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Beneficial effects of dual TORC1/2 inhibition on chronic experimental colitis

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ABSTRACT

Background and aim: AZD8055, a new immunosuppressive reagent, a dual TORC1/2 inhibitor, had been used successfully in animal models for heart transplantation. The aim of this study was to evaluate the effects and mechanisms of AZD8055 on chronic intestinal inflammation.

Methods: Dextran sulfate sodium (DSS) - induced chronic colitis was used to investigate the effects of AZD8055 on the development of colitis. Colitis activity was monitored by body weight assessment, colon length, histology and cytokine profile analysis.

Results: AZD8055 treatment significantly alleviated the severity of colitis, as assessed by colonic length and colonic damage. In addition, AZD8055 treatment decreased the colonic CD4+ T cell numbers and reduced both Th1 and Th17 cell activation and cytokine production. The percentages of Treg cells in the colon were also expanded by AZD8055 treatment. Furthermore, AZD8055 effectively inhibited mTOR downstream proteins and signal transducer and activator of transcription related proteins in CD4+ T cells of intestinal lamina propria.

Conclusions: These findings increased our understanding of DSS-induced colitis and shed new lights on mechanisms of digestive tract chronic inflammation. Dual TORC1/2 inhibition showed potent anti-inflammatory and immune regulation effects by targeting critical signaling pathways. The results supported the strategy of using dual mTOR inhibitor to treat inflammatory bowel disease.

1. Introduction

Inflammatory bowel disease (IBD) is a group of immune mediated inflammatory disease including ulcerative colitis (UC) and Crohn's disease (CD), characterized by chronic or recurring non-specific inflammatory disorder in the digestive tract mucosa. Although the pathogenesis of IBD is considered as multi-factorial, evidence has indicated that genetic predisposition coupled with environmental factors can result in dysfunctional immune responses [1,2]. The abnormal immune response leads to excessive activation and differentiation of T helper (Th) cell subsets or deficiency of regulatory T (Treg) cells, resulting in continuous immune disorder and uncontrolled intestinal inflammation, which can seriously influence the patient's quality of life and their longevity [3,4].

CD4+ T helper cells are the main drivers in the disease process when the intestinal immune balance is perturbed [5]. Th subsets, involving Th1, Th2, Th17, and Treg cells, have been shown to be critically involved in the pathogenesis of IBD in clinical and experimental animal studies [4,6–8]. Differentiation and function of Th subsets are regulated by intricate network of cytokines and transcription factors. The Janus kinase (JAK)-signal transducers and activators of transcription (*STAT*) pathway is one of the significant pathways that involves in regulating Th subsets differentiation and functions [9,10]. Briefly, the differentiation of IFN-γ-producing-Th1 cells depends on downstream transcription factors STAT4 and T-box expressed in T cells (T-bet) in the presence of Interleukin-2 (IL-12) [9]. Th2 cells which secrete IL-4, IL-5, and IL-13 can differentiate in the presence of IL-4. In the Th2 cell differentiation, transcription factors STAT6 and GATA Binding Protein 3 (GATA3) are major master regulators [9]. IL-17-secreting Th17 cells, characterized by the expression of intracellular RAR-related orphan receptor γt (RORγt), can be activated by IL-6 stimulation and subsequent activation of STAT3 [11,12]. In addition, Treg cells, defined by expression of the transcription factor forkhead box P3 (Foxp3), can suppress effector T cells, thus playing a pivotal role in peripheral

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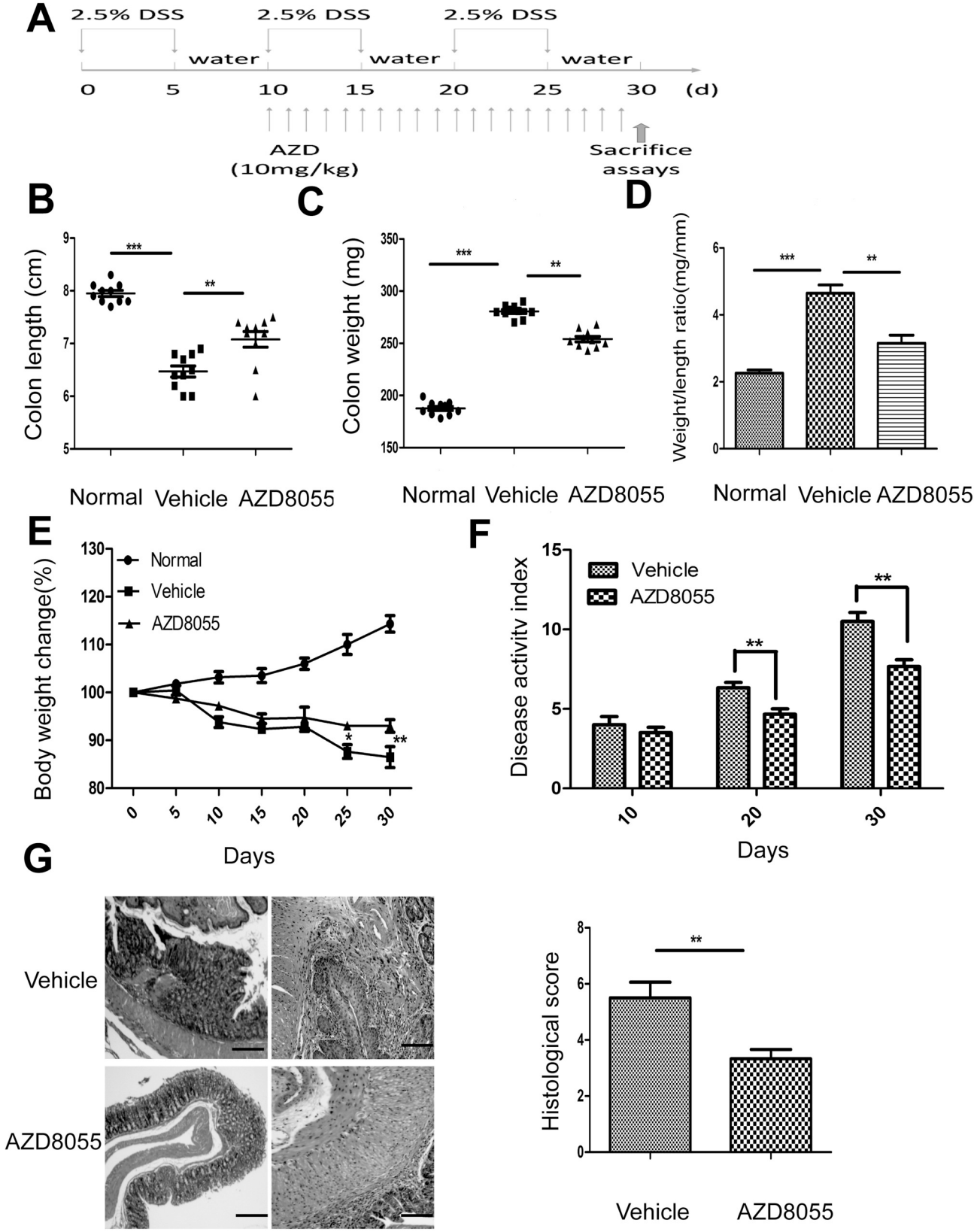

Fig. 1. AZD8055 attenuated DSS-induced chronic colitis. (1) Normal control group (Normal group, n = 10); (2) Vehicle-treated DSS mice (Vehicle group, n = 10); (3) AZD8055-treated DSS mice (AZD8055 group, n = 10). The mice were sacrificed after 3 cycles of DSS at day 30. (A) The workflow of induction of chronic colitis. (B) The length of colon of each group. (C) The weight of colon after removal of the feces. (D) The weight/length ratio of the colon. (E) Body weights were recorded at day 5, 10, 15, 20, 25, 30 and calculated as percentage of the initial weight. (F) Disease activity index (DAI) score was calculated at day 10, 20, 30. (G) Histological observations of colon sections with H&E staining. Scale bars:100 μm. Data represented mean ± SEM of 10 mice per group. All experiments were duplicated for three times. Statistical significance was assessed by one-way ANOVA (B-E) and student's *t*-test (F and G). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

tolerance, preventing autoimmune disease and chronic inflammation [13,14].

Mammalian target of rapamycin (mTOR), including mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2), can regulate the differentiation of T cells by integrating environmental clues including growth factors, amino acid, insulin and co-stimulatory molecule engagement [15,16]. Moreover, the Nuclear Factor-kappaB (NF-κB) pathway is identified as one of the key regulators in this immunological setting of IBD [17]. It has been shown that NF-κB signaling is dependent on mTOR and mTOR inhibition can block NF-κB activation [18–20]. mTORC1 phosphorylates downstream proteins ribosomal protein S6 kinases (p70S6K) and eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1), both of which are involved in protein translation [21]. mTORC2 phosphorylates protein kinase B (PKB, also known as AKT) at the site of Ser473, increasing its enzymatic activity [22,23]. Rapamycin (mTORC1inhibitor) treatment could reduce the number of T helper cells in lamina propria and blocked lymphocytic IFN-γ release, hence ameliorating IL-10 knock out murine colitis [24]. AZD8055 is an ATP-competitive dual mTOR inhibitor, which can block phosphorylation of the mTORC1 downstream substrates p70S6K and 4E-BP1, as well as phosphorylation of the mTORC2 downstream substrates AKT^{S473} and glycogen synthase kinase-3 beta (GSK-3β) [24]. Recent findings demonstrate that dual mTOR inhibition AZD8055 was more effective than mTORC1 inhibition by rapamycin in blocking T cell proliferation and also more significantly prolonged allograft survival in experimental organ transplantation [25].

Although the T cell transfer model of colitis is preferred to study adaptive immune responses within colitis, this model doesn't give comprehension to the pathological T cells in healthy or wild type animals [26]. Dextran sulfate sodium (DSS)-induced colitis, a well-established model, can be applied in all backgrounds of mice and can also respond to many drugs used to treat IBD, which resembles human IBD [27–29]. Chronic experimental colitis is induced by multiple cycles of DSS administration. In this model, Th cell responses play dominant roles and JAK/STAT signaling pathway is central in mediating these cytokines produced by Th cells [26,30,31]. Whether AZD8055 has effect on the development of chronic colitis has not been fully elaborated. In this study, we investigated the potential therapeutic effects of AZD8055 on DSS-induced chronic experimental colitis model and explored the potential mechanisms involved.

2. Materials and methods

2.1. Mice

6–8 weeks old male wild-type C57BL/6 mice were purchased from Shanghai Laboratory Animal Center (SLAC) and maintained in a specific pathogen-free environment in the Research Center for Experimental Medicine of Shanghai Ruijin Hospital. All animal experiments were preformed and approved by the Ethical Committee on Animal Experiments at Shanghai Ruijin Hospital. All endeavors were made to alleviative suffering.

2.2. Drug administration

AZD8055, purchased from StemcellTM, was diluted in sterile emulsifiers and administrated at the dose of 10 mg/kg/d intra-peritoneally (i.p.). AZD8055 was administrated at the beginning of second cycle of DSS (day 10) (treatment protocol). The vehicle (sterile emulsifiers) in an equal volume was injected in control DSS mice accordingly.

2.3. Induction of chronic colitis and assessment of colonic lesions

Chronic colitis was induced in mice by oral administration of 2.5% (*w/v*) DSS (MW 36,000–50,000, MP Biomedical) dissolved in tap water and administrated for three cycles of DSS (5 days DSS followed by 5 days of tap water). Control health mice received normal drinking water throughout. The drugs were administrated at the beginning of second cycle of DSS (day 10). The workflow was shown in Fig. 1A. All mice were monitored including weight loss, diarrhea, rectal bleeding. The disease activity index score (DAI) was described previously [32]. On day 30, the mice were sacrificed and the entire colon was removed, cleaned and weighted. The distal colon length and weight were measured and scored. Difference in weight was considered to be the water content.

2.4. Histological examination

Colonic specimens were embedded in paraffin and subjected to hematoxylin and eosin (H&E) staining for evaluating the severity of colitis. The microscopic score of inflammation was calculated as edema, crypt loss, ulceration, mono- and poly-morphonuclear cells infiltration. The sum of these histological active disease scores were expressed as total microscopic score as previously described [32]. The analysis was performed by two investigators who were blinded to the experiment.

2.5. Preparation of lamina propria lymphocytes

The lamina propria (LP) lymphocytes (LPLs) in the large intestines were collected as described with some modification [32]. Briefly, the colon was opened longitudinally, thoroughly washed in ice-cold PBS, and cut into 1 cm segments. Epithelial cells were removed by incubating pieces of 1 cm segments for 25 min at 37 °C in Hank's Balanced Salt Solution (HBSS) supplemented with 4% FBS and 5 mM EDTA (Sigma-Aldrich). The cell suspension was passed through a 100 μm filter and then cut in 1 mm pieces with scissors, and incubated for 30 min with magnetic stirrer at 37 °C in complete medium RPMI 1640 supplemented with 4% FBS and 1 mg/mL collagen type IV (R&D) and Dnase I (Sigma-Aldrich). After digestions, tissues were passed through a 40 μm filter, and mononuclear cells were isolated by density centrifugation over a 40/80% Percoll discontinuous solution (GE Healthcare) for centrifuging at 2500 rpm for 25 min at room temperature. LPLs were collected at the interface of the Percoll gradient, washed, and suspended in RPMI 1640 containing 4% FBS.

2.6. Intracellular staining and flow cytometry

Mesenteric lymph nodes and lamina propria lymphocytes were isolated and prepared as mononuclear cells from mice. Cells were surface stained with anti-CD3-APC, anti-CD4-FITC, anti-CD8-PE, anti-B220-PE, anti–CD11c-FITC (ebioscience, San Diego, CA) in the presence of Fc blocking antibodies. For intracellular staining, mononuclear cells were stimulated with PMA (50 ng/mL, sigma) and Ionomycin (1 μg/mL, Tocris) and BFA (1:1000, eBioscience) for 6 h. Cells were washed, fixed, permeabilized with Cytofix/Cytoperm buffer (BD Biosciences) and intracellular stained with antibodies against IFN-γ/IL-17/Foxp3 (ebioscience, San Diego, CA). All labeled cells were detected using FCM on the FACSAN Flow Analyzer. The results were evaluated with FlowJo v10.

2.7. Quantitative real-time polymerase chain reaction

Total RNA was extracted from the tissues using the TRIzol reagent (Invitrogen), according to the manufacturer's instructions. RNA concentration was assayed by measuring absorbance at 260 nm, and purity was evaluated from the 260/280 ratio of absorbance using NanoDrop ND1000 (Thermo Scientific). cDNA synthesis was performed using a Prime Script RT reagent Kit (Takara). Real-time polymerase chain reaction (PCR) was performed on QuantiTect SYBR Green PCR Kit. Triplicate measurements were performed and HPRT was used as endogenous control. Results were then analyzed by ΔΔCT-method. The

Table 1
Sequence of primer pairs used in real-time quantitative PCR.

Gene name	Forward primer(5′ → 3′)	Reverse primer(5′ → 3′)
TNF-α	CTCTTCAAGGGACAAGGCTG	CTCTTCAAGGGACAAGGCTG
IL-6	CCAATGCTCTCCTAACAGA	TGTCCACAAACTGATATGC
IFN-γ	CAGCAACAACATAAGCGTC	CTCAAACTTGGCAATACTC
IL-10	AGGGCACCCAGTCTGAGAACA	CGGCCTTGCTCTTGTTTTCAC
IL-4	AACGAGGTCACAGGAGAAGG	TCTGCAGCTCCATGAGAACA
IL-17	CCAGGGAGAGCTTCATCTGT	CTTGGCCTCAGTGTTTGGAC
HPRT	TCAACGGGGGACATAAAAGT	TGCATTGTTTTACCAGTGTCAA
T-bet	AGCAAGGACGGCGAATGTT	GGGTGGACATATAAGCGGTTC
GATA3	GGAAAGCTGGTTCGGAGGCA	GCCGATTCATTCGGGCTCAG
RORγt	GGAGCTCTGCCAGAATGAGC	CAAGGCTCGAAACAGCTCCAC
Foxp3	GAAAGAGCACAT-TCCAGAGTTC	ATGGCCCAGCGGATGAG
IL-23p19	TGTGCCCCGTATCCAGTGT	CGGATCCTTTGCAAGCAGAA

primer sequences are listed in Table 1.

2.8. Western blot analysis

The tissues and cells were lysed in RIPA buffer with Protease/Phosphatase Inhibitor Cocktail (Cell signaling Technology) on ice. The supernatants were collected after centrifugation at 12000 rpm at 4 °C for 30 min. The protein concentrations were measured by BCA assay (Thermo Fisher Scientific) according to the manufacturer's instructions. Then equal amounts of proteins were subjected to SDS/PAGE electrophoresis and blotting. PVDF membranes were blocked by 10% milk in TBST and then incubated with primary antibody. The primary antibodies were purchased from Cell Signaling Technology (except as specifically otherwise noted). Secondary antibodies were used according to manufacturers' instructions. Immuno-reactivity was visualized using ECL (Life Technologies). The protein levels were quantified with Image J software and standardized by calculating the ratio of target to GAPDH mean intensity.

2.9. ELISA assay for cytokines

The entire colon was removed from the distal end of the cecum to the rectum, cut longitudinally, washed with PBS and incubated overnight with complete medium in the 6-well plate. The supernatant levels of interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) were measured using mouse ELISA kits (eBioscience) according to the protocol provided by the manufacturer. The plates were read at 450 nm using a Microplate Reader (Bio-Rad). All experiments were performed at least three times.

2.10. Statistical analyses

For statistical tests, Prism 5.0 (GraphPad Software, SanDiego, CA, USA) was used. Data was presented as means ± SEM from three independent experiments. Statistical analysis was determined by Student's *t*-test and one-way analysis of variance (one-way ANOVA). Statistically significant differences were reported as $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$.

3. Results

3.1. AZD8055 attenuated DSS-induced chronic colitis

The DSS-induced chronic colitis model has been widely used to test the efficacy of preventive and therapeutic agents in IBD [33]. The DSS-induced chronic colitis model and drug treatment protocol were shown in Fig. 1A. The length of the colon in the AZD8055-treated DSS mice was significantly longer compared with vehicle-treated DSS mice (Fig. 1B). The weight of the colon in the AZD8055-treated DSS mice was significantly lower compared with vehicle-treated DSS mice (Fig. 1C). A significant decrease of the ratio of weight to length in the colon was observed in the AZD8055-treated DSS mice compared with vehicle -treated DSS mice ($P < 0.01$) (Fig. 1D). Furthermore, compared with vehicle-treated DSS mice, AZD8055-treated DSS mice showed markedly lower loss of original weight (Fig. 1E). Meanwhile, AZD8055-treated DSS mice displayed distinctly lower DAI score (Fig. 1F). Histological analysis of colon tissue sections from vehicle-treated DSS mice showed typical inflammation. While AZD8055 administration remarkably attenuated intestinal inflammation in DSS mice (Fig. 1G). Taken together, AZD8055 could ameliorate the symptoms of DSS-induced chronic colitis.

3.2. AZD8055 suppressed markers of inflammation in DSS-induced chronic colitis

The NF-κB pathway was identified as one of the key regulators in this immunological setting of IBD [17]. Supernatants levels of TNF-α, IL-1β and IL-6 were significantly reduced in AZD8055-treated DSS mice compared with those from vehicle-treated DSS mice (Fig. 2A). In addition, increased phosphorylation of IκBα and p65 and decreased expression of IκBα were observed in vehicle-treated DSS mice compared with normal control mice. While phosphorylation of IκBα and p65 levels were restored in AZD8055 treatment group (Fig. 2B). These results demonstrated that AZD8055 could block NF-κB pathway, thus probably suppressing inflammation.

3.3. AZD8055 blocked Th1/Th17 responses and promoted Treg cell development

Mononuclear cells from the mesenteric lymph nodes (mLN) and lamina propria lymphocytes (LPL) of vehicle- or AZD8055- treated DSS mice were analyzed by flow cytometry for surface expression of CD4, CD8, B220 and CD11c to differentiate between T cells, B cells and macrophages or dendritic cells infiltration. The percentages of CD4 + T cells both in mLN ($P < 0.01$) and LPL ($P < 0.01$) as well as percentages of CD8 + T cells in LPL ($P < 0.05$) were significantly decreased by AZD8055 treatment (Fig. 3A–C). Nevertheless, the percentages of CD8 + T cells in mLN and B220 + B cells and CD11c + macrophages/dendritic cells in mLN and LPL remained unaltered (Fig. 3A–C). The reduction of CD4 + T cells in mLN and LPL impelled us to investigate which subsets among these cells were affected by AZD8055 treatment. As shown in Fig. 4, the percentages and absolute numbers of Th1 cells (CD4 + IFN-γ + cells) and Th17 cells (CD4 + IL-17 + cells) were significantly decreased in AZD8055-treated DSS mice compared with vehicle- treated DSS mice both in mLN and LPL (Fig. 4A–C). Furthermore, the percentages and absolute numbers of Treg cells (CD4 + Foxp3 + cells) were increased in AZD8055-treated DSS mice compared with vehicle-treated DSS mice both in mLN and LPL (Fig. 4A-C). Therefore, AZD8055 could block Th1/Th17 responses and promote Treg cell development in chronic colitis.

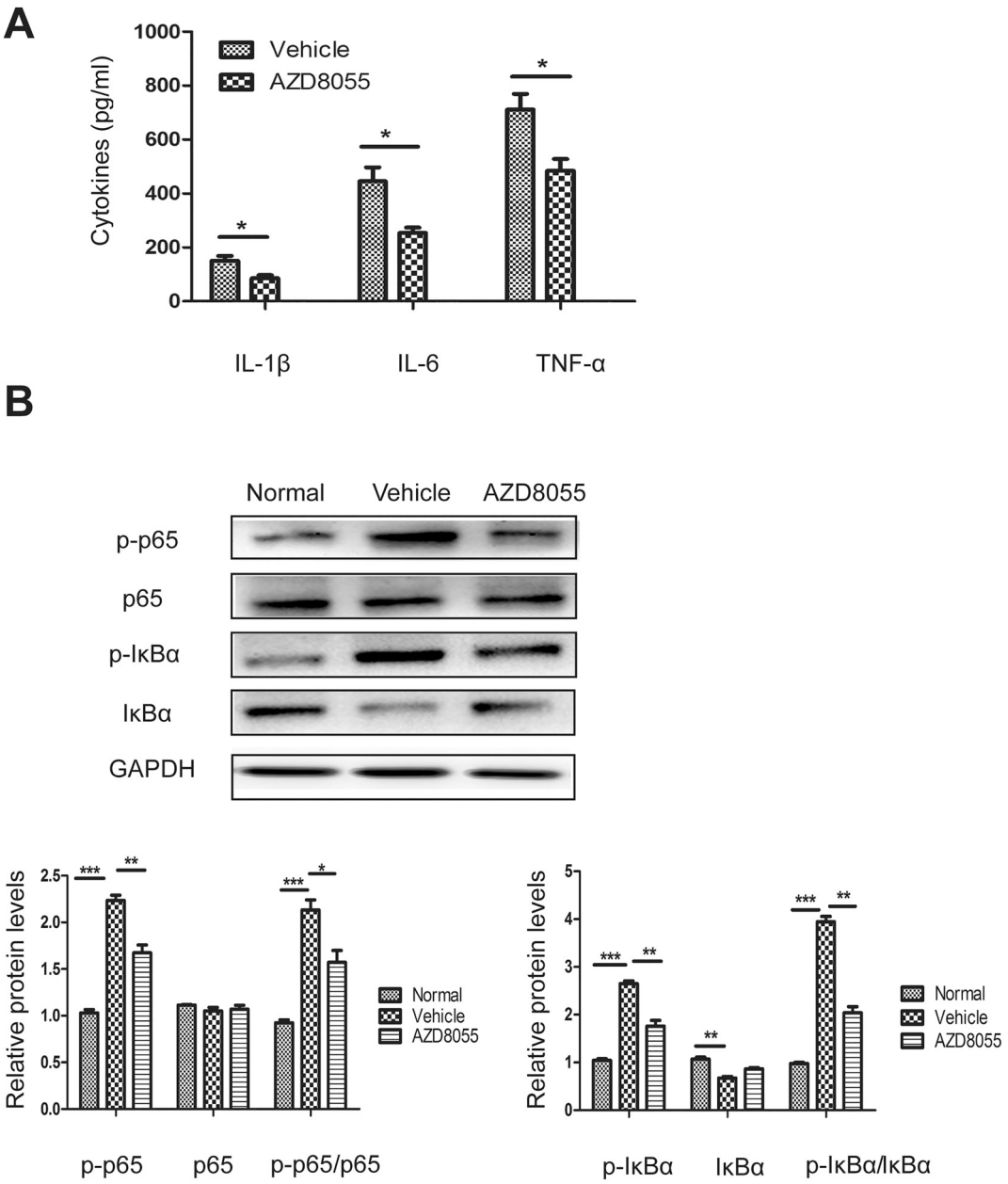

Fig. 2. AZD8055 suppressed NF-κB signaling pathway. (A) Supernatants derived from colonic specimens were analyzed for the level of indicated cytokines. (B) Colonic specimens were analyzed for phosphorylated and total IκBα and p65 levels by immunoblotting. Densitometry analysis of immunoblotting was also shown. Data represented mean ± SEM of 3 independent experiments, n = 3. Statistical significance was assessed by Student's *t*-test (A) and one-way ANOVA (B). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

3.4. AZD8055 inhibited mTOR and JAK/STAT signaling pathway in LP-CD4+ T cells

mTOR downstream pathway and JAK/STAT signaling cascades are reported to be key regulators of the differentiation and function in T cells [9]. CD4+ T cells in the LPL were purified from vehicle- or AZD8055-treated DSS mice. Western blot analysis revealed that phosphorylation of p70S6K, 4E-BP1 (downstream of mTORC1) and AKT^{S473}, GSK-3β (downstream of mTORC2) were reduced by AZD8055 treatment (Fig. 5A). No changes in total p70S6K, 4E-BP1, AKT^{S473}, and GSK-3β protein levels were noticed after any treatment. In addition, as shown in Fig. 5B, consistent with the observation of a reduction in Th1 and Th17 cells, there was a decreased expression of p-STAT4 for Th1 cells and p-STAT3 for Th17 cells. However, p-STAT6 was not affected obviously by AZD8055 treatment. No changes in total STAT4, STAT6 and STAT3 were observed after any treatment. Furthermore, the expression of key transcription factors, T-bet for Th1 cells and RORγt for Th17 cells were significantly decreased while Foxp3 for Treg cells were increased by AZD8055 treatment in the same CD4+ T cell preparations, but not GATA3 for Th2 cells (Fig. 5C). The data collectively demonstrated that AZD8055 could inhibit Th1 and Th17 cells probably through mTOR and JAK/STAT signaling pathways.

3.5. AZD8055 inhibited CD4+ T cell related cytokines and transcription factors in the colon

In the colon tissues of mice, Th1 and Th17 cell related cytokines IFN-γ mRNA and IL-17A mRNA expressions were markedly reduced and their respective mRNA expressions of transcription factor T-bet and RORγt were also suppressed in AZD8055-treated DSS mice compared

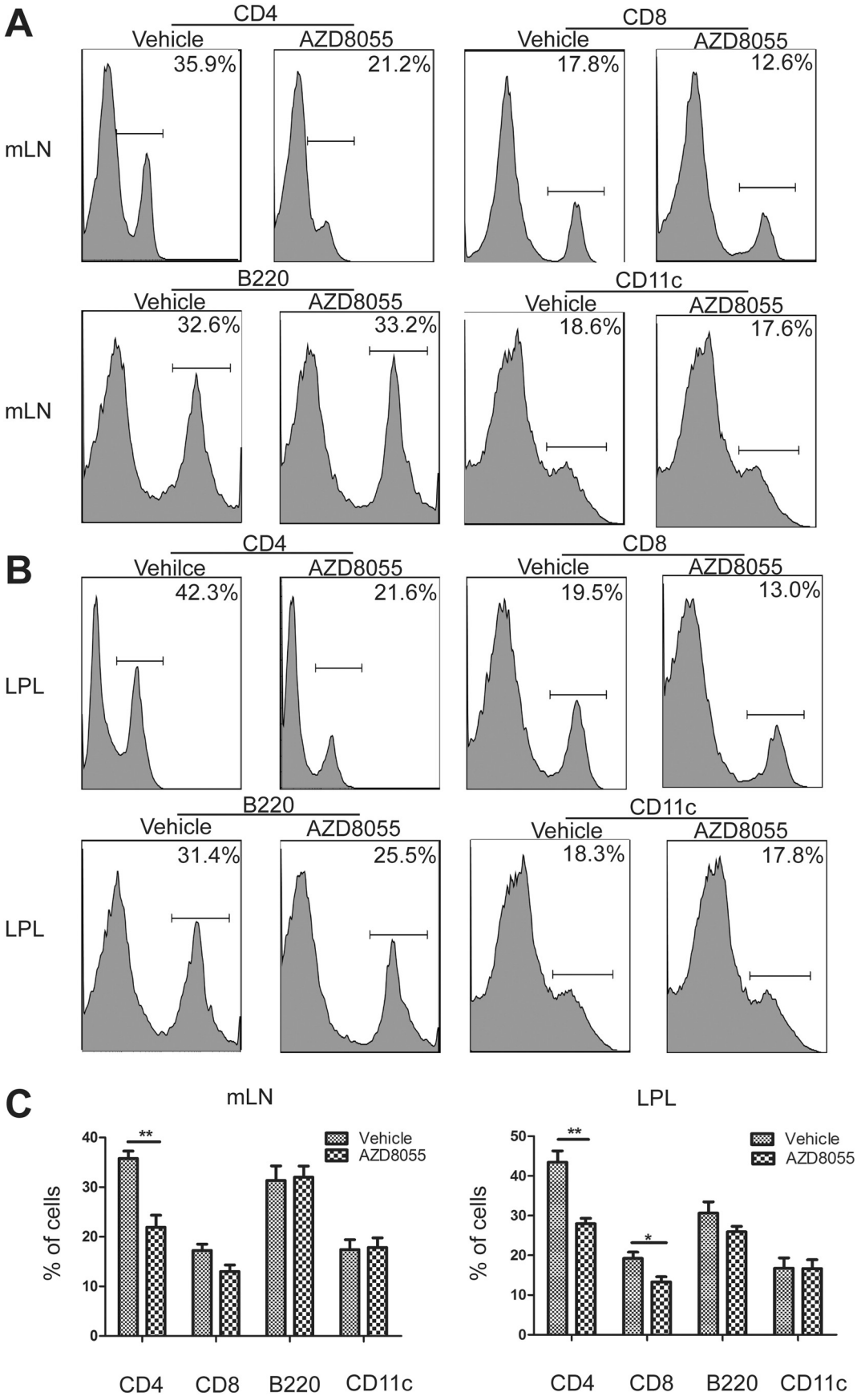

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Fig. 3. the effects of AZD8055 on the T cell, B cell and macrophages/dendritic cells infiltration in the mesenteric lymph nodes (mLN) and lamina propria lymphocytes (LPL). (A) Cells isolated from mLN were analyzed for expression of CD4, CD8, B220 in the lymphocyte gate and CD11c in total mononuclear cell gate by flow cytometry. (B) Cells isolated from LPL were analyzed by flow cytometry. (C) Percentages of cells positively expressed with these antigens in mLN (Left) or LPL (Right) are represented. Data represented mean ± SEM of 3 mice per group. All experiments were duplicated for three times. Statistical significance was assessed by t-test. $^{*}P < 0.05$, $^{**}P < 0.01$.

with vehicle-treated DSS mice (Fig. 6A and C). However, Treg cell related cytokine IL-10 mRNA and transcription factor Foxp3 mRNA expressions were increased by AZD8055 treatment (Fig. 6D and E). As far as Th2 response, levels of IL-4 mRNA and transcription factor GATA3 expressions, showed no statistically significant change by AZD8055 treatment (Fig. 6B and E). These results revealed that DSS exposure resulted in prominent Th1 and Th17 responses.

3.6. Effect of AZD8055 on activation of STAT3 signaling in colon of DSS-induced chronic colitis

mTOR and STAT signaling pathways are key regulators of the differentiation and function of cells in the immune system [34]. STAT3 signaling has emerged as a crucial regulator of the differentiation of Th17 cells [35]. As shown in Fig. 7, the colonic mRNA expression of genes associated with STAT3 signaling pathway, including IL-23p19, TNF-α and IL-6, were notably up-regulated in vehicle-treated DSS mice compared with normal mice, while their expressions were notably down-regulated in AZD8055-treated DSS mice (Fig. 7A–C). In addition, the protein level of phospho-mTOR in the colon was dramatically increased upon cycles of DSS treatment in vehicle-treated DSS mice compared with normal mice, suggesting the potential role of mTOR in intestinal inflammation and regeneration. While the protein level of phospho-mTOR in the colon was decreased in AZD8055 treatment (Fig. 7D and E). Moreover, the protein level of phospho-STAT3 was markedly decreased after AZD8055 treatment (Fig. 7D and E). The above results indicated that the effect of AZD8055 on Th17 response might through STAT3 signaling pathway.

4. Discussion

mTOR signaling pathway plays indispensable roles in health and disease through distinct mechanism [36]. Dys-regulation of mTOR signaling pathway is commonly observed in human inflammatory diseases and cancers [37,38]. Therefore, drugs that target mTOR could be interesting candidate to treat immune mediated inflammatory diseases like IBD. Previous studies have observed that an increased mTOR activity was found in IBD patients [39]. A case reported that use of sirolimus (mTORC1 inhibitor) had sustained improvement in severe refractory Crohn's disease patient symptoms [40]. And inhibition of mTOR signaling pathway benefited patients with chronic IBD [40]. Ogino H et al. reported that regulatory T cells expanded by rapamycin (mTORC1 inhibitor) in vitro could suppress colitis in an experimental mouse model [41]. Yin et al. demonstrated that rapamycin could alleviate the perpetuation of TNBS-induced colitis by promoting the differentiation of Treg cells and inhibiting the generation of Th17 cells [42].

However, the mechanism and activation of mTOR pathway in IBD have not been clearly investigated. In order to investigate the effect and mechanism of AZD8055, chronic DSS-induced mouse model was used. The DSS-induced colitis model shares similar gene expression as IBD patients, being sensitive to common IBD therapeutics, and also displays T cell accumulation in the inflamed colon similar to what is found in IBD patients [26,43–45]. AZD8055 is a selective ATP-competitive inhibitor of mTOR kinase with an IC50 of 0.8 nmol/L [46]. There was no adverse effects by intra-peritoneal injection of AZD8055 in our model (data not show), which was consistent with the report that AZD8055 could suppress allograft rejection by suppressing T cell proliferation and no adverse effects on either renal function or wound healing were found [25]. Our previous study showed that 7 days treatment with AZD8055 was already effective in acute colitis (4% DSS). In the current study, we found that longer treatment with AZD8055 could significantly reduce colonic mucosal inflammation as investigated by clinical symptoms and histological examination in chronic colitis (3 cycles of 2.5% DSS) (Fig. 1).

mTOR is a key intracellular regulator of the immune system, and mTOR inhibition may have an immunosuppressive activity probably by modulating differentiation of CD4+ T cells [47]. CD4+ T cells play an important role in the initiation and progression of IBD. Following antigenic stimulation, mTOR signaling can promote the differentiation of Th1, Th2 and Th17 cells, and suppress the generation of Treg cells in the naïve T cells [48]. T cell-mediated immunity is associated with pathogenesis of chronic colitis induced by multiple cycles of DSS or in the recovery phase of exaggerated colitis [49,50]. The DSS-induced chronic colitis model has recently been considered as a good animal model of colitis mediated by Th1/Th17 CD4+ T effector cells [51,52]. In our chronic model of DSS-induced colitis, the results favored an increase in the percentages of Th1 and Th17 cells. The increased mRNA expressions of cytokine levels and transcription factors in the colon also favored an induction of Th1/Th17 responses. Besides, our study demonstrated that AZD8055 treatment could decrease Th1 and Th17 response and promoted Treg cells. As far as Th2 response, the level of IL-4 was only slightly elevated in DSS-induced chronic model, but it couldn't be inhibited by AZD8055 treatment. The transcription factor GATA3 also remained unchanged. In our observation, there was less evidence of any effect of AZD8055 on Th2 response. However, the effect of AZD8055 on Th2 response could be tested in a prominent Th2 model like the oxazolone colitis. In the case of oxazolone induced chronic colitis model, IL-4 was increased in the early phase followed by IL-13 elevation in the chronic phase [53].

mTOR/STAT pathways play critical role in the control and generation of immune responses. It has been established that phosphorylation of STAT3 expression leads to increased levels of pro-inflammatory cytokines such as TNF-α, IL-6 and IL-23 [54]. These cytokines are also required for the development of DSS-induced chronic colitis [55]. And our data presented here support this conclusion. STAT3 has emerged as an important regulator of the differentiation of Th17 cells. mTOR/STAT3 signaling also play important role in the differentiation of Th17 cells [56]. Furthermore, there exists crosstalk between mTOR and STAT3 signaling pathway which is a crucial pathway in colitis associated cancer [57]. In the current study, we observed that AZD8055 treatment significantly reduced the pro-inflammatory cytokines (IL-23, TNF-α, IL-6) and the expression of phosphorylation of STAT3 and phosphorylation of mTOR in the colon (Fig. 7). mTOR and STAT signaling cascades are reported to be key regulators of the differentiation and function of cells of the immune system [34]. The effect for AZD8055 on pathogenic Th1 and Th17 cells were also controlled by its influence on the JAK/STAT family members, as AZD8055 suppressed p-STAT4 for Th1, p-STAT3 for Th17 cell differentiation in the LP-CD4+ T cells (Fig. 5). The data collectively demonstrated that AZD8055 suppressed Th1 and Th17 cells probably through the JAK/STAT pathway. But whether this is a direct or indirect effect of AZD8055 on JAK/STAT activation needs to be further determined.

Moreover, mTOR is essentially required for activating NF-κB and the role of mTOR inhibition on modulating NF-κB pathways was also described in lymphoma and other cell types [18–20]. NF-κB pathway activation has been observed in many inflammatory and autoimmune

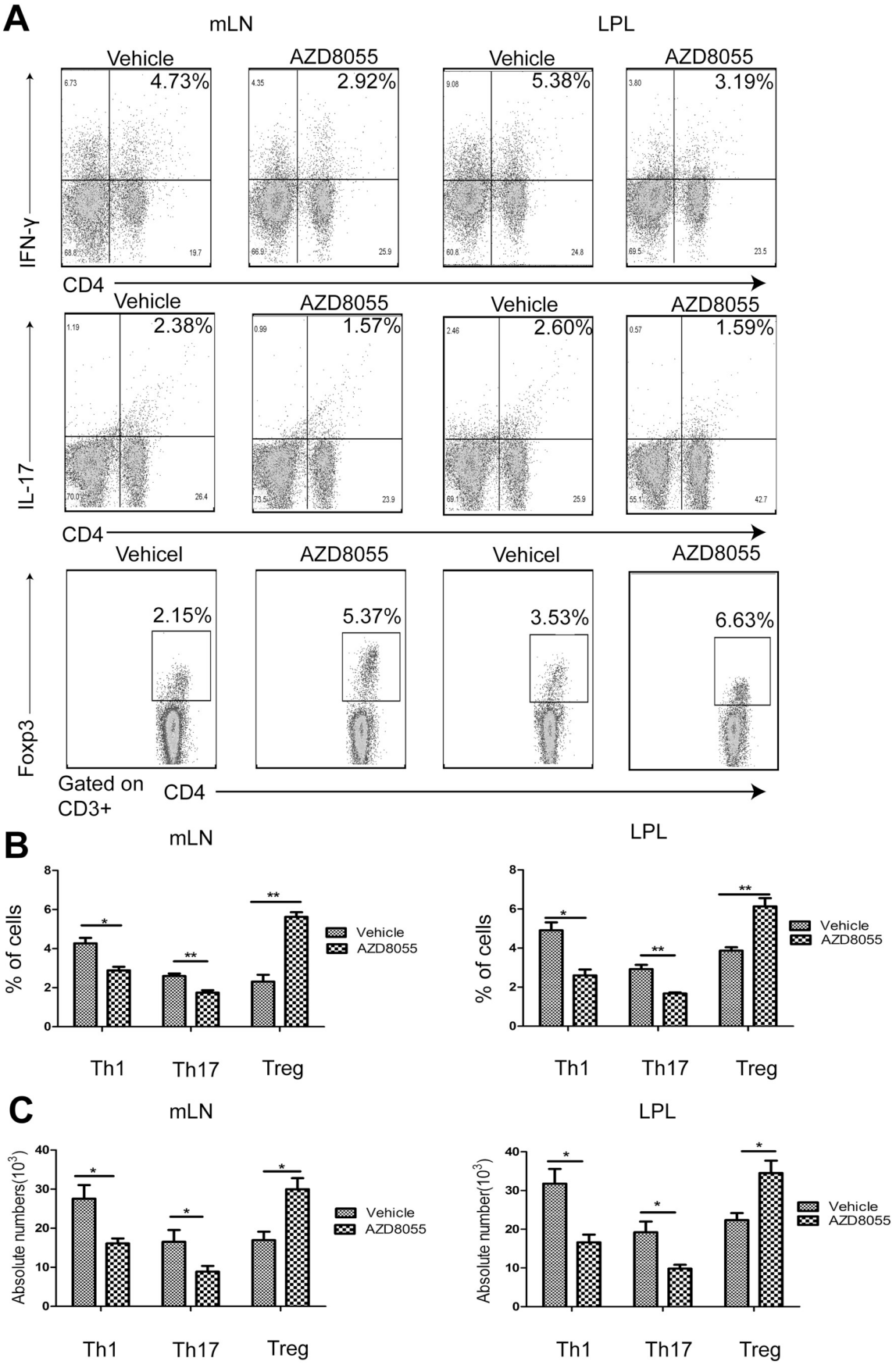

Fig. 4. the percentages and absolute numbers of cytokine-producing T cells. Lymphocytes were isolated from mLN and LPL and analyzed by flow cytometry on day 30. (A) Subsets of Th1/Th17 and Treg cells were analyzed by surface staining of CD4 and intracellular staining of IFN-γ, IL-17 and Foxp3 in mLN (left) and LPL (right). (B) The percentages of cells positive expression with these antigens in mLN (left) or LPL (right). (C) The absolute numbers of Th1/Th17 and Treg cells in mLN (left) or LPL (right). Data represented mean ± SEM of 3 independent experiments, n = 3. Statistical significance was assessed by Student's *t*-test. $^{*}P < 0.05$, $^{**}P < 0.01$.

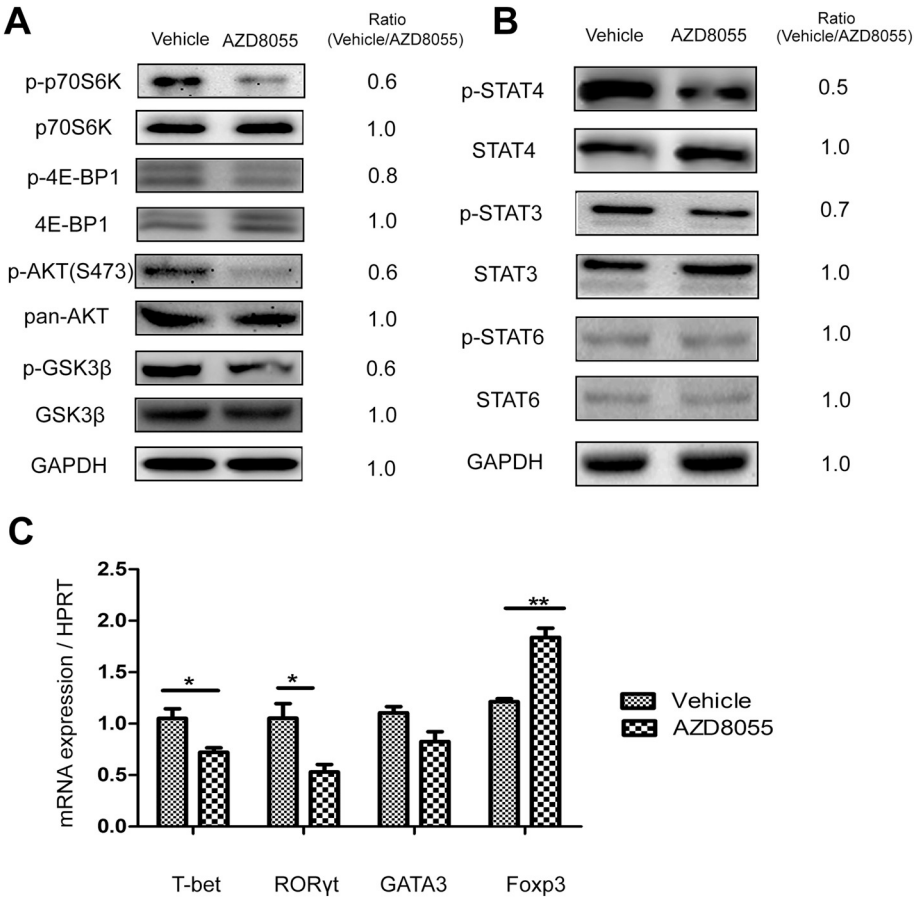

Fig. 5. the effects of AZD8055 on expression of mTOR and JAK/STAT signaling pathway. Purified CD4+ T cells were isolated in the lamina propria from vehicle- or AZD8055-treated DSS mice. (A) CD4+ T cells from LPL were analyzed by immunoblotting for phosphorylation of indicated proteins in the mTOR pathway. (B) The same CD4+ T cell preparations were analyzed in the JAK/STAT pathway. (C) The same CD4+ T cell preparations were analyzed for the mRNA abundance of T-bet, RORγt, GATA3 and Foxp3 by real-time PCR. Data are shown as mean ± SEM from 3 pooled independent experiments (n = 9). Statistical significance was assessed by t-test. $^{*}P < 0.05$, $^{**}P < 0.01$.

responses. The chronic mucosal inflammation in IBD was caused by hyper-activation of immune cells, which produce high levels of pro-inflammatory cytokines like TNF-α, IL-1β, IL-6, leading to colonic tissue damage. The NF-κB pathway was identified as one of the key regulators in this process [17]. The observation of present study demonstrated that AZD8055 treatment could suppress abnormal NF-κB activation through the decreasing phosphorylation nuclear targeting of p65 and phosphorylation level of IκBα and also declined NF-κB related pro-inflammatory genes in the colon (Fig. 2).

mTOR inhibition perhaps establishes long-term immune tolerance by inhibiting the differentiation and function of effector T cells and expanding Treg cells. This mechanism is different from that of other immune suppressants including FK506 and cyclosporine, which mainly block Ca2+ signaling and calcineurin activation downstream of TCR stimulation [58]. CD4+ T cells are considered to be the major initiators of IBD when the immune balance is perturbed. Many subsets of CD4+ T cells such as Th1, Th2, Th17, Th9 and Th22 cells have been recognized as players in perpetuating chronic intestinal inflammation [5]. In future studies, it would be interesting to test the effects of AZD8055 on Th9 and Th22 cells in DSS-induced chronic colitis. It would be also interesting to combine or compare therapies with other immunosuppressive agents like mTORC1 inhibitor, mTORC2 inhibitor, AKT inhibitor and PI3K inhibitor. The classical mTORC1 inhibitor, rapamycin, strongly suppresses the functions of p70S6K (downstream of mTORC1), but has a weak influence on the phosphorylation of the other major mTORC1 targets, like 4E-BP1 [59]. In addition, rapamycin eliminates mTORC1-mediated feedback inhibition of PI3K–AKT signaling, which attenuates its therapeutic effects, as reflected by the disappointing therapeutic outcomes in patients with cancer [60,61]. Although it is generally accepted that rapamycin is a specific inhibitor of mTORC1, prolonged rapamycin treatment can inhibit mTORC2 in some, but not all, cell lines or tissues [23,62,63]. Compared with rapamycin, dual mTOR inhibition AZD8055 could completely inhibit mTORC1 activity, including the rapamycin-resistant phosphorylation of 4E-BP1, as well as strongly block mTORC2 activity, which is less likely to activate the feedback loop [46]. The immunosuppressive effect of mTOR inhibition was obvious in patients or mouse models of immune-mediated inflammatory diseases, such as systemic lupus erythematosus and rheumatoid arthritis [24,64,65]. In our study, we could observe not only that AZD8055 could block mTORC1 and mTORC2 activity in the CD4+T cells isolated from lamina propria, but also suppress the generation of Th1 and Th17 cells and promote Treg cells in the DSS-induced chronic colitis. To further confirm the physiological roles of mTOR and JAK/STAT signaling pathway in DSS-induced chronic colitis, the use of sophisticated genetic systems need to elucidate the ultimate molecular and cellular specificities to demonstrate the underlying processes.

Additionally, in ulcerative colitis (UC), increased apoptosis and proliferation were observed probably due to a recurrence-remission cycle, which was considered to be a sign of increased susceptibility to colorectal cancer [66]. Iwamoto et al. indicated that during the onset of UC, the death of epithelial cells by apoptosis was an early event and occurred mainly in crypts of involved and adjacent uninvolved areas of active UC [67]. AZD8055 accounted for the greater inhibition of cell proliferation and greater induction of autophagy compared to rapamycin, in some cancer cell lines, even cell death [68,69]. However, cell death was observed in company with autophagy but sometimes, in the absence of autophagy [68]. Autophagy is genetically linked with IBD. Pott et al. demonstrated that autophagy within the intestinal epithelium maintained barrier integrity and limited inflammation by protecting the cells from TNF-induced apoptosis in a model of chronic colitis [70,71]. In our research, we found that AZD8055 could partially ameliorate the histological symptoms in the DSS-induced colitis. It was reasonable that the repair/proliferation processes might catch up with the progression of DSS-induced apoptosis during chronic inflammation. This possibility

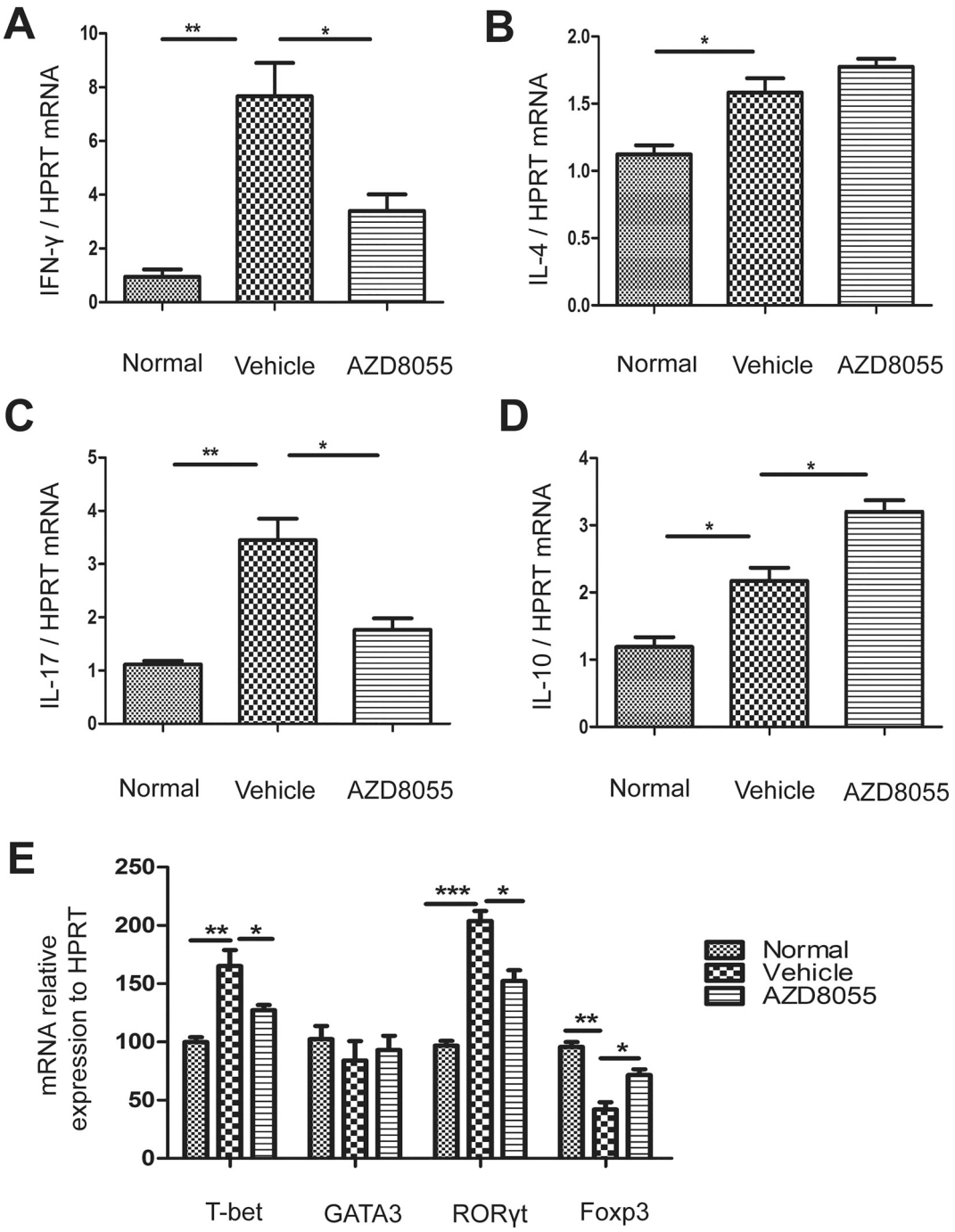

Fig. 6. the effects of AZD8055 on colonic mRNA expressions of Th1- (A), Th2- (B), Th17- (C) and Treg-associated (D) effective cytokines and mRNA expressions of the respective transcription factors (E) of DSS-induced chronic colitis in mice. Data represented mean ± SEM of 3 independent experiments, n = 3. Statistical significance was assessed by one-way ANOVA. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

was supported by recent data demonstrating that colonic epithelial mTORC1 promoted the development of UC through COX-2-mediated Th17 responses. The results showed that RPTOR (essential mTORC1 component) depletion reduced the expression of COX-2, IL-6, and IL-23, as well as Th17 infiltration in the colon, thus ameliorating UC [72]. However, another study showed that mTORC1 inhibition impaired intestinal cell proliferation and induced cell apoptosis in DSS-induced acute colitis, thus augmenting colitis symptoms and increasing mortality in mice [73]. The different effects of mTOR inhibition are mainly owing to pathogenic differences between chronic colitis and acute tissue damage. Refractory and chronic colitis was regarded as an autoimmune disease due to hyperactivity of the adaptive immune system. While, in acute colitis involved an acute cell damage that disrupted the mucosal integrity, which did not develop to an autoimmune response stage [73].

Furthermore, mucosal macrophages also play important role in the development of colitis. In the mucosa of IBD patients as well as in animal models of colitis, there were accumulations of pro-inflammatory macrophages [74]. Pathogen-derived molecules or mediators could impel macrophages polarized into M1 or M2 phenotypes [75,76].However, different phenotypes of macrophages had disparate consequences on the severity of murine colitis. M2-polarized macrophages protected mice from DSS-induced colitis, while M1-polarized macrophages resulted in pathological process [77,78]. Additionally,

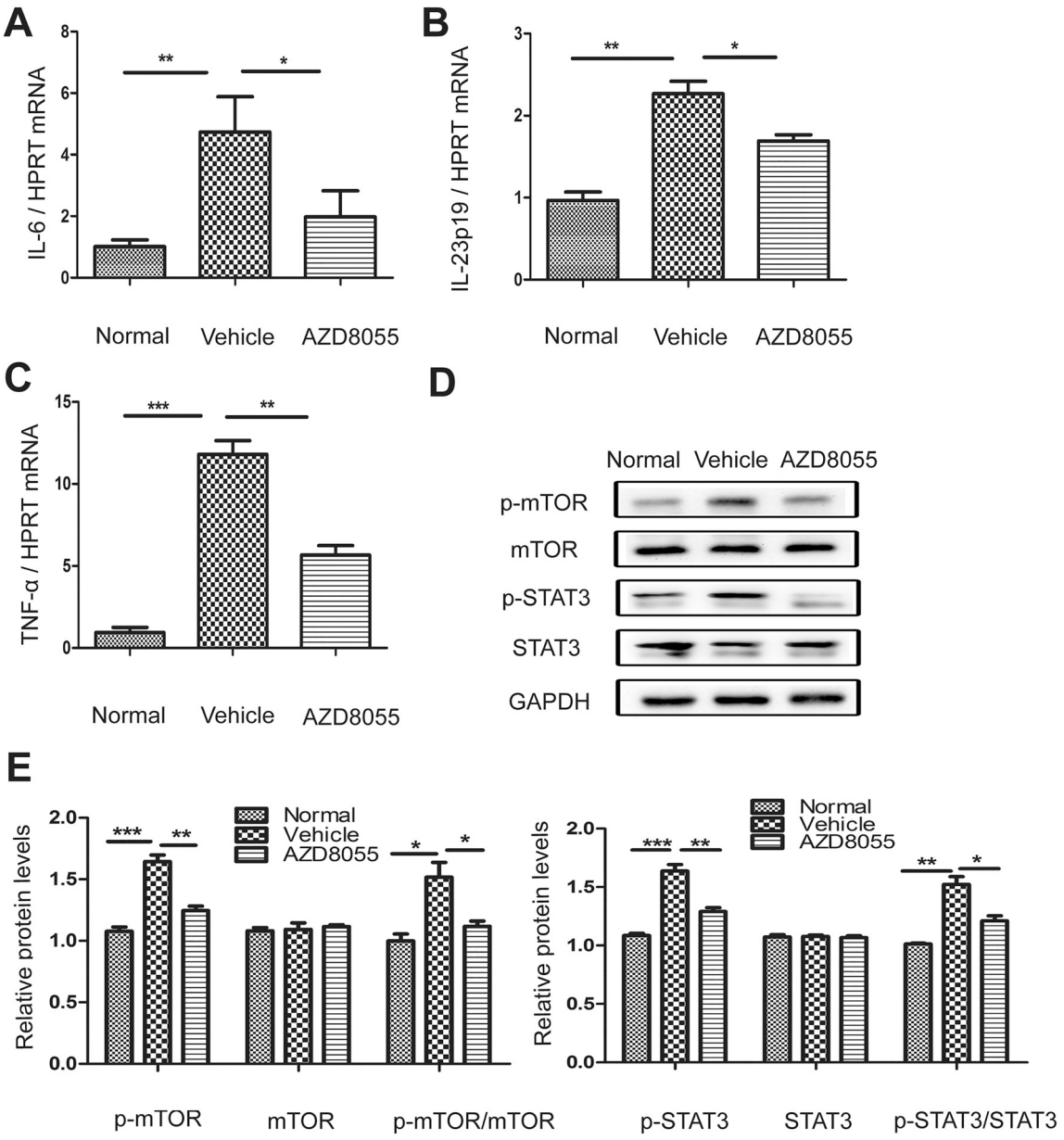

Fig. 7. the effects of AZD8055 on the activation of STAT3 related signaling pathway in colon of DSS-induced chronic colitis in mice. Colonic mRNA expression of interleukin-6 (IL-6) of each group (A), interleukin-23p19 (IL-23p19) of each group (B), and tumor necrosis factor-α (TNF-α) of each group (C). (D) Colonic mTOR and STAT3 activation of colon tissue of normal, vehicle-, or AZD8055-treated mice. (E) Densitometry analysis of immunoblotting was also shown. Data represented mean ± SEM of 3 mice per group. All experiments were duplicated for three times. Statistical significance was assessed by one-way ANOVA. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

recent evidence showed that Myo1F could induce macrophage polarization to a pro-inflammatory M1 phenotype as well as stimulate the production and secretion of IL-1β by stimulating PI3K/AKT/STAT signaling in the DSS-induced colitis model [79]. While Myo1F deficiency or treatment with mTOR inhibitor AZD8055 suppressed macrophage polarized into M1 phenotype and IL-1β secretion by affecting STAT signaling pathway [79]. In the future studies, it is also interesting to demonstrate the effects and mechanisms of AZD8055 on mucosal macrophages in DSS-induced chronic colitis.

Taken together, dual mTOR inhibition AZD8055 alleviated the perpetuation of chronic DSS-induced colitis, and inhibited the generation of pathogenic Th1 and Th17 cells and promoted Treg cells. The possible mechanism was probably through decreasing NF-κB pathway activation and suppression of mTOR/STAT signaling pathway. The continued expansion of our understanding of mTOR signaling will provide therapeutic opportunities for managing IBD.

Abbreviations

IBD	Inflammatory bowel disease
CD	Crohn's colitis
UC	Ulcerative colitis
Th cells	Helper T cells
Th1 cells	T helper 1 cells
Th2 cells	T helper 2 cells
Th17 cells	T helper 17 cells
Treg cells	Regulatory T cells
JAK	janus kinase
STAT	signal transducer and activator of transcription
IFN-γ	Interferon-gamma

T-bet	T-box expressed in T cells
GATA3	GATA Binding Protein 3
RORγt	RAR-related orphan receptor γt
Foxp3	forkhead box P3
mTOR	mammalian target of Rapamycin
mTORC1	mTOR complex 1
mTORC2	mTOR complex 2
ATP	Adenosine triphosphate
DSS	dextran sulfate sodium
p70S6K	Ribosomal protein S6 kinase
4E-BP1	*4E Binding Protein 1*
AKT	also known protein kinase B
DMSO	Dimethyl sulfoxide
HPRT	hypoxanthine-guanine phosphoribosyl transferase
DAI	disease activity index score
PBS	phosphate buffered saline
PMA	phorbol myristate acetate
RAPA	Rapamycin
TNF-α	tumor necrosis factor alpha
IL-1β	Interleukin-1 beta
mLN	mesenteric lymph nodes
LPL	lamina propria lymphocytes
NF-κB	Nuclear factor-kappaB

Conflict of interest

The authors have no conflicts of interest.

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