Biochemical Pharmacology 80 (2010) 1708–1717

Contents lists available at ScienceDirect

Biochemical Pharmacology

journal homepage: www.elsevier.com/locate/biochempharm

AMPK agonist downregulates innate and adaptive immune responses in TNBS-induced murine acute and relapsing colitis

Aiping Bai [a,b], Allan G. Ma [a,c], Michael Yong [a], Carolyn R. Weiss [a,c], Yanbing Ma [a], Qingdong Guan [a,c], Charles N. Bernstein [d,e], Zhikang Peng [a,c,*]

[a] Department of Pediatrics and Child Health, University of Manitoba, Winnipeg, Canada
[b] Department of Gastroenterology, The First Affiliated Hospital, Nanchang University, Nanchang City, China
[c] Department of Immunology, University of Manitoba, Winnipeg, Canada
[d] University of Manitoba IBD Clinical and Research Centre, Winnipeg, Manitoba, Canada
[e] Department of Internal Medicine, University of Manitoba, Winnipeg, Manitoba, Canada

ARTICLE INFO

Article history:
Received 26 May 2010
Accepted 13 August 2010

Keywords:
AMP-activated protein kinase
AMPK
5-Aminoimidazole-4-carboxamide ribonucleoside
AICAR
TNBS-induced colitis
Macrophage
T help cell

ABSTRACT

AMP-activated protein kinase (AMPK), a cellular energy sensor, has been reported to participate in modulating inflammatory responses, but its role in intestinal inflammation remains unclear. IBD has been characterized by excessive innate and adaptive immune responses. Here, the roles of 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR), an agonist of AMPK, in regulating immune responses of experimental colitis were investigated. *In vitro* effects of AICAR on LPS-induced macrophage activation and Th1 and Th17 differentiation, as well as *in vivo* effects of AICAR in mice with 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced colitis, were explored. In acute colitis, daily AICAR treatment commenced 2 days after TNBS delivery (day 1), while in relapsing colitis, AICAR treatment commenced after three weekly TNBS administrations. Colon inflammation, production of proinflammatory cytokines and NF-κB activation in colon tissues, and Th1 and Th17 cell populations in lamina propria mononuclear cells (LPMCs) and mesenteric lymph node cells (MLNs) were assayed. Results show that AICAR significantly inhibited *in vitro* LPS-induced macrophage activation and Th1 and Th17 cell differentiation. Administration of AICAR was therapeutically effective in ameliorating acute and relapsing experimental colitis, as shown by reduced body weight loss and significant attenuation in colon histological inflammation. Moreover, this treatment inhibited NF-κB activation in macrophages, and reduced levels of TNF, Th1- and Th17-type cytokines, and Th1 and Th17 cell populations in LPMCs and MLNs. AICAR-initiated AMPK activation may act as a central downregulator in ongoing innate and adaptive immune responses of murine colitis, providing a novel therapeutic approach in the treatment of IBD.

1. Introduction

Inflammatory bowel disease (IBD), referring mainly to Crohn's disease and ulcerative colitis, is a serious intestinal disorder. Although the etiology of IBD remains unclear, it is strongly suggested that dysregulated immune responses play an important role in the development of IBD [1]. After exposure to the abundant intestinal bacterial antigens or environmental factors, innate immune cells in intestinal mucosa, including macrophages, are activated, leading to the overproduction of proinflammatory cytokines such as TNF, IL-12, and IL-23 [2,3]. Immune cells such as macrophages, together with these proinflammatory cytokines, contribute to maintaining the uncontrolled inflammatory response, eventually leading to the intestinal tissue damage seen in IBD [4,5]. Recently, T helper type 1 (Th1) cells and interleukin (IL)-17-producing T helper (Th17) cells have been demonstrated to play an important role in IBD [6]. Both IFN-γ and IL-17 are highly expressed in the inflamed mucosa of IBD patients [7,8], and inhibiting the production of these Th1 and Th17 cytokines leads to the attenuation of human IBD and animal colitis models [9,10]. Downregulating these immune responses, which include excessive activation of macrophages and Th1 and Th17 responses, can

Abbreviations: AICAR, 5-aminoimidazole-4-carboxamide ribonucleoside; AMP, adenosine monophosphate; AMPK, AMP-activated protein kinase; DSS, dextran sodium sulfate; ELISA, enzyme-linked immunosorbent assay(s); IBD, inflammatory bowel disease; IFN, interferon; IL, interleukin; iNOS, inducible nitric oxide synthase; LPMC, lamina propria mononuclear cell; mAb, monoclonal antibody; MLNs, mesenteric lymph node cells; NF-κB, nuclear factor κB; p-AMPK, phosphorylated-AMPK; RORγt, RA orphan receptor γ; T-bet, T-box expressed in T cells; Th, T-helper cell; TNBS, 2,4,6-trinitrobenzene sulfonic acid; TNF, tumor necrosis factor.

* Corresponding author at: Department of Pediatrics and Child Health, University of Manitoba, 532-715 McDermot Avenue, Winnipeg, Manitoba, Canada R3E 3P4. Tel.: +1 204 789 3815; fax: +1 204 789 3937.
E-mail addresses: zpeng@ms.umanitoba.ca, zpeng@mich.ca (Z. Peng).

0006-2952/$ – see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.bcp.2010.08.009

successfully ameliorate IBD, as indicated by clinical and experimental research [11–14].

5-Aminoimidazole-4-carboxamide ribonucleoside (AICAR) is an intermediate in the generation of inosine monophosphate, which acts as an agonist of adenosine monophosphate(AMP)-activated protein kinase (AMPK) [15,16]. Although AICAR has initially been shown to be involved in the regulation of glucose uptake and fatty acid and cholesterol synthesis, there is recent evidence that via activation of AMPK, AICAR participates in modulating inflammatory responses. For example, AICAR diminishes nuclear translocation of NF-κB and inhibits the production of proinflammatory cytokines such as IFN-γ and TNF, thus, downregulating *in vivo* inflammation in different animal models such as experimental autoimmune encephalomyelitis [17], LPS-induced lung injury [18], and respiratory virus-induced airway inflammation [19]. Whether AICAR influences excessive innate and adaptive immune responses during the development of IBD, however, has not been explored. In the present study, for the first time, we examined the role of AICAR as an agonist of AMPK in the downregulation of immune responses during the development of acute and relapsing murine colitis induced by 2,4,6-trinitrobenzene sulfonic acid (TNBS).

2. Materials and methods

2.1. Animals

Seven- to eight-week-old female C57Bl/6 and BALB/c mice were purchased from Charles River Laboratories (Saint-Constant, PQ, Canada). The mice were maintained in standard animal cages under specific pathogen-free conditions in the animal facility at the Central Animal Care Services, University of Manitoba, Canada. Mice were treated in accordance with a protocol approved by the Institutional Animal Research Review Committee of the University of Manitoba.

2.2. Peritoneal macrophage culture

Peritoneal macrophages were isolated from normal mice, and adjusted to 1.0×10^6 cells/mL in Dulbecco's modified Eagle's medium containing 10% FCS and 2 mM glutamine. The cells were treated with different doses of AICAR for 0.5 h, followed by 100 ng/mL lipopolysaccharide (LPS, from *Escherichia coli* 0111:B4; Sigma) stimulation.

2.3. T cell differentiation in vitro

1.0×10^6 cells/mL $CD4^+$ T cell sorted from splenocytes were stimulated for 4 days with plate-bound antibodies to CD3 (145-2C11, 4 μg/mL, R&D Systems) and soluble antibodies against CD28 (PV-1, 2 μg/mL, R&D Systems). Polarization of T cells into Th1 or Th17 cells was performed with the above antibodies in the presence of recombinant mouse IL-12 (10 ng/mL, eBioscience) and anti-IL-4 (10 μg/mL, eBioscience) for Th1, and human transforming growth factor (TGF)-β (5 ng/mL, eBioscience), recombinant mouse IL-6 (20 ng/mL, eBioscience), and IL-23 (10 ng/mL, eBioscience) for Th17. For flow cytometry analysis, polarized Th17 or Th1 cells were cultured for a further 5 h in the presence of phorbol 12-myristate 13-acetate (PMA, 50 ng/mL), ionomycin (500 ng/mL). Brefeldin A (10 μg/mL) was added during the final 4 h.

2.4. Evaluation of AICAR in 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced acute colitis

In TNBS-induced acute colitis, TNBS was administered on day 1 with minor changes to previously described methods [20,21]. Briefly, C57Bl/6 mice were lightly anaesthetized and 2.5 mg of TNBS (Sigma, St Louis, MO, USA) in 50% ethanol (vol/vol) were slowly administered for a total volume of 0.1 mL per mouse. Normal control mice received 0.1 mL of 0.9% saline alone using the same technique. In the preventive study, mice were intraperitoneally injected with AICAR (500 mg/kg body weight) immediately after the delivery of TNBS (TNBS + AICAR group). This was repeated daily until the mice were sacrificed on day 3. In the intervention study, daily AICAR treatment commenced 2 days after the TNBS delivery until mice were sacrificed on day 7. TNBS mice that received TNBS administration were intraperitoneally injected with 0.1 mL of saline, daily.

2.5. Evaluation of ARCAR in TNBS-induced relapsing colitis

Relapsing colitis was induced with TNBS using previously described methods [22] with minor changes. Five escalating doses of TNBS consisting of 0.5 mg, 0.75 mg, 1.0 mg, 1.25 mg, and 1.5 mg TNBS in 50% ethanol (vol/vol) were administered respectively for a total volume of 0.1 mL per mouse at weekly intervals (at days 1, 8, 15, 22 and 29). Control mice were given intrarectal administration with 0.1 mL of 0.9% saline alone. In the treatment group (TNBS + AICAR), after the third administration of TNBS, the mice were intraperitoneally injected with AICAR (500 mg/kg body weight) daily on days 16–19, 23–26, and 30 (Fig. 8A), while the TNBS group of mice were intraperitoneally injected with 0.1 mL saline at the same days as the treatment group. Two days after the last TNBS or ethanol injection, mice were sacrificed.

2.6. Histology analyses

Paraffin sections of the colon were stained with H&E, and histological scores were performed to grade the degree of colonic inflammation from 0 to 4 using previously described scoring systems for hapten-induced colitis [23].

2.7. Culture of mesenteric lymph node cells (MLNs) and lamina propria mononuclear cells (LPMCs)

MLNs were removed aseptically and gently crushed to prepare single-cell suspensions in Hanks balanced salt solution (HBSS; Sigma, St Louis, MO, USA). The suspensions were washed and resuspended in complete RPMI 1640 medium (RPMI 1640 containing 10% fetal bovine serum, 2 mmol/L glutamine, 25 mmol/L HEPES buffer, and 100 U/mL penicillin, 100 μg/mL streptomycin). LPMCs were isolated from freshly obtained colonic specimens using a modified method, described previously [10]. In brief, the colonic specimens were washed in HBSS–calcium–magnesium free solution, and then incubated in HBSS containing 0.75 mM EDTA (Sigma) and 1 mM DTT (Sigma) at 37 °C for 30 min to remove the epithelium. The tissues were digested further in RPMI 1640 (HyClone, Logan, UT, USA) containing 400 U/mL colleagenase IV (Sigma) and 0.01 mg/mL DNase I (Sigma) in a shaking incubator at 37 °C. This step was repeated 2–3 times. The cells released from the tissues were purified by a 40–100% percoll (Pharmacia Biotech, Piscataway, NJ, USA) gradient. LPMCs were enriched in T cells by incubating LPMCs in petri dishes for 3 h at 37 °C to remove adherent cells.

MLNs and T cell-enriched LPMCs were cultured in 96-well round-bottom plates at 1.0×10^6 cells/mL of complete medium with or without plate-coated murine anti-CD3 antibody (8 μg/mL, R&D Systems) and soluble anti-CD28 antibody (1 μg/mL, R&D Systems) for 48 h.

2.8. Intracellular cytokine staining and flow cytometry analysis

1.0×10^6 T cell-enriched LPMCs or MLNs were simulated with phorbol myristate acetate (PMA; Sigma Chemical Co.) and ionomycin (Sigma Chemical Co.) for 5 h at 37 °C. Brefeldin A (10 μg/mL; Sigma Chemical Co.) was added during the final 4 h of

stimulation. The cells were harvested, preincubated with an Fcγ receptor blocking mAb (CD16/32; 2.4G2) for 15 min, and incubated with FITC-anti-CD4 mAbs for surface staining for 30 min. The cells were then subjected to intracellular cytokine staining, using PE-anti-INF-γ mAbs and APC-anti-IL-17 mAbs for 60 min at 4 °C, according to the manufacturer's instructions (eBioscience Inc., San Diego, CA). The stained cells were analyzed by using a FACS Calibur flow cytometer (Becton Dickinson, Mountain View, CA).

2.9. Cytokines measured by enzyme-linked immunosorbent assays (ELISA)

Cytokine levels in culture supernatants or colonic homogenates were assayed by ELISA, following the manufacturer's instructions (BD PharMingen, San Diego, CA). In short, polyclonal rat anti-mouse cytokine antibodies were used as capturing antibodies and biotinylated polyclonal rat anti-mouse cytokine antibodies were used for detection. Streptavidin-HRP and tetramethylbenzidine sulfonate were added as color indicators and plates were read at 405 nm.

2.10. Western blot

Proteins were extracted from colonic tissues or cultured cells by using a lysis buffer (0.1 M PBS pH 7.4 containing 1% deoxycholic acid sodium, 0.2% SDS, and protease inhibitors). The proteins of the samples were separated by SDS-PAGE and then electrophoretically transferred to polyvinylidene difluoride membranes. The membranes were incubated with primary antibodies, which included rabbit anti-phospho-NF-κB P65, anti-iNOS, anti-β-actin (from Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit anti-AMPK-α1 (Thr172), or anti-phospho-AMPK-α1 (Thr172) (from Cell Signaling Technology). After incubating with secondary antibodies, the immunoreactive bands were visualized using an enhanced chemiluminescence method (Amersham Biosciences, Buckinghamshire, UK). Rabbit anti-β-actin was used as an inner control.

2.11. Real-time PCR

Total RNA was extracted from colonic tissues or from cultured cells by Trizol reagent (Invitrogen, Milan, Italy) and retrotranscribed into complementary DNA (cDNA). PCR was performed using a Syber green based PCR using iQ SYBR mix (Bio-Rad, Hercules, CA). Real-time PCR was performed, and the primers sequences were as follows: RORγ forward: 5′-ACA CCG AGG GCT TAA CAA GAC ACT-3′, reverse: 5′-TGT GTG GTT GTT GGC ATT GTA GGC-3′; IL-23 P19 forward: 5′-TGC ACC AGC GGG ACA TAT GAA TCT-3′, reverse: 5′-TGT TGT CCT TGA GTC CTT GTG GGT-3′; IL-12 P35 forward: 5′-AAG TCT GCC GGC TAT CCA GAC AAT-3′, reverse: 5′-AAC TGA GGT GGT TTA GGA GGG CAA-3′; T-bet forward: 5′-AGC CAG CCA AAC AGA GAA GAC TCA-3′, reverse: 5′-AAT GTG CAC CCT TCA AAC CCT TCC-3′; TNF forward: 5′-TTC CGA ATT CAC TGG AGC CTC GAA-3′, reverse: 5′-TGC ACC TCA GGG AAG AAT CTG GAA-3′; inducible nitric oxide synthase (iNOS) forward: 5′-CTG CTG GTG GTG ACA AGC ACA TTT-3′, reverse: 5′-ATG TCA TGA GCA AAG GCG CAG AAC-3′; 18S ribosomal RNA (rRNA) forward: 5′-CGC CGC TAG AGG TGA AAT TC-3′, reverse: 5′-TTG GCA AAT GCT TTC GCT C-3′. The expression of the genes was analyzed using TaqMan-based assays (Applied Biosystems, Darmstadt, Germany) and calculated relative to the housekeeping gene 18s rRNA using the ΔΔCt algorithm.

2.12. Statistical analysis

Differences between experimental groups were assessed by one-way ANOVA, the Tukey–Kramer multiple comparisons test (for multiple groups), or Student's *t*-test (for comparisons between two groups). $p < 0.05$ was considered to be statistically significant.

3. Results

3.1. AICAR inhibits LPS-induced macrophage activation in vitro

To investigate AMPK activation induced by AICAR in macrophages, we identified the expressions of AMPK and phosphorylated-AMPK (p-AMPK, a marker of AMPK activation) in macrophages incubated with different doses of AICAR by Western blot. As shown in Fig. 1A, treatment of macrophages with AICAR increases AMPK phosphorylation dose-dependently, an effect that reaches maximal levels with the highest concentration of 1 mM AICAR. The expression of p-AMPK is undetectable in cells without the addition of AICAR.

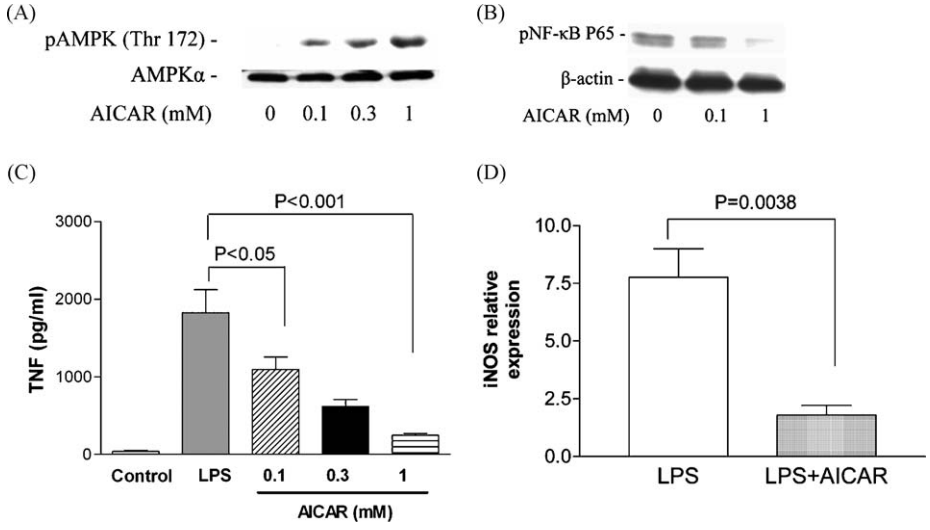

Fig. 1. AICAR *in vitro* inhibits LPS-induced macrophage activation. (A) Phosphorylated-AMPK (p-AMPK) and AMPK. Peritoneal macrophages were cultured and treated with different doses of AICAR. Two hours later, the expressions of p-AMPK and AMPK were detected by Western blot. (B) Phosphorylated-NF-κB (pNF-κB) P65. Peritoneal macrophages were pretreated with different doses of AICAR 30 min before addition of LPS (100 ng/mL). The expression of pNF-κB P65 was detected by Western blot 1.5 h after LPS activation. (C) TNF. The levels of TNF in the supernatants, collected 24 h after LPS stimulation, were assayed by ELISA ($n = 3$ per group). (D) iNOS. The expression of iNOS in AICAR pretreated cells was determined by real-time PCR 3 h after LPS treatment ($n = 4$ per group).

LPS-induced macrophage activation was characterized by the increase of NF-κB activation, iNOS induction, and TNF production (Fig. 1B–D). When the macrophages were treated with AICAR, the expression of phosphorylated-NF-κB P65, and TNF production were inhibited in a dose-dependent pattern (Fig. 1B and C). Treatment with 1 mM AICAR achieves the highest inhibitory effect on TNF production, with significantly downregulated iNOS expression, as shown by real-time PCR (Fig. 1D).

3.2. AICAR inhibits the in vitro differentiation of Th1 and Th17 cells

To investigate AMPK activation in naïve T cells, AICAR was added and incubated with naïve T cells and the expressions of AMPK and phosphorylated-AMPK were detected by Western blot. AICAR administration increased AMPK phosphorylation in a time-dependent pattern: phosphorylation of AMPK was induced immediately in the cells by AICAR, beginning within 30 min after the addition of AICAR and reaching maximum levels after 2 h. This expression was sustained for at least 24 h (Fig. 2A).

As AICAR successfully induced AMPK activation in naïve T cells, we next investigated the *in vitro* effects of AICAR on Th1 and Th17 cell differentiation, the two main Th-type responses during the process of IBD [24]. AICAR was added to the culture from the outset of Th1 or Th17 cell differentiation. The differentiation of Th1 and Th17 cells was significantly inhibited by AICAR administration, as detected by flow cytometry (Fig. 2B and D). As well, the expression of T-bet and RORγt, two key transcriptional factors of Th1 and Th17 cell [25,26], was also significantly downregulated, as detected by real-time PCR (Fig. 2C and E).

3.3. AICAR attenuates TNBS-induced acute colitis

First, we evaluated whether AICAR is effective in a preventive study using a mouse model of hapten-driven acute colitis. Mice with colitis exhibited weight loss right after induction of TNBS colitis, while AICAR-treated mice rapidly regained weight, resulting in a higher final body weight than untreated mice at day 3 ($p < 0.05$) (Fig. 3A). Macroscopically, the colon was markedly shorter in untreated mice than in AICAR-treated mice (Fig. 3B). Histopathologically, untreated colitis mice showed marked infiltration of inflammatory cells, reduction of goblet cells and crypts, focal ulcerations and/or extensive destruction of the mucosal layer, and lymphoid aggregates, whereas AICAR-treated mice showed mild infiltration of inflammatory cells in the mucosa and minimal reduction of goblet cells and crypts (Fig. 3C). Furthermore, TNF, INF-γ, and IL-17 levels in colon tissue were significantly reduced in AICAR-treated mice compared with untreated colitis mice (Fig. 3D). The results suggest that AICAR significantly inhibits the inflammatory process of TNBS-induced acute colitis.

3.4. AICAR inhibits macrophage activation in TNBS-induced acute colitis

Transcription factors of the NF-κB family play an important role in the regulation of genes involved in inflammation [27,28]. In IBD, NF-κB activation is mainly found in macrophages of inflamed intestinal mucosa [29], and it can induce the expression of iNOS and heavy production of proinflammatory cytokines such as TNF and IL-1β [30,31]. As shown in Fig. 4, in untreated mice, p-AMPK is not detectable, but pNF-κB, iNOS expression, and production of

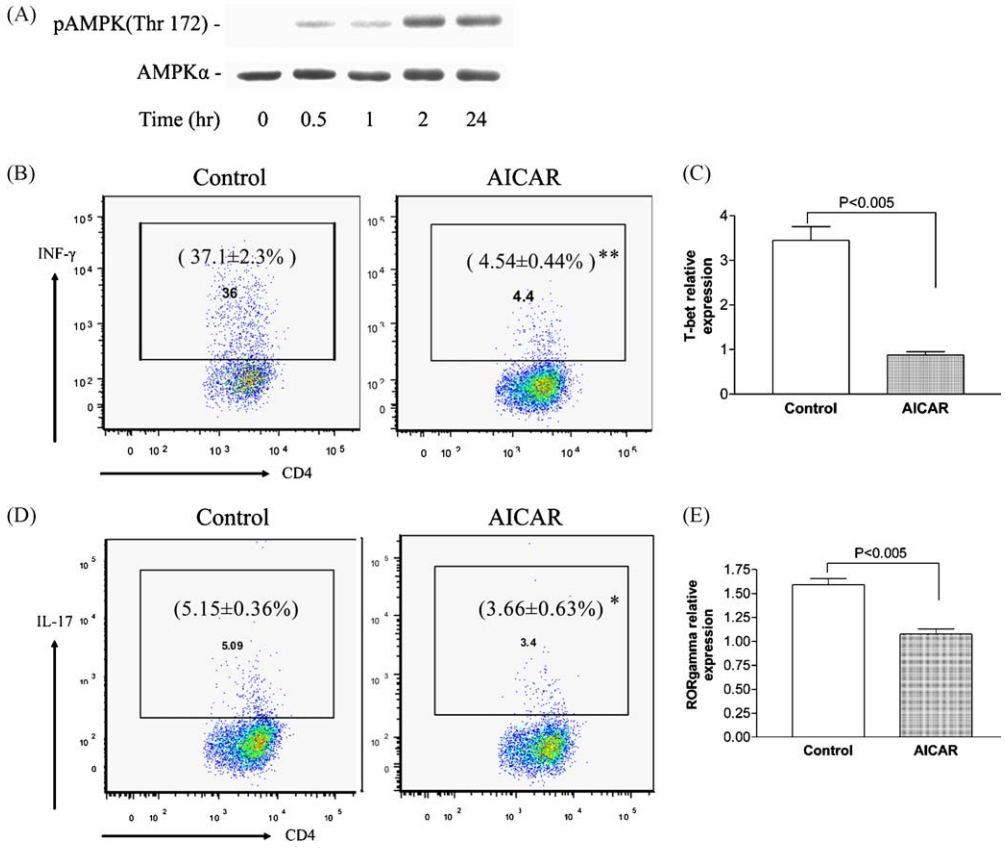

Fig. 2. AICAR *in vitro* inhibits Th1 and Th17 cell differentiation. Naïve T cells were treated with 1 mM AICAR. The expressions of p-AMPK and AMPK were detected by Western blot at the indicated times after AICAR treatment (A). Th1 cell and Th17 cell differentiations were induced respectively for 4 days. Th1 and Th17 cell populations were detected by flow cytometry (B and D; $n = 4$ per group, $^{*}p < 0.05$, $^{**}p < 0.001$, compared with Control), and the expressions of T-bet and RORγ were determined by real-time PCR (C and E; $n = 3$ per group).

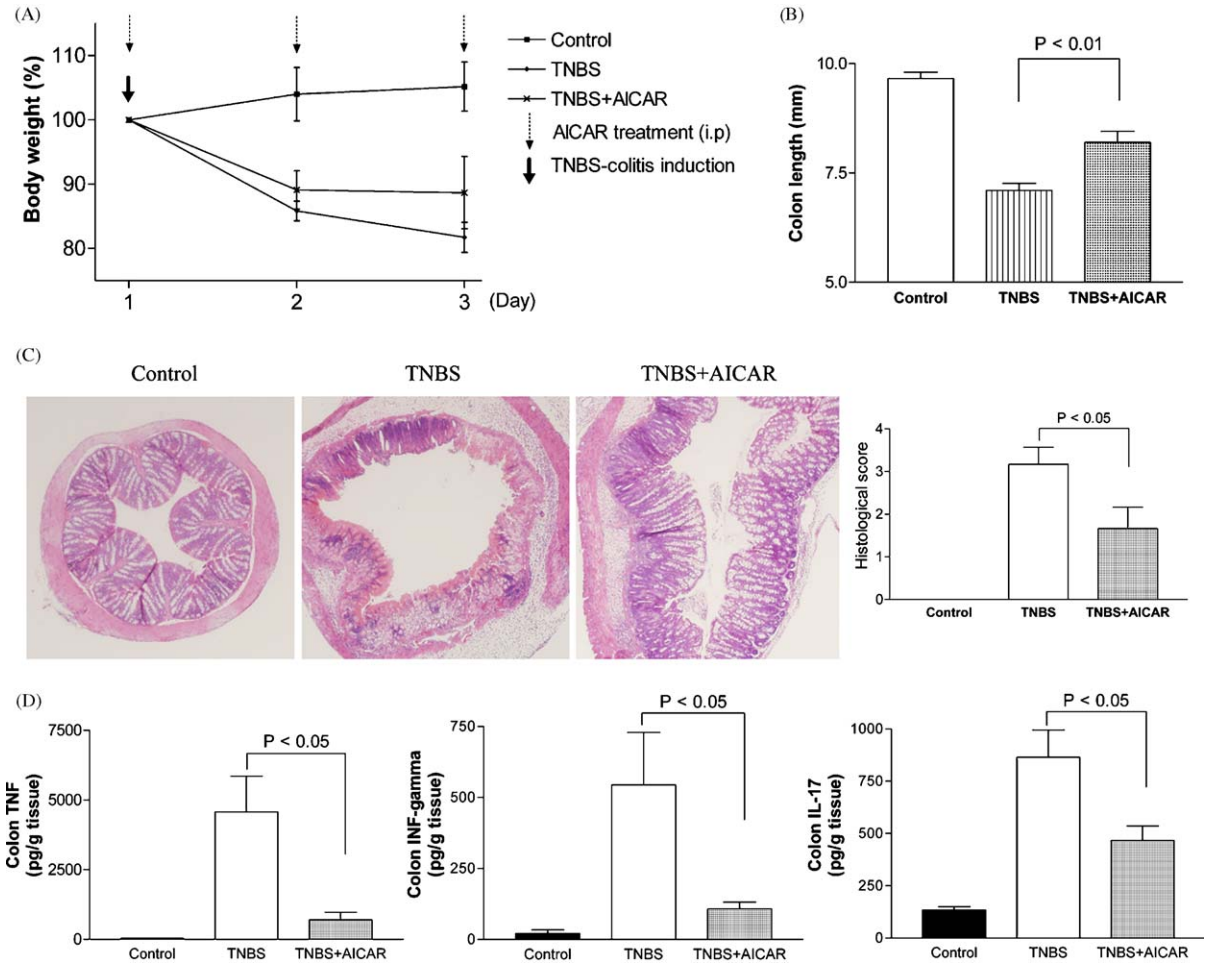

Fig. 3. AICAR treatment attenuates TNBS-induced acute colitis. Colitis was induced by intrarectal administration of 2.5 mg TNBS. AICAR was given i.p. immediately after TNBS administration and repeated daily until the mice were sacrificed on day 3. (A) Body weight changes (n = 10). (B) Colon length. (C) Colon inflammation. Colon sections were stained with H&E (magnification 40×) (left) and inflammation scores were evaluated (right). (D) The levels of TNF, INF-γ, and IL-17 in colonic homogenates determined by ELISA (n = 5).

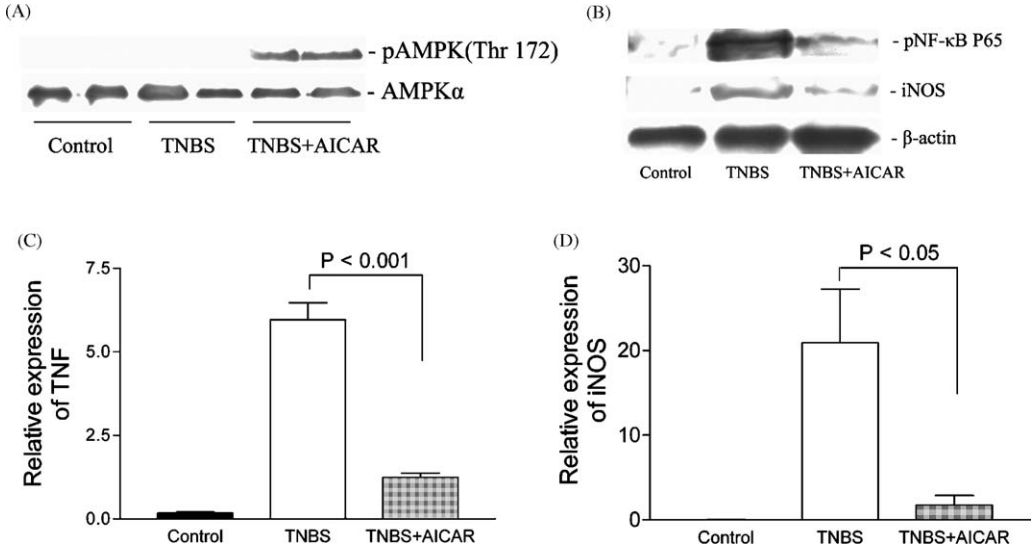

Fig. 4. AICAR treatment reduces colon inflammatory responses of TNBS-induced acute colitis. TNBS colitis was induced at day 1, and AICAR was given i.p. immediately after TNBS administration and repeated daily until the mice were sacrificed on day 3. The expressions of p-AMPK/AMPK, pNF-κB P65, iNOS and β-actin were identified by Western blot analysis (A and B), and the expressions of iNOS and TNF were identified by real-time PCR (C and D, n = 5 per group).

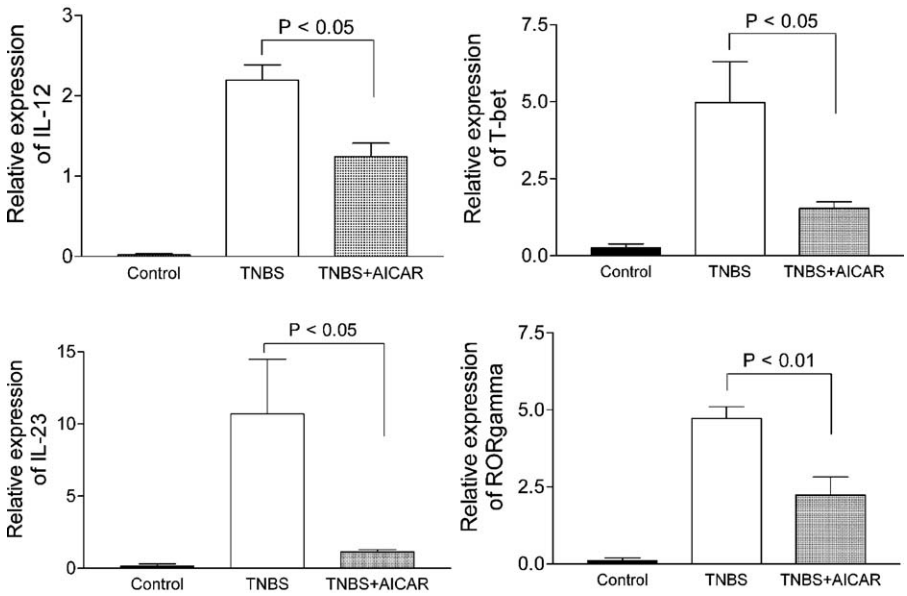

Fig. 5. Th1 and Th17 cytokine expressions are downregulated in acute TNBS-induced colitis by AICAR treatment. TNBS was intrarectally administered at day 1 and AICAR was given i.p. immediately after TNBS administration and repeated daily until the mice were sacrificed on day 3. The expressions of IL-12 (p35), IL-23 (p19), and the transcriptional factors such as T-bet and RORγt were identified by real-time PCR ($n = 5$ per group).

proinflammatory cytokines such as TNF in colon tissues are significantly increased. AICAR treatment induced, to a high degree, the expression of p-AMPK in the colon tissues of AICAR-treated mice (Fig. 4A). This was accompanied by significantly inhibited NF-κB activation and, thus, downregulated iNOS expression and TNF production in colon tissues of mice in comparison with mice without AICAR treatment (Fig. 4B–D). These results collectively suggest that AICAR has a broad inhibitory effect on the inflammatory properties of macrophages.

3.5. AICAR inhibits Th1 and Th17 levels and polarization in TNBS-induced acute colitis

TNBS-induced acute colitis is characterized as having predominant Th1 and Th17 cytokine responses [20,21]. To define whether AICAR inhibits both Th1 and Th17 cell responses of TNBS-induced acute colitis, we examined the expression of the IL-12/IFN-γ pathway and the IL-23/IL-17 pathway by real-time PCR. High levels of IL-12, IL-23, T-bet, and RORγt were detected in the colons of colitis mice receiving no treatment, while AICAR-treated mice showed inhibited expression of those molecules (Fig. 5).

To confirm the *in vivo* effects of AICAR treatment on $CD4^+$ T cells differentiation, we performed intracellular staining of LPMCs and MLNs by membrane CD4 and intracellular INF-γ or IL-17 double staining in order to detect Th1 or Th17 cell population. The administration of AICAR decreased the numbers of both IFN-γ-producing cells and IL-17-producing cells in LMPCs and MLNs ($p < 0.05$) (Fig. 6), in agreement with the result of inhibited production of INF-γ and IL-17 in colon tissues. This result indicates that AICAR may inhibit Th1 and Th17 cell responses, and, thus, downregulate IFN-γ and IL-17 production.

3.6. AICAR ameliorates established TNBS-induced acute colitis

Next, to study the therapeutic effect of AICAR on established TNBS-induced acute colitis, AICAR treatment was given 2 days after the TNBS delivery. Mice with colitis exhibited weight loss over the course of the experiment, while AICAR-treated mice rapidly regained weight after AICAR administration, resulting in a higher final body weight than untreated mice at days 6 and 7 ($p < 0.05$) (Fig. 7A). Also, the colon was markedly shorter in untreated mice than in AICAR-treated mice (Fig. 7B). These changes were correlated with the macroscopic and histological scores of the colon (Fig. 7C) and proinflammatory cytokine levels such as TNF, INF-γ, and IL-17 in colon tissues of different mice groups (Fig. 7D). AICAR treatment also induced increased p-AMPK expression in colon tissues (data not shown). The results suggest that AICAR treatment successfully ameliorates TNBS-induced acute colitis.

3.7. AICAR ameliorates established TNBS-induced relapsing colitis

Because IBD is a chronic disease with a high relapse rate, we then assessed whether AICAR treatment also affected the course of TNBS-induced relapsing colitis. During the process of TNBS-induced relapsing colitis, the loss of body weight in untreated mice was first observed just after each TNBS delivery. In contrast, the mice receiving AICAR treatment showed less of a body weight loss (Fig. 8B). Moreover, AICAR treatment improved the macroscopic and histological appearance of the colon wall, with longer colon length (Fig. 8C), lower histological scores (Fig. 8D), and lower TNF, INF-γ, and IL-17 productions in colon tissues (Fig. 8E), and increased p-AMPK expression (data not shown), compared to those of untreated colitis mice. This provides direct evidence that AICAR treatment ameliorates TNBS-induced relapsing colitis.

4. Discussion

Recently, it has been reported that AMPK activation induced by AICAR can inhibit NF-κB activation and, thus, downregulate TNF production of macrophages [32]. The effectiveness of AICAR was shown for certain inflammatory diseases in murine models such as LPS-induced lung injury [18] and autoimmune encephalomyelitis [17]. In IBD, intestinal macrophages localized in the subepithelial region and intestinal lamina propria are the main source for proinflammatory cytokines, including TNF and IL-1β [2]. Because macrophages represent the principal inflammatory cells in the mucosal microenvironment and contribute significantly to the tissue damage involved in IBD [4], we studied the inhibitory effect of AMPK activation initiated by AICAR on macrophages during the

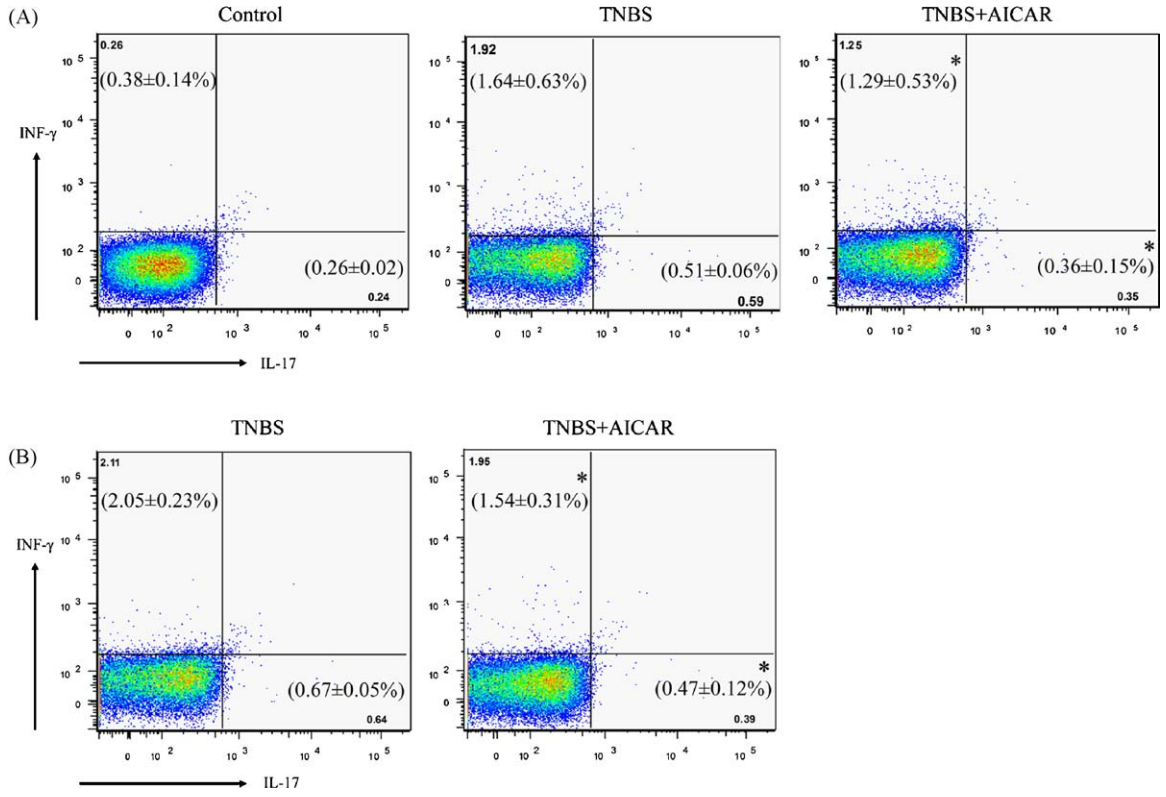

Fig. 6. AICAR treatment inhibits Th1 and Th17 cell responses in TNBS-induced acute colitis. Colitis was induced by intrarectal administration of 2.5 mg TNBS. AICAR was given i.p. immediately after TNBS administration and repeated daily until the mice were sacrificed on day 3. MLNs and LPMCs were isolated respectively from control, TNBS, and TNBS + AICAR groups immediately after the mice were sacrificed. (A) Th1 (INF-γ) or Th17 cell population in MLNs determined by flow cytometry ($n = 4$ per group). (B) Th1 or Th17 cell population in LPMCs determined by flow cytometry ($n = 4$ per group). $^*p < 0.05$, compared with TNBS group.

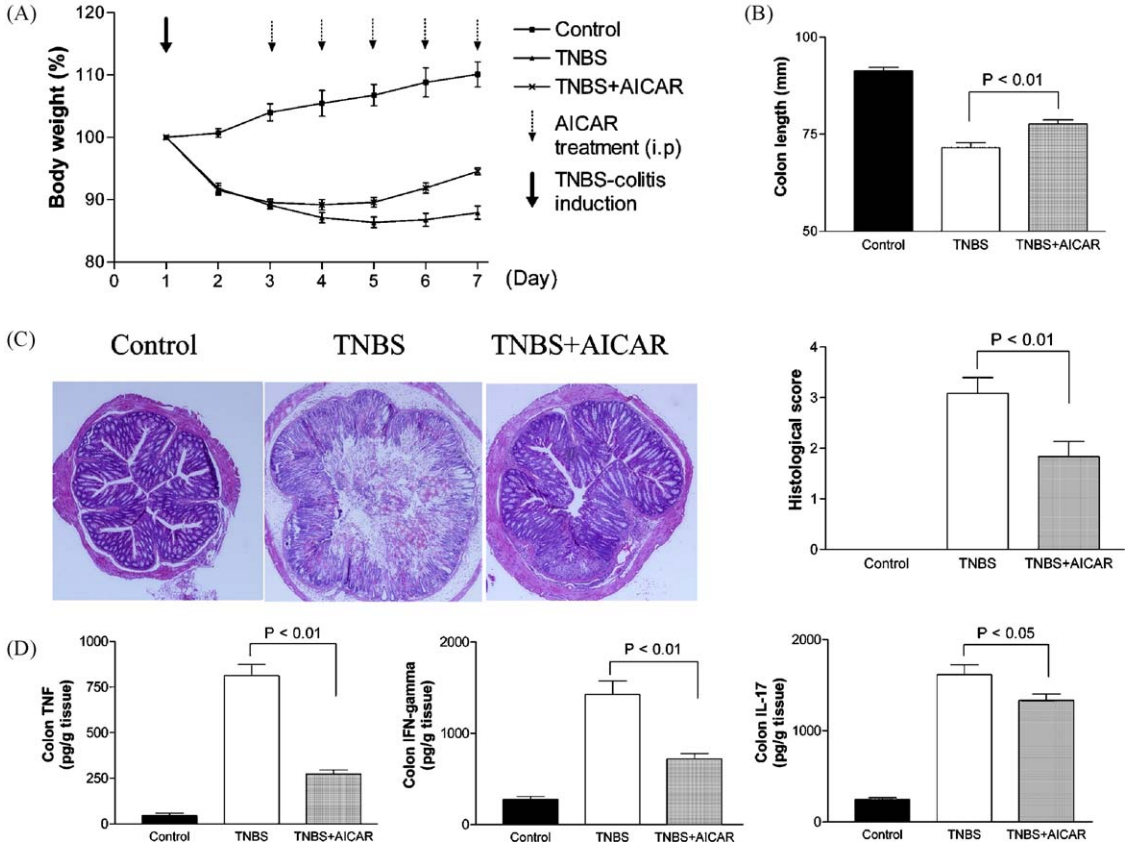

Fig. 7. AICAR treatment, starting 2 days after TNBS administration (days 3–7), attenuates ongoing acute colitis. Colitis was induced by intrarectal administration of 2.5 mg TNBS at day 1. AICAR was given i.p. daily from days 3 to 7. (A) Body weight ($n = 12$). (B) Colon length. (C) Colon inflammation. Colon sections were stained with H&E (magnification 40×) (left) and inflammation scores were evaluated (right). (D) The levels of TNF, INF-γ, and IL-17 in colonic homogenates determined by ELISA ($n = 6$).

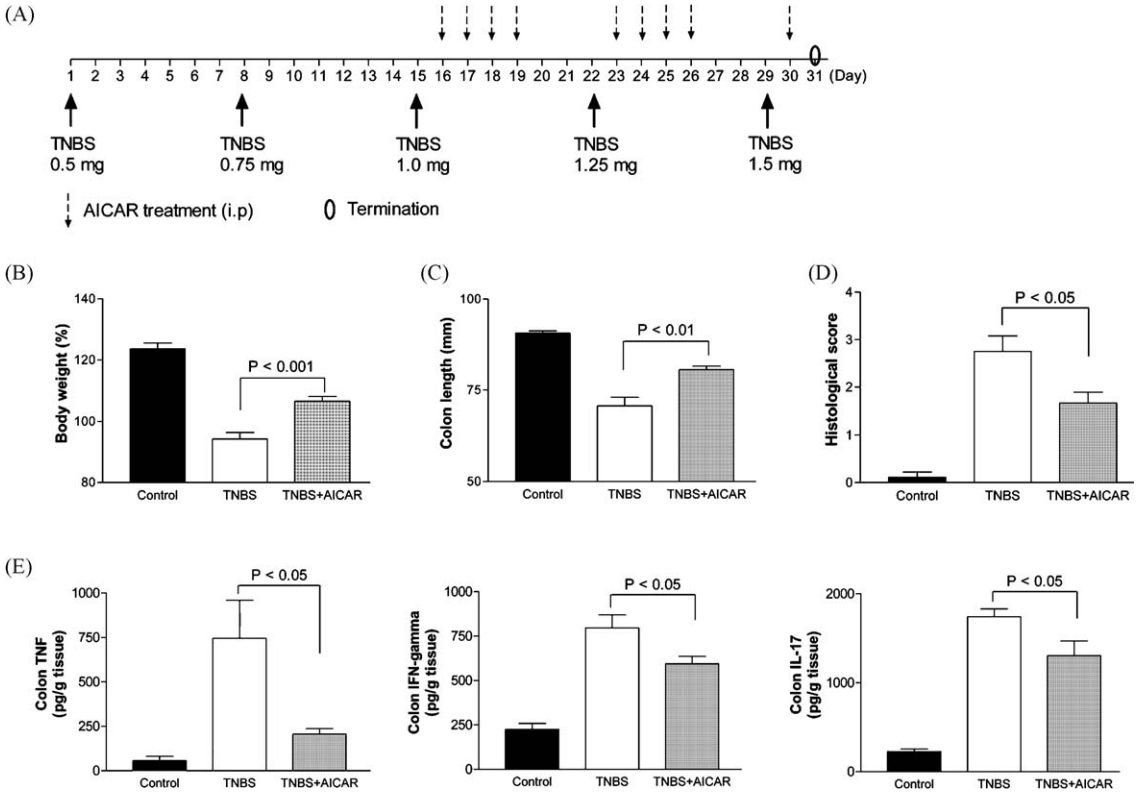

Fig. 8. AICAR treatment, commencing after three TNBS administrations, significantly ameliorates ongoing relapsing colitis. (A) Protocol. Relapsing colitis was induced by five escalating doses of TNBS administration at a 1-week interval. AICAR treatment was given i.p. after the third TNBS administration at the indicated days. (B) Body weight (at day 31, $n = 12$). (C) Colon length. (D) Colon inflammation. Colon sections were stained with H&E (magnification 40×) (left) and inflammatory scores were evaluated (right). (E) The levels of TNF, INF-γ, and IL-17 in colon homogenates determined by ELISA ($n = 6$).

process of murine colitis. In both the *in vitro* and *in vivo* experiments, we found that AICAR administration inhibited NF-κB activation, which subsequently resulted in a down-regulation of iNOS expression in macrophages and an inhibition of TNF production, a key proinflammatory cytokine in the pathogenesis of IBD. Overall, AICAR administration resulted in an amelioration of experimental colitis, indicating that one of the mechanisms for the anti-inflammatory effect of AICAR is through the inhibition of innate immune responses driven by macrophages.

IBD has been characterized by excessive Th1 and Th17 cell responses [24]. The dysregulated Th1 and Th17 cell responses lead to alterations in mucosal cytokines expression, including increased IFN-γ and IL-17 expression, two key mediators in IBD. Promotion of Th1 and Th17 cell responses induced by IL-21 [22] and IL-23 [7] leads to the exacerbation of colitis, while inhibition of Th1 and Th17 cell responses by monoclonal antibodies or an IgG fusion protein to the p40 subunit of IL-12 and IL-23 improves IBD and murine colitis [9,33]. Thus, blocking these cytokines has been investigated as a potential therapy for Th1- and Th17-mediated autoimmune diseases. Activation of immune cells requires high metabolic demands and is associated with increased glycolysis and energy consumption [34,35]. As an important cellular energy sensor that plays a role in maintaining systemic and cellular energy balance [36], AMPK activation is largely responsible for immune cell function. Since predominant Th1 and Th17 responses also require high metabolic demands, it is speculated that the excessive Th1 and Th17 responses occurring in IBD can be inhibited by AICAR-initiated AMPK activation. To confirm this, we first studied the *in vitro* effect of AMPK activation initiated by AICAR on Th1 and Th17 cell differentiation and discovered that AICAR downregulated the expressions of T-bet and RORγt, subsequently impairing Th1 and Th17 cell differentiation. Secondly, to analyze the *in vivo* immune responses in mice with colitis, we used both TNBS-induced acute and relapsing murine colitis models, which have shown predominant Th1 and Th17 cell responses [20–22] and have been used in the study of IBD, especially Crohn's disease, by many investigators. In the *in vivo* evaluation, we found that AICAR inhibited adaptive immune responses during the process of colitis, with reduced Th1 and Th17 cell populations in the lamina propria and mesenteric lymph nodes. This was associated with the inhibition of Th1-type cytokines IFN-γ and IL-12, Th1-associated transcription factor T-bet, Th17-type cytokines IL-17 and IL-23 and Th17-associated transcription factor RORγt. Our data provides novel evidence that AICAR can not only inhibit macrophage-driven proinflammatory processes, but also dampen *in vitro* and *in vivo* adaptive immune responses.

AICAR has been used extensively as an agonist of AMPK [16–19,37–39]. In the intracellular milieu, AICAR is phosphorylated by adenosine kinase to AICA riboside monophosphate (ZMP), which mimics AMP and activates AMPK without altering the cellular levels of ATP, ADP, or AMP [15]. Binding of ZMP to AMPK produces allosteric alterations in AMPK, making the enzyme a better substrate for the upstream serine/threonine kinase 11 (STK11, also called LKB1) [40]. This may be the reason that AICAR has been widely used in many studies. To date, many experiments have demonstrated that the effect of AICAR is dependent on AMPK activation. Peairs et al. studied AICAR (1 mM)-induced activation of AMPK in mesangial cells and found that the effect of AICAR was thoroughly blocked by pretreatment with 5′-iodotubercidin (0.1 μM), an adenosine kinase inhibitor [41] implying that blocking AMPK activation can prohibit the action of AICAR. This was further supported by studies with AMPK-deficiency cells or mice in which the effect of AICAR is totally abolished, including inhibiting prostacyclin synthase nitration in diabetes [42], initiat-

ing relaxation of aortic rings and vasorelaxation [43], and inducing glucose uptake in skeletal muscle [44]. These studies provide strong evidence that AMPK is indispensable for AICAR action. Up to now, only one study has declared that the anti-inflammatory effects of AICAR are independent of AMPK activity [45]. They found that pretreatment with 5′-iodotubercidin (0.3 μM) could prevent AICAR (3 mM)-mediated AMPK phosphorylation in RAW264.7 cells, a macrophage cell line, but did not reverse the suppressive effects of AICAR on LPS-induced gene expression in the cells. We performed a similar experiment on the effect of AICAR in LPS-induced TNF production of mouse peritoneal macrophage and found that pretreatment of 5′-iodotubercidin (0.3 μM) significantly abolished different doses of AICAR (0.1 mM and 1 mM)-induced TNF production (data not shown). Collectively, the fact that AICAR-induced inhibition of immune responses or inflammation is mediated through an AMPK activity-dependent pathway has been widely accepted.

To date, as an agonist of AMPK, AICAR has been clinically used in the treatment of type 2 diabetic patients for the reduction of blood glucose concentrations [37,46], in obese females for stimulating fatty acid oxidation [47], and in healthy men to determine whether AICAR stimulates muscle glucose uptake in humans [48]. In addition, no side effects of AICAR have been reported. As IBD is an immunological disease by nature, targeting excessive cytokines with monoclonal antibodies (mAb) such as infliximab (an mAb against TNF) has currently become a highly promising approach and achieved great success clinically [49]. However, this therapy only targets one single cytokine at a time which may not be enough for the control of the disease. This mAb therapy also has significant side effects of infusion reactions and the development of antibodies against the infused mAb, which reduce the effectiveness of the treatment [50,51]. Compared with current mAb therapy, AICAR acts as a central immune regulator on both innate and adaptive immune responses. More importantly, AMPK has no apparent impact on lymphocyte responses under normal physiological conditions [52]. This makes the new strategy more attractive for use in humans.

In summary, our data provide evidence that AICAR, an agonist of AMPK, may act as a central regulator in the immune response by inhibiting NF-κB activation in macrophages, impairing Th1 and Th17 cells differentiation, and, consequently, reducing proinflammatory cytokines production. Furthermore, the findings of this report show that, for the first time, AICAR administration inhibits excessive immune responses, leading to the amelioration of both acute and relapsing colitis in mice. These findings suggest that an AICAR-initiated AMPK activation strategy may offer a promising alternative to our current approaches of managing IBD.

Conflict of interest

The authors state that there is no conflicting financial interest.

Acknowledgments

The authors sincerely thank Ms. Terry Enno for providing histological examination and Ms. Xiaoling Gao for providing flow cytometry analysis. This study was supported by grants from the Canadian Institutes of Health Research (ROP-92387 to ZP), the National Natural Scientific Foundation of China (30860108 to AB), and UCB Canada (to CNB), and conducted using facilities of the Manitoba Institute of Child Health, Inc. AB is the recipient of a postdoctoral fellowship from the Manitoba Institute of Child Health, Inc. CNB is supported in part by a Research Scientist Award of the Crohn's and Colitis Foundation of Canada and the Bingham Chair in Gastroenterology.

References

[1] Xavier RJ, Podolsky DK. Unravelling the pathogenesis of inflammatory bowel disease. Nature 2007;448:427–34.
[2] Kamada N, Hisamatsu T, Okamoto S, Sato T, Matsuoka K, Arai K, et al. Abnormally differentiated subsets of intestinal macrophage play a key role in Th1-dominant chronic colitis through excess production of IL-12 and IL-23 in response to bacteria. J Immunol 2005;175:6900–8.
[3] Bai AP, Ouyang Q. Probiotics and inflammatory bowel diseases. Postgrad Med J 2006;82:376–82.
[4] Mahida YR. The key role of macrophages in the immunopathogenesis of inflammatory bowel disease. Inflamm Bowel Dis 2000;6:21–33.
[5] Ogata H, Hibi T. Cytokine and anti-cytokine therapies for inflammatory bowel disease. Curr Pharm Des 2003;9:1107–13.
[6] Ahern PP, Izcue A, Maloy KJ, Powrie F. The interleukin-23 axis in intestinal inflammation. Immunol Rev 2008;226:147–59.
[7] Kobayashi T, Okamoto S, Hisamatsu T, Kamada N, Chinen H, Saito R, et al. IL23 differentially regulates the Th1/Th17 balance in ulcerative colitis and Crohn's disease. Gut 2008;57:1682–9.
[8] Fujino S, Andoh A, Bamba S, Ogawa A, Hata K, Araki Y, et al. Increased expression of interleukin 17 in inflammatory bowel disease. Gut 2003;52:65–70.
[9] Fuss IJ, Becker C, Yang Z, Groden C, Hornung RL, Heller F, et al. Both IL-12p70 and IL-23 are synthesized during active Crohn's disease and are down-regulated by treatment with anti-IL-12 p40 monoclonal antibody. Inflamm Bowel Dis 2006;12:9–15.
[10] Bai A, Lu N, Guo Y, Chen J, Liu Z. Modulation of inflammatory response via alpha2-adrenoceptor blockade in acute murine colitis. Clin Exp Immunol 2009;156:353–62.
[11] Mannon PJ, Fuss IJ, Mayer L, Elson CO, Sandborn WJ, Present D, et al. Anti-interleukin-12 antibody for active Crohn's disease. N Engl J Med 2004;351:2069–79.
[12] Andou A, Hisamatsu T, Okamoto S, Chinen H, Kamada N, Kobayashi T, et al. Dietary histidine ameliorates murine colitis by inhibition of proinflammatory cytokine production from macrophages. Gastroenterology 2009;136:564–74. e562.
[13] Kamada N, Hisamatsu T, Okamoto S, Chinen H, Kobayashi T, Sato T, et al. Unique CD14 intestinal macrophages contribute to the pathogenesis of Crohn disease via IL-23/IFN-gamma axis. J Clin Invest 2008;118:2269–80.
[14] Mucida D, Park Y, Kim G, Turovskaya O, Scott I, Kronenberg M, et al. Reciprocal TH17 and regulatory T cell differentiation mediated by retinoic acid. Science 2007;317:256–60.
[15] Hardie DG. AMP-activated/SNF1 protein kinases: conserved guardians of cellular energy. Nat Rev Mol Cell Biol 2007;8:774–85.
[16] Su RY, Chao Y, Chen TY, Huang DY, Lin WW. 5-Aminoimidazole-4-carboxamide riboside sensitizes TRAIL- and TNF{alpha}-induced cytotoxicity in colon cancer cells through AMP-activated protein kinase signaling. Mol Cancer Ther 2007;6:1562–71.
[17] Nath N, Giri S, Prasad R, Salem ML, Singh AK, Singh I. 5-Aminoimidazole-4-carboxamide ribonucleoside: a novel immunomodulator with therapeutic efficacy in experimental autoimmune encephalomyelitis. J Immunol 2005;175:566–74.
[18] Zhao X, Zmijewski JW, Lorne E, Liu G, Park YJ, Tsuruta Y, et al. Activation of AMPK attenuates neutrophil proinflammatory activity and decreases the severity of acute lung injury. Am J Physiol Lung Cell Mol Physiol 2008;295:L497–504.
[19] Kim TB, Kim SY, Moon KA, Park CS, Jang MK, Yun ES, et al. Five-aminoimidazole-4-carboxamide-1-beta-4-ribofuranoside attenuates poly (I:C)-induced airway inflammation in a murine model of asthma. Clin Exp Allergy 2007;37:1709–19.
[20] Boirivant M, Amendola A, Butera A, Sanchez M, Xu L, Marinaro M, et al. A transient breach in the epithelial barrier leads to regulatory T-cell generation and resistance to experimental colitis. Gastroenterology 2008;135:1612–23. e1615.
[21] Zhang Z, Zheng M, Bindas J, Schwarzenberger P, Kolls JK. Critical role of IL-17 receptor signaling in acute TNBS-induced colitis. Inflamm Bowel Dis 2006;12:382–8.
[22] Fina D, Sarra M, Fantini MC, Rizzo A, Caruso R, Caprioli F, et al. Regulation of gut inflammation and th17 cell response by interleukin-21. Gastroenterology 2008;134:1038–48.
[23] Bai A, Guo Y, Lu N. The effect of the cholinergic anti-inflammatory pathway on experimental colitis. Scand J Immunol 2007;66:538–45.
[24] Nielsen OH, Kirman I, Rudiger N, Hendel J, Vainer B. Upregulation of interleukin-12 and -17 in active inflammatory bowel disease. Scand J Gastroenterol 2003;38:180–5.
[25] Bowen H, Kelly A, Lee T, Lavender P. Control of cytokine gene transcription in Th1 and Th2 cells. Clin Exp Allergy 2008;38:1422–31.
[26] Afzali B, Lombardi G, Lechler RI, Lord GM. The role of T helper 17 (Th17) and regulatory T cells (Treg) in human organ transplantation and autoimmune disease. Clin Exp Immunol 2007;148:32–46.
[27] Dejardin E. The alternative NF-kappaB pathway from biochemistry to biology: pitfalls and promises for future drug development. Biochem Pharmacol 2006;72:1161–79.
[28] Guha M, Mackman N. LPS induction of gene expression in human monocytes. Cell Signal 2001;13:85–94.

[29] Rogler G, Brand K, Vogl D, Page S, Hofmeister R, Andus T, et al. Nuclear factor kappaB is activated in macrophages and epithelial cells of inflamed intestinal mucosa. Gastroenterology 1998;115:357–69.
[30] Ding A, Hwang S, Lander HM, Xie QW. Macrophages derived from C3H/HeJ (Lpsd) mice respond to bacterial lipopolysaccharide by activating NF-kappa B. J Leukoc Biol 1995;57:174–9.
[31] Atreya I, Atreya R, Neurath MF. NF-kappaB in inflammatory bowel disease. J Intern Med 2008;263:591–6.
[32] Sag D, Carling D, Stout RD, Suttles J. Adenosine 5′-monophosphate-activated protein kinase promotes macrophage polarization to an anti-inflammatory functional phenotype. J Immunol 2008;181:8633–41.
[33] Stallmach A, Marth T, Weiss B, Wittig BM, Hombach A, Schmidt C, et al. An interleukin 12 p40-IgG2b fusion protein abrogates T cell mediated inflammation: anti-inflammatory activity in Crohn's disease and experimental colitis in vivo. Gut 2004;53:339–45.
[34] Frauwirth KA, Thompson CB. Regulation of T lymphocyte metabolism. J Immunol 2004;172:4661–5.
[35] Maciver NJ, Jacobs SR, Wieman HL, Wofford JA, Coloff JL, Rathmell JC. Glucose metabolism in lymphocytes is a regulated process with significant effects on immune cell function and survival. J Leukoc Biol 2008;84:949–57.
[36] Kemp BE, Stapleton D, Campbell DJ, Chen ZP, Murthy S, Walter M, et al. AMP-activated protein kinase, super metabolic regulator. Biochem Soc Trans 2003;31:162–8.
[37] Boon H, Bosselaar M, Praet SF, Blaak EE, Saris WH, Wagenmakers AJ, et al. Intravenous AICAR administration reduces hepatic glucose output and inhibits whole body lipolysis in type 2 diabetic patients. Diabetologia 2008;51:1893–900.
[38] Guo D, Hildebrandt IJ, Prins RM, Soto H, Mazzotta MM, Dang J, et al. The AMPK agonist AICAR inhibits the growth of EGFRvIII-expressing glioblastomas by inhibiting lipogenesis. Proc Natl Acad Sci USA 2009;106:12932–7.
[39] Myerburg MM, King Jr JD, Oyster NM, Fitch AC, Magill A, Baty CJ, et al. AMPK agonists ameliorate sodium and fluid transport and inflammation in CF airway epithelial cells. Am J Respir Cell Mol Biol 2009.
[40] Hardie DG. AMP-activated protein kinase as a drug target. Annu Rev Pharmacol Toxicol 2007;47:185–210.
[41] Peairs A, Radjavi A, Davis S, Li L, Ahmed A, Giri S, et al. Activation of AMPK inhibits inflammation in MRL/lpr mouse mesangial cells. Clin Exp Immunol 2009;156:542–51.
[42] Xie Z, Zhang J, Wu J, Viollet B, Zou MH. Upregulation of mitochondrial uncoupling protein-2 by the AMP-activated protein kinase in endothelial cells attenuates oxidative stress in diabetes. Diabetes 2008;57:3222–30.
[43] Goirand F, Solar M, Athea Y, Viollet B, Mateo P, Fortin D, et al. Activation of AMP kinase alpha1 subunit induces aortic vasorelaxation in mice. J Physiol 2007;581:1163–71.
[44] Jorgensen SB, Viollet B, Andreelli F, Frosig C, Birk JB, Schjerling P, et al. Knockout of the alpha2 but not alpha1 5′-AMP-activated protein kinase isoform abolishes 5-aminoimidazole-4-carboxamide-1-beta-4-ribofuranosidebut not contraction-induced glucose uptake in skeletal muscle. J Biol Chem 2004;279:1070–9.
[45] Kuo CL, Ho FM, Chang MY, Prakash E, Lin WW. Inhibition of lipopolysaccharide-induced inducible nitric oxide synthase and cyclooxygenase-2 gene expression by 5-aminoimidazole-4-carboxamide riboside is independent of AMP-activated protein kinase. J Cell Biochem 2008;103:931–40.
[46] Babraj JA, Mustard K, Sutherland C, Towler MC, Chen S, Smith K, et al. Blunting of AICAR-induced human skeletal muscle glucose uptake in type 2 diabetes is dependent on age rather than diabetic status. Am J Physiol Endocrinol Metab 2009;296:E1042–1048.
[47] Steinberg GR, Smith AC, Van Denderen BJ, Chen Z, Murthy S, Campbell DJ, et al. AMP-activated protein kinase is not down-regulated in human skeletal muscle of obese females. J Clin Endocrinol Metab 2004;89:4575–80.
[48] Cuthbertson DJ, Babraj JA, Mustard KJ, Towler MC, Green KA, Wackerhage H, et al. 5-Aminoimidazole-4-carboxamide 1-beta-D-ribofuranoside acutely stimulates skeletal muscle 2-deoxyglucose uptake in healthy men. Diabetes 2007;56:2078–84.
[49] Owczarek D, Cibor D, Szczepanek M, Mach T. Biological therapy of inflammatory bowel disease. Pol Arch Med Wewn 2009;119:84–8.
[50] Baert F, Noman M, Vermeire S, Van Assche G, D'Haens G, Carbonez A, et al. Influence of immunogenicity on the long-term efficacy of infliximab in Crohn's disease. N Engl J Med 2003;348:601–8.
[51] Baert F, De Vos M, Louis E, Vermeire S. Immunogenicity of infliximab: how to handle the problem? Acta Gastroenterol Belg 2007;70:163–70.
[52] Mayer A, Denanglaire S, Viollet B, Leo O, Andris F. AMP-activated protein kinase regulates lymphocyte responses to metabolic stress but is largely dispensable for immune cell development and function. Eur J Immunol 2008;38:948–56.