We found that mTORC1 activity was immediately induced in the colonic epithelial cells after DSS treatment (Fig. 1A). This is consistent with the report that mTOR is activated in intestinal ischemia- and irradiation-induced intestinal regeneration (37, 38). Inhibition of mTORC1 activity by either rapamycin treatment or haploinsufficiency of Rheb in mice resulted in increased apoptosis and decreased cell proliferation, leading to more severe tissue damage in our DSS- or TNBS-induced colitis models. mTORC1 activates S6K1 and phosphorylates 4EBP1 to stimulate protein biosynthesis in response to nutrition stimulation. We found that, upon intestinal injury, the S6K1 was activated, but phospho-4EBP1 levels were unchanged (Fig. 2C, Supplemental Fig. 3), suggesting these two key downstream components of mTORC1 play distinct roles in intestinal regeneration. It is reported that rapamycin efficiently inhibits the activity of S6K1, but not 4EBP1 phosphorylation (37). We showed that, in the DSS-induced colitis model, the mice were dead after treatment of rapamycin (Fig. 1B), suggesting that S6K1 is probably the key player downstream of mTORC1. Similar results have been reported in intestinal ischemia/reperfusion injury and LPS-induced lung injury, further supporting our speculation (38, 39). Recently, Fuller et al. (40), using multiple genetically engineered mouse alleles, found that S6K1/2, but not 4EBP1/2, was involved in intestinal regeneration and demonstrated that rapamycin functioned mainly through the mTORC1-S6K pathway rather than through the 4EBP1/2-EIF4E branch. These reports, together with our current study, suggest that mTORC1 plays an essential role in epithelial cells during acute injury mainly through the effector S6K1. Besides S6K1-induced translational elongation, we also found that S6K1-mediated Stat3 activity was critical for stimulation of cell proliferation and inhibition of apoptosis during intestinal regeneration.

Aberrant activation of mTORC1 has been tightly linked to colorectal polyposis and tumors in both humans and mice (41–43). Inhibition of mTORC1 activity through genetic or pharmaceutical approaches has been demonstrated to be efficient at reducing cancer progression (22, 44), suggesting a tumorogenic role of mTORC1 signaling in the intestine. However, the function and molecular mechanism of mTORC1 in tissue regeneration in response to acute inflammation are not well characterized. It is increasingly important to comprehensively understand the role of mTORC1 in acute injury, because adverse clinical effects have been reported associated with sirolimus therapy, especially in the case of lung transplants (25, 45, 46). In the studies based on animal models, rapamycin treatment has been demonstrated to result in acute renal failure, liver regeneration, and lung injury (39, 47, 48). In the current study, we demonstrated the positive contribution of mTORC1-mediated S6K1-Stat3 activation in colon tissues toward recovery from acute experimental colitis in mice. Our finding further reinforces the complexity of mTORC1 functions in cancer and tissue regeneration, which will have potential implications for the clinical use of mTORC1 inhibitors in colorectal cancer patients without acute IBD. In addition, the improvement of the symptoms by amino acids–induced mTORC1 activation will have potential applications in disease treatment.

Acknowledgments

We thank Meizhen Liu for technical assistance. We also thank the members in Dr. Liu and Dr. Li’s group for comments and advice.

Disclosures

The authors have no financial conflicts of interest.

References


mTORC1 PROTECTS AGAINST ACUTE BOWEL INJURY


