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Original Article

Synergic effect of combined melatonin and tofacitinib on ameliorating dextran sulfate sodium-induced colitis in rat—role of JAKs/STAT, cell-stress signaling, and inflammatory-immune reaction

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Abstract: Background: This study investigated whether the combined treatment of melatonin and tofacitinib offers enhanced protection against dextran sulfate sodium-induced acute colitis (AC) in rats. Using CCD-18Co fibroblasts and a rat colitis model, we assessed the anti-inflammatory, anti-apoptotic, and immunomodulatory effects of the combination therapy. Methods: CCD-18Co cells were grouped as A1 (CCD-18Co), A2 (CCD-18Co + lipopolysaccharide (LPS)), A3 (CCD-18Co + LPS + Melatonin), A4 (CCD-18Co + LPS + Tofacitinib), or A5 (CCD-18Co + LPS + melatonin + tofacitinib). Sprague-Dawley rats were categorized into groups 1 (normal control), 2 (AC), 3 (AC + melatonin), 4 (AC + tofacitinib), and 5 (AC + melatonin + tofacitinib), and the colons were harvested 14 days after AC induction. Key findings: Cell viability at time points of 24, 48, and 72 h was the highest in A1, lowest in A2, and progressively increased from A3 to A5 (all $P < 0.0001$). The protein expression levels of inflammatory, DNA-damaged, and autophagic (ratio of LC3-BII to LC3-BI) biomarkers displayed identical patterns of apoptosis among the groups (all $P < 0.0001$). Additionally, the blood stool, colon leakage, and colon injury scores were the lowest in group 1, highest in group 2, and significantly progressively reduced from groups 3 to 5 (all $P < 0.0001$). The protein expression of the Janus kinase family-signal transducer and activator of transcriptions/cell-stress signaling, inflammation, oxidative stress, DNA-damaged, apoptotic biomarkers, and cellular expression of immune and inflammatory factors exhibited an identical pattern of colon injury scores among the groups. Conclusions: Combined melatonin-tofacitinib treatment effectively protected the colon against dextran sulfate sodium-induced damage, mainly through the suppression of Janus kinase family-signal transducer and activator of transcriptions/cell-stress signaling, inflammation, and oxidative stress.

Keywords: Acute colitis, dextran sulfate sodium, Janus kinase family, oxidative stress, cell-stress signaling

Introduction

Ulcerative colitis (UC) is an idiopathic, non-infectious disease of recurrent idiopathic intestinal inflammation that affects many people worldwide; its prevalence is gradually increasing, indicating that it is the most common form of inflammatory bowel disease worldwide [1, 2].

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Over 90% of the UC lesions spread continuously from the rectum to the large intestine. Common clinical manifestations include diarrhea, abdominal pain, fever, and bloody stools, which cause weight loss and anemia. The exact cause of the disease is currently unknown, but it is speculated to be related to factors such as genetics, immune system disorders, intestinal flora imbalance, diet, stress, etc. [3-7]. Other studies have shown that immune regulatory disorders may play an important role in its pathogenesis [6, 8].

The current treatment for UC is the administration of anti-inflammatory drugs such as aminosalicylates and corticosteroids to suppress inflammatory reactions. Biological agents (including anti-tumor necrosis factor (TNF), anti-integrin, and anti-interleukin (IL)-12/IL-23) [5] have been used for the treatment of UC. However, at least 25-50% of patients are not responsive to the above treatment, or UC has repeated attacks on patients regardless of treatment [9], which implies that the treatment of UC is still a formidable challenge for clinicians. These issues [5, 9] raise the consideration that when the abovementioned drugs are ineffective for the treatment of UC, new therapeutic interventions should be developed immediately.

Tofacitinib is a newly developed small-molecule drug that orally inhibits Janus kinase (JAK) [10, 11]. In 2012, the US Food and Drug Administration approved tofacitinib for the treatment of rheumatoid arthritis [12]. In 2018, the adoption of tofacitinib expanded, and tofacitinib component drugs were used to treat UC [10, 12]. Janus kinase is composed of tyrosine kinases, i.e., JAK1, JAK2, JAK3, and tyrosine kinase 2 (TYK2). In vitro studies have shown that tofacitinib inhibits JAK1, JAK2, and JAK3 [10, 12] and partially inhibits TYK2 [13, 14] in cell cultures. Additionally, other in vivo experimental results have shown that tofacitinib preferentially inhibits JAK1 and JAK3 [13], blocks cytokines (such as IL-2 and IL-6) that depend on JAK1 and JAK3, and reduces the release of inflammatory substance ILs [14-16]. Furthermore, abundant data have previously demonstrated that tofacitinib effectively blocks the γ-chain cytokines (IL-2, IL-4, IL-15, and IL-21) and can also inhibit the expression of interferon-γ, IL-6, IL-12, and IL-23 signal transduction [17-19]. These issues raise the hypothesis that tofacitinib has therapeutic potential for the treatment of UC, especially in patients refractory to traditional treatment.

Melatonin, first discovered in the pineal gland tissue of cattle [20], was confirmed to exist in the intestinal mucosa of rats in 1977, with the highest content in the rectum, followed by the colon [21], and has been shown to play a vital role in the physiology and pathology of the gastrointestinal tract [22, 23]. Intriguingly, studies have shown that patients with active UC have notably lower plasma melatonin levels than those in remission [24]. In the gastrointestinal tract, melatonin activity is controlled by melatonin receptors melatonin 2,5-hydroxytryptamine, and cholecystokinin 2 receptors. Additionally, high doses of melatonin can reduce intestinal peristalsis [25]. Additionally, melatonin treatment has been shown to effectively suppress dextran sulfate sodium (DSS)-induced UC in mice, primarily by suppressing inflammation and antioxidant effect [26-28]. Furthermore, clinical studies have demonstrated that adjuvant melatonin therapy could maintain remission in patients with UC [29, 30]. Our recent study has demonstrated that a combination of melatonin and adipose-derived mesenchymal stem cell-derived exosomes notably improved DSS-induced AC in rats [31]. These issues raised the hypothesis that the combination of melatonin and tofacitinib would offer a synergistic effect in protecting the colon against DSS-induced AC in rats.

Materials and methods

Ethics

All animal procedures were approved by the Institute of Animal Care and Use Committee at Kaohsiung Chang Gung Memorial Hospital (Affidavit of Approval of Animal Use Protocol No. 2020121803) and were performed in accordance with the Guide for the Care and Use of Laboratory Animals. Animals were housed in an Association for Assessment and Accreditation of Laboratory Animal Care International (Frederick, MD, USA)-accredited animal facility at Kaohsiung Chang Gung Memorial Hospital under temperature (22 ± 1°C), humidity (55 ± 5%), and light (12:12/light: dark photocycle, lights on at 08:00). Standard laboratory rat chow and tap water were provided ad libitum. All animals were allowed to acclima-

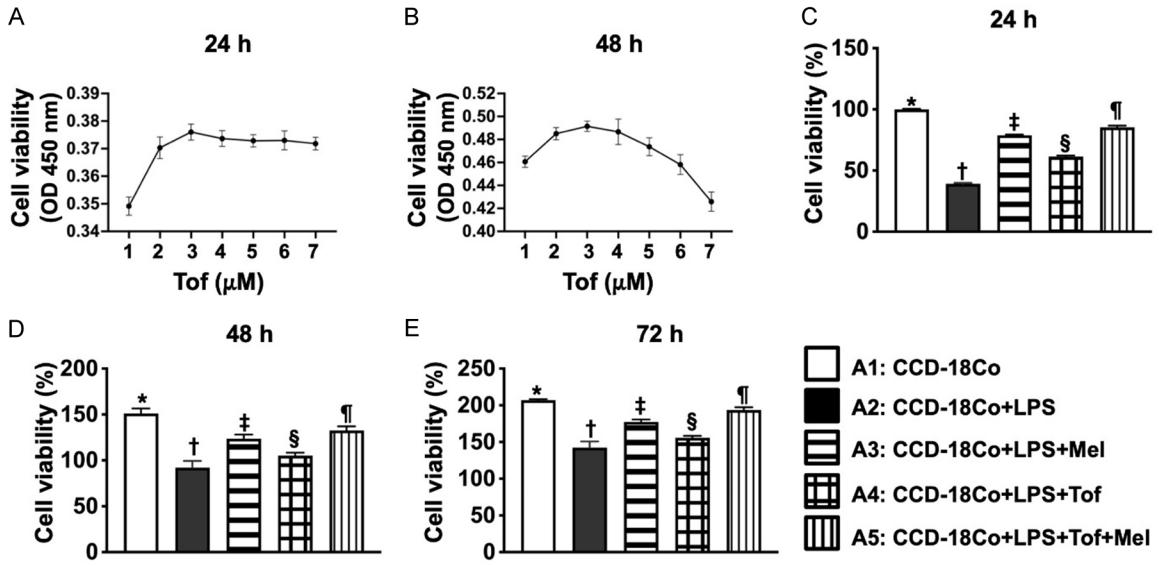


Figure 1. To test the suitable therapeutic concentration of tofacitinib on protecting the CCD-18Co cell against lipopolysaccharide (LPS) damage. (A) Illustrating the cell viability of CCD-18Co cells at time point of 24 h undergoing stepwise-increased concentrations (i.e., 0, 1, 2, 3, 4, 5, 6 and 7 μM) of Tofacitinib treatment. The result showed that the cell viability at this time interval did not show great difference among these different concentrations. (B) By 48 after cell culturing, the cell viability was remarkably suppressed when the concentration of tofacitinib ≥ 5 μM. (C-E) Analytical results of cell viability at time points of 24 (C) 48 (D) and 72 (E) hours, * vs. other group with different symbols (†, ‡, §, ¶), $P < 0.0001$. All statistical analyses were performed by one-way ANOVA, followed by Bonferroni multiple comparison post hoc test (n = 6 for each group). Symbols (*, †, ‡, §, ¶) indicate significance for each other (at 0.05 level). LPS = lipopolysaccharide.

tize to the animal facility for 14 days prior to experimental manipulation.

Cell culture and in vitro studies

The CCD-18Co (ATCC CRL-1459) cell line derived from human normal colon cells was grown at 37°C under 5% CO_2 in 10-cm plastic dishes containing 10 mL of Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 1% non-essential amino acids, 100 μg/mL streptomycin, and 100 units/mL penicillin.

To elucidate the suitable dose of tofacitinib for the in vitro study, the CCD-18Co cells were treated by stepwise increased concentrations (i.e., 1, 2, 3, 4, 5, 6, and 7 μM) of tofacitinib. The results of cell viability showed that the suitable dose of tofacitinib for cell viability after 48 h of cell culturing was identified to be 3.0 μM. Thus, this dose of tofacitinib was used in subsequent in vitro studies (**Figure 1**).

To elucidate the therapeutic impact of melatonin and tofacitinib, the CCD-18Co cell line was categorized into groups A1 (CCD-18Co), A2 (CCD-18Co + lipopolysaccharide (LPS) (50 ng/mL treated for 6 h)), A3 (CCD-18Co + LPS + tofacitinib (3 μM treated for 48 h)), A4 (CCD-18Co + LPS + melatonin (100 μM treated for 48 h)), and A5 (CCD-18Co + LPS + melatonin + tofacitinib), respectively. The melatonin and LPS doses used in the present in vitro study were based on those used in our previous study [31].

Animal grouping and treatment strategy in AC

Pathogen-free adult male Sprague-Dawley rats (n = 44) weighing 300-325 g (Charles River Technology, BioLASCO Taiwan Co., Ltd., Taiwan) were used in the present study.

Animals were categorized into group 1 (normal control, n = 6), group 2 (5 days of 5% DSS in drinking water used for AC induction, n = 11), group 3 (melatonin (50 mg/kg/day for five days, i.e., administered from days to 6-10 after AC induction), n = 9), group 4 (tofacitinib (30 mg/kg/day for five days, i.e., administered from day 6 to 10 after AC induction), n = 9), and group 5 (melatonin + tofacitinib treatment for five days after AC induction for total five days, n

= 9), respectively. The melatonin dose used in this study was based on that used in our previous study [31]. The tofacitinib dose used in this study was based on a previous report [32] with some modifications.

DSS-induced animal model of AC in rats

DSS is a water-soluble, negatively charged sulfated polysaccharide with a highly variable molecular weight ranging from 5 to 1400 kDa. The most severe form of murine colitis, which closely resembles human UC, can be induced by the administration of 40-50 kDa DSS in drinking water. The mechanism by which DSS induces intestinal inflammation is likely the result of damage to the epithelial monolayer lining of the large intestine, which allows the dissemination of pro-inflammatory intestinal contents (e.g., bacteria and their products) into the underlying tissue. The DSS-induced AC model is popular in inflammatory bowel disease research because of its rapidity, simplicity, reproducibility, controllability, and morphological and symptomatic resemblance to UC in humans. The procedure and protocol for AC induction were based on our previous report [31].

Cell proliferation assay

First, 1×10^4 CCD-18Co cells were plated in 24-well plates. After being treated with different doses of tofacitinib and melatonin, cells were stained with MTT solution (Sigma, St. Louis, MO, USA) and incubated for 1-2 h. Finally, the formazan crystals were solubilized in 200 μL of dimethyl sulfoxide (Sigma, St. Louis, MO, USA) and measured at 560 nm.

Flow cytometric analysis for determining the apoptosis of CCD-18Co cells

Apoptosis was analyzed using the Annexin V/propidium iodide (PI) assay and 4',6-diamidino-2-phenylindole staining. An Annexin V/PI staining kit (Annexin Apoptosis Detection Kit I; BD Biosciences, Heidelberg, Germany) was used for the Annexin V/PI assay. Cells were harvested under various conditions and suspended in phosphate-buffered saline for the assay. Cells were suspended in 1 mL of fluorescein isothiocyanate (FITC)-annexin V solution, and PI was added to a final concentration of 1 μg/mL. Samples were analyzed using flow cytometry (Becton Dickinson, San Jose, CA, USA). The green fluorescence of FITC was measured at 530 ± 20 nm, and the red fluorescence was measured at 600 nm.

Histopathological scoring assessment of colon injury

The criteria for assessing mucosal damage in the colon were based on our previous report with minimal modifications [31]. Briefly, colon specimens were fixed in 4% paraformaldehyde, and the crypt-villus orientation was maintained during the embedding procedure. Sections of 4 μm thickness were stained with hematoxylin and eosin (H&E). The degree of colonic injury was evaluated using a light microscope by two senior technicians blinded to the treatment protocol.

Briefly, the injury score of the colon was categorized from 0 to 5 based on the following criteria: grade 0, mucosa with normal villi; grade 1, development of subepithelial space at the villus apex, frequently associated with capillary congestion; grade 2, scattered epithelial denudation on villus tips; grade 3, denuded tips with exposed lamina propria and villus blunting; grade 4, epithelial shedding from both the apex and mid-region of the villi, associated with shortened and widened villus structure and exposure of dilated capillaries; and grade 5, complete destruction of villi and disintegration of the lamina propria with ulceration.

Immunohistochemical (IHC) and immunofluorescent (IF) staining

The procedure and protocol for IF staining have been described in detail in our previous studies [31, 33]. For IHC and IF staining, rehydrated paraffin sections were first treated with 3% H_2O_2 for 30 min and then incubated with ImmunoBlock reagent (BioSB, Santa Barbara, CA, USA) for 30 min at room temperature. Sections were then incubated with primary antibodies against CD3 (1:400, Abcam), CD4 (1:400, Genetex), gamma-phosphorylated histone H2AX (γ-H2AX; 1:1000, Abcam), and matrix metalloproteinase (MMP-9) (1:200, Invitrogen), whereas sections incubated with irrelevant antibodies served as controls. Three sections of kidney specimens from each rat were analyzed. For quantification, three randomly selected high-power fields (200× or 400× for

IHC and IF studies, respectively) were analyzed in each section. The mean number of positively stained cells per high-power field for each animal was determined by summing all numbers divided by 9.

Western blot analysis

The procedure and protocol for western blot analysis were based on our previous reports [31, 33]. Briefly, equal amounts (50 μg) of protein extract were loaded and separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis with acrylamide gradients. After electrophoresis, the separated proteins were transferred onto polyvinylidene difluoride membranes (Amersham Biosciences, Amersham, UK). Non-specific sites were blocked by incubating the membrane in a blocking buffer (5% nonfat dry milk in T-TBS (TBS containing 0.05% Tween 20)) overnight. The membranes were incubated with the following primary antibodies (nuclear factor (NF)-κB (1:1000, Cell Signaling Technology), NADPH oxidase (NOX1; 1:4000, Sigma), NOX2 (1:4000, Sigma), NOX4 (1:4000, Abcam), cleaved (c)-caspase3 (1:1000, Cell Signaling Technology), cleaved poly (ADP-ribose) polymerase (1:1000, Cell Signaling Technology), MMP-9 (1:4000, Abcam), TNF-α (1:1000, Cell Signaling Technology), IL-1β (1:1000, Cell Signaling Technology), γ-H2AX (1:1000, Cell Signaling Technology), phosphatidylinositol 3-kinase (1:1000, Cell Signaling Technology), protein kinase B (Akt; 1:1000, Cell Signaling Technology), mechanistic target of rapamycin (mTOR; 1:1000, Cell Signaling Technology), JAK2 (1:1000, Cell Signaling Technology), STAT3 (1:4000, Abcam), and actin (1:10000, Merck)) for 1 h at room temperature. Horseradish peroxidase-conjugated anti-rabbit immunoglobulin IgG (1:2000; Cell Signaling Technology, Danvers, MA, USA) was used as the secondary antibody for one-hour incubation at room temperature. The washing procedure was repeated eight times within 1 h. The immunoreactive bands were visualized using enhanced chemiluminescence (Amersham Biosciences) and exposed to Biomax L film (Kodak, Rochester, NY, USA). For quantification, enhanced chemiluminescence signals were digitized using the Labwork software (UVP, Waltham, MA, USA).

For each western blot experiment, protein lysates from six animals per group were used to ensure biological replicates. β-actin was consistently used as a loading control and for normalization. When blots for multiple proteins were obtained from the same gel and sample set, the same β-actin images were used across the corresponding panels.

Fluorescein-based (FITC-dextran 4 kDa) gut permeability assay

To assess gut permeability in vivo, a fluorescein-based assay was performed, as previously described [31, 33]. On day 14, after AC induction, the lumen of the colonic segment (10-15 cm) of each group of animals was carefully clamped. The 4-kDa FITC-conjugated dextran (FD4; Sigma), dissolved in Krebs buffer and protected from light, was immediately administered into the lumen of the clamped jejunal segment at a final concentration of 0.5 mg/mL. One hour after colon clamping, the clamp was released, and the abdominal wall was closed. After 60 min of colon clamping, arterial blood (0.5 mL) was sampled, from which plasma was obtained. The fluorescence intensity of the plasma was measured at an excitation/emission wavelength of 496/520 nm using a plate reader. The concentration (mg/mL) of FD4 in the plasma was calculated using a standard curve.

Statistical analysis

Quantitative data are expressed as the mean ± SD. Statistical analyses were performed using ANOVA, followed by Bonferroni multiple comparison post-hoc tests. The SAS statistical software for Windows version 8.2 (SAS Institute, Cary, NC, USA) was used. Statistical significance was set at $P < 0.05$.

Results

*To test the suitable therapeutic concentration of tofacitinib on protecting the CCD-18Co cell against LPS-induced damage (**Figure 1**)*

First, to elucidate the optimal dose of tofacitinib therapy with the lowest side effects, the CCD-18Co cell line (i.e., normal human colon cells) was treated with stepwise increasing concentrations (i.e., 1, 2, 3, 4, 5, 6, and 7 μM) of tofacitinib. The results demonstrated that at the early stage (24 h), cell viability, as determined using the MTT assay, was similar among the different concentrations of tofacitinib

(**Figure 1A**). However, at the time point of 48-h cell culture, the cell viability was remarkably suppressed when the concentration of tofacitinib was increased up to 5 μM and even remarkably suppressed when the dosage of tofacitinib was increased to more than 5 μM (**Figure 1B**). Thus, the dosage of 3.0 μM tofacitinib was used for the in vitro study in the current study.

Next, we tested whether 3.0 μM tofacitinib treatment could suppress or ensure cell proliferation during cell culture and LPS treatment, i.e., an endotoxin indicated inflammation, CCD-18Co cells were categorized into A1 (CCD-18Co cells), A2 (CCD-18Co cells + LPS (50 ng/ml treated for 6 h)), A3 (CCD-18Co cells + LPS + tofacitinib (3.0 μM treated for 48 h)), A4 (CCD-18Co cells + LPS + melatonin (100 μM treated for 48 h)), and A5 (CCD-18Co + LPS + tofacitinib + melatonin). The results of the MTT assay demonstrated that at 24 h (**Figure 1C**), 48 h (**Figure 1D**), and 72 h (**Figure 1E**), the cell proliferation capacity was the highest in A1, the lowest in A2, significantly higher in A5 than in A3 and A4, and significantly lower in A4 than in A3. Our findings suggest that low doses of tofacitinib (i.e., 3.0 μM) had a notably protective effect rather than a suppressive effect in high doses of tofacitinib (i.e., ≥ 5.0 μM) during cell culture treated with LPS.

*Impact of melatonin and tofacitinib treatment on protecting the cells against LPS-induced apoptosis, DNA damage, and autophagy (**Figure 2**)*

Furthermore, we wanted to determine whether combined melatonin and tofacitinib treatment would offer a synergistic effect on protecting the cells against LPS-induced cell apoptosis, DNA damage, and autophagy; the CCD-18Co cells were categorized into A1 to A5, as shown in **Figure 1**.

By time interval of 72 h cell culturing, the flow cytometric analysis demonstrated that the early apoptosis (Annexin V^+/PI^-) of CCD-18Co cells was the lowest in A1, highest in A2, and significantly progressively reduced from A3 to A5 (**Figure 2A-F**), whereas the late apoptosis (Annexin V^+/PI^+) in these cells was the lowest in A1, highest in A2, significantly higher in A4 than in A3 and A5 and significantly higher in A3 than in A5 (**Figure 2G**).

When we examined protein levels, we found that the expression of cleaved caspase 3 (**Figure 2H**) and cleaved PARP (**Figure 2I**), two indices of apoptosis, displayed a similar pattern of early apoptosis among the groups.

Additionally, the protein levels of γ-H2AX (**Figure 2J**), an indicator of DNA damage, and the ratio of protein LC3-BII to protein LC3-BI (**Figure 2K**), an index of autophagy, also displayed a similar pattern of early and late apoptosis among the groups.

Our findings suggest that the combination of melatonin and tofacitinib is superior to monotherapy in protecting CCD-18Co cells against LPS-induced death and autophagy.

*Impact of melatonin and tofacitinib treatment on alleviating the LPS-induced pro-inflammatory cytokines (**Figure 3**)*

To elucidate whether combined melatonin and tofacitinib treatment was superior to a single therapy for ameliorating LPS-induced upregulation of pro-inflammatory cytokines in cultured CCD-18Co cells, the cells were categorized into groups A1 to A5, as shown in **Figure 1**.

The results of western blot analysis demonstrated that the protein levels of IL-17, IL-1β, NF-κB, TNF-α, IL-6, interferon-γ, and MMP-9, seven indicators of inflammation, were the lowest in A1 and significantly and progressively reduced from A2 to A5, implicating that combined melatonin and tofacitinib was superior to monotherapy in suppressing LPS-induced inflammatory reaction.

*Body weight and stool analysis and impact of melatonin and tofacitinib on alleviating the gut permeability by day 14 after AC induction by SSD (**Figure 4**)*

In the animal model study, we found that the incidence of bloody stools by day 8 was significantly higher in group 2 (AC) than in groups 1 (sham control), 3 (AC + melatonin), 4 (AC + tofacitinib), and 5 (AC + melatonin + tofacitinib), but there was no difference among the latter four groups. However, by day 14 after AC induction, this parameter did not differ among the five groups.

Additionally, the ratio of body weight on day 14 to baseline was significantly higher in group 1 than in groups 2-5; however, there was no difference among the latter four groups.

Furthermore, the mortality rate by day 14 after AC induction was 0% (0/6) in group 1, 27.3%

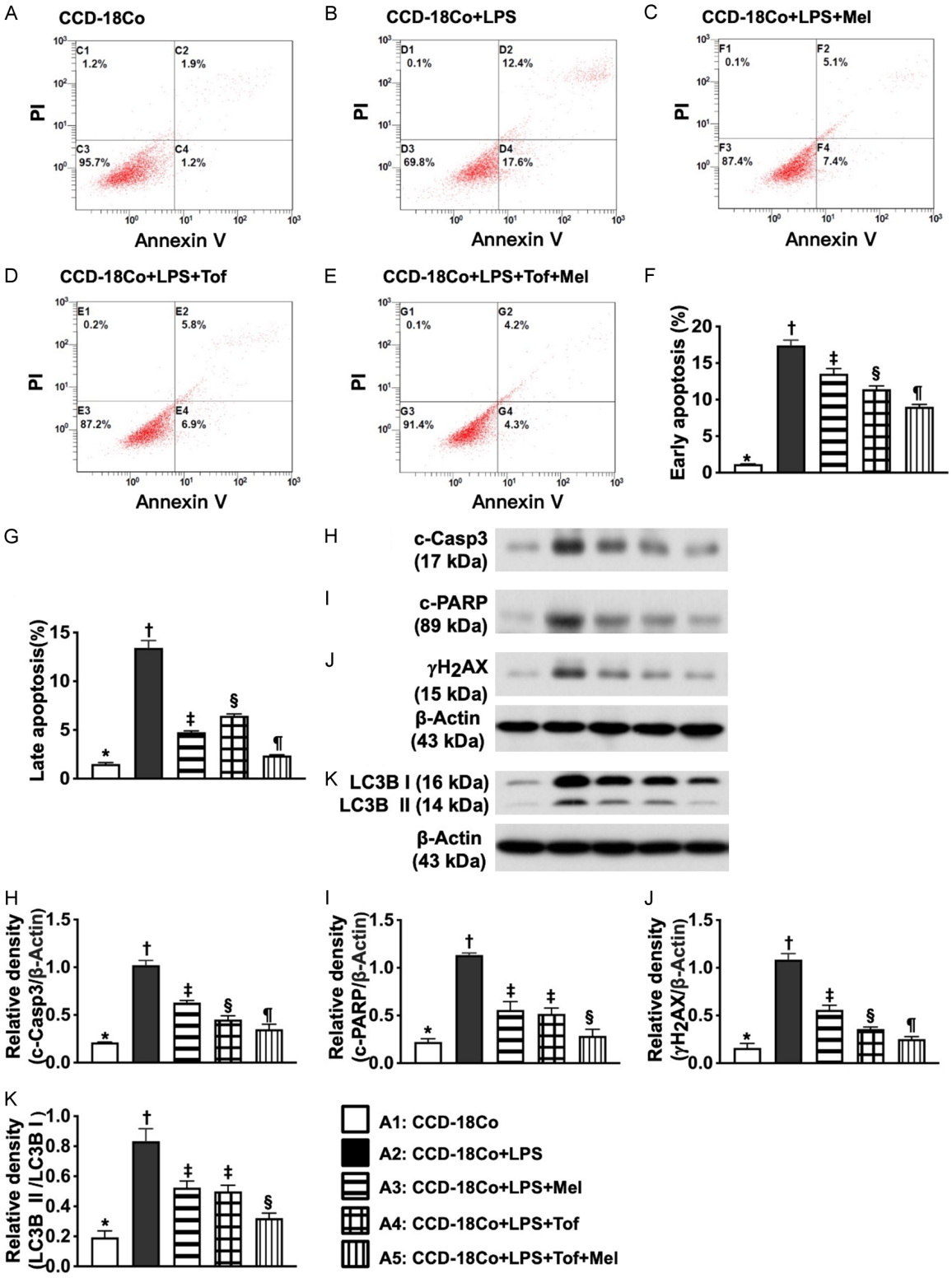


Figure 2. Impact of tofacitinib and melatonin treatment on protecting the cells against LPS-induced apoptosis, DNA-damage and autophagy. (A-E) Illustrating the flow cytometric analysis for assessment of early and late apoptosis. (F and G) Represented analytical results of early (annexin V^+/PI^-) (F) and late (annexin V^+/PI^+) (G) apoptosis, * vs. other group with different symbols (†, ‡, §, ¶), $P < 0.0001$ (n = 6). (H) Protein expression of cleaved caspase 3 (c-Casp3), * vs. other group with different symbols (†, ‡, §, ¶), $P < 0.001$. (I) Protein expression of cleaved poly ADP-ribose polymerase (c-PARP), * vs. other group with different symbols (†, ‡, §), $P < 0.001$. (J) Protein expression of γ-H2AX, * vs. other group with different symbols (†, ‡, §, ¶), $P < 0.001$. (K) Protein expression of the ratio of LC3-BII to protein LC3-BI, * vs. other group with different symbols (†, ‡, §), $P < 0.001$ (n = 3 for each group).

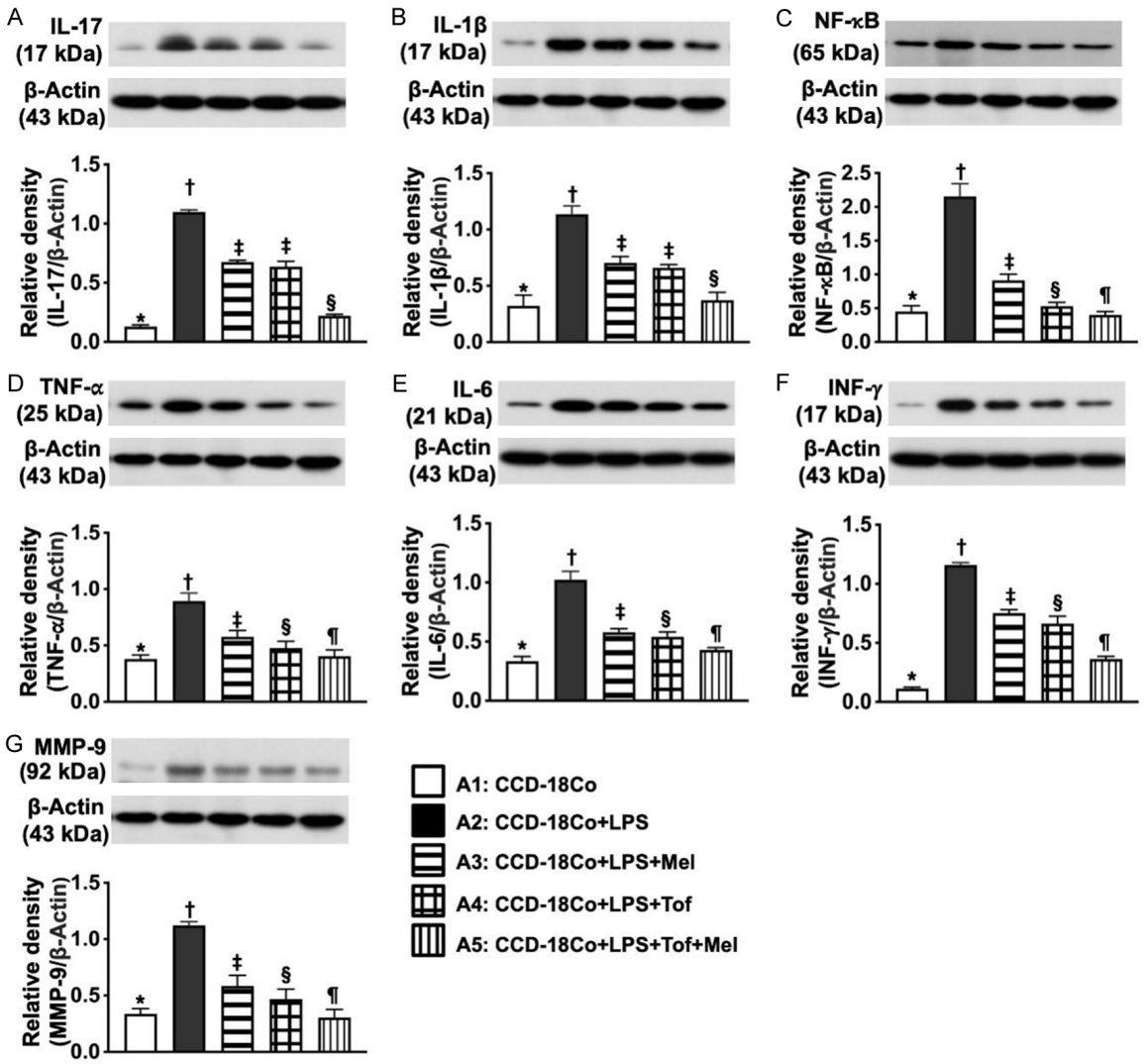


Figure 3. Impact of tofacitinib and melatonin treatment on suppressing LPS-induced proinflammatory cytokines. (A-G) Represented the protein expressions of interleukin (IL)-17 (A), IL-1β (B), nuclear factor (NF)-κB (C), tumor necrosis factor (TNF)-α (D), IL-6 (E), interferon (IFN)-γ (F) and matrix metalloproteinase (MMP)-9 (G), * vs. other group with different symbols (†, ‡, §, ¶), $P < 0.001$. n = 3 for each group. LPS = lipopolysaccharide.

(3/11) in group 2, 0% (0/9) in group 3, 0% (0/9) in group 4, and 11.1% (1/9) in group 5. Thus, this parameter did not differ among the five groups (P > 0.12).

Furthermore, the key finding of the present study was that the fluorescein-based gut permeability assay (i.e., mucosal barrier damage test) demonstrated that the circulatory concentration of FD4 was the lowest in group 1, highest in group 2, and significantly reduced in groups 3 to 5.

Our findings suggest that combined melatonin and tofacitinib therapy is superior to monotherapy in protecting the colon against SSD-induced damage.

*Impact of combined melatonin and tofacitinib therapy on attenuating the colon injury and cellular expression of DNA damage by day 14 after AC induction by SSD (**Figure 5**)*

To assess whether combined melatonin and tofacitinib therapy would attenuate colon damage, H&E and IF staining were performed in the present study. The results of H&E staining showed that the colon injury score was the lowest in group 1, the highest in group 2, significantly lower in group 5 than in groups 3 and 4,

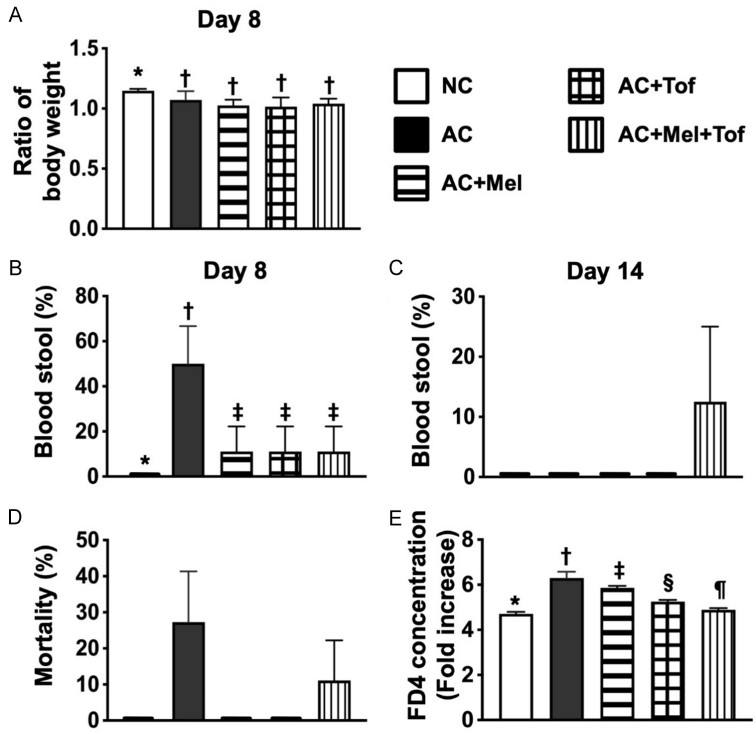


Figure 4. Body weight and stool analysis and gut permeability by day 14 after AC induction by SSD. (A) Ratio of body weight (BW) at day 14 after AC induction to the baseline BW, * vs. †, $P < 0.001$. (B) Incidence of bloody stool by day 8, * vs. other group with different symbols (†, ‡), $P < 0.001$. (C) The incidence of bloody stool by day 14, $P > 0.5$. (D) The accumulated mortality rate, $P > 0.12$. (E) Fluorescein-based gut permeability assay (i.e., mucosal barrier damage test) demonstrated that the FD4 concentration was significantly increased in AC group than in other groups. Analytical result of FD4 concentration (i.e., fold increase) in plasma, * vs. other group with different symbols (†, ‡), $P < 0.001$. n = 6-10 for each group.

and significantly higher in group 3 than in group 4.

Additionally, IF microscopic findings demonstrated that γ-H2AX+ cells in the colon tissue, an index of DNA-damaged biomarker, displayed an identical pattern of colon injury scores among the groups.

Our findings suggest that the combined melatonin and tofacitinib therapy is superior to either treatment alone in protecting the colon from SSD-induced damage.

*Impact of combined melatonin and tofacitinib therapy on regulating the cell proliferation signaling and the components of Janus kinase/STAT by day 14 after AC induction by SSD (**Figure 6**)*

It is well recognized that the Janus kinase family is a critical component of the cytokine signaling cascade that participates in the pathogenesis of AC. Western blotting was performed to elucidate whether combination therapy with melatonin and tofacitinib effectively suppressed the Janus kinase family and cell proliferation signaling in colon specimens.

The results demonstrated that the protein expression levels of JAK1, JAK2, and TYK2, three components of Janus kinase, and STAT3, the downstream signaling of Janus kinase for continuing Janus kinase signal transduction, were the lowest in group 1 and significantly and progressively reduced from groups 1 to 5, indicating that combined melatonin and tofacitinib treatment was superior to monotherapy in suppressing the Janus kinase family and its downstream transduction signaling pathway.

When examining biomarkers of cell proliferation signaling, we found that p-PI3K, p-AKT, and p-mTOR exhibited a pattern identical to that of the Janus kinase family.

Our findings highlight that SSD-induced AC notably elicited cell proliferation signaling and Janus kinase/STAT components to cause colon damage, which was alleviated by melatonin-tofacitinib therapy.

*Impact of combined melatonin and tofacitinib therapy on attenuating the apoptosis, DNA damage, and oxidative stress by day 14 after AC induction by SSD (**Figure 7**)*

We wanted to verify whether melatonin and tofacitinib therapy would efficaciously attenuate the protein levels of apoptosis, DNA damage, and oxidative stress in the colon tissues; the western blot analysis was once again used in the present study.

The results showed that the protein expression of cleaved caspase 3 and cleaved PARP, two indicators of apoptosis, and protein expres-

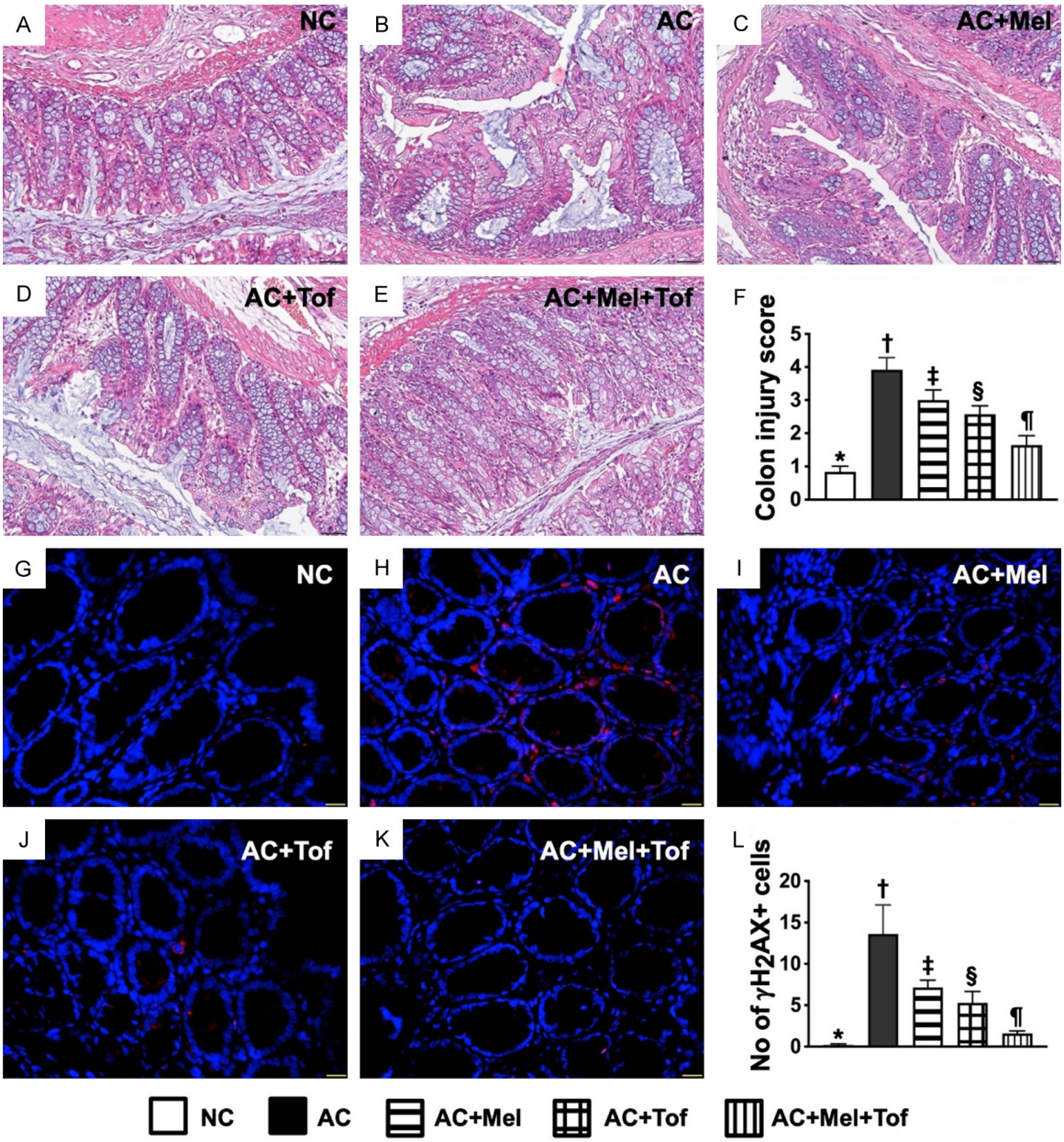


Figure 5. Impact of combined tofacitinib and melatonin therapy on attenuating the colon damage and cellular level of DNA damage by day 14 after AC induction by SSD. (A-E) Illustrating the light microscopic finding (200×) of H&E stain for identification of the pathology of colon injury scores. (F) Analytical result of colon injury score, * vs. other groups with different symbols (†, ‡, §, ¶), $P < 0.0001$. Scale bars in right lower corner represent 50 μm. (G-K) Illustrating the IF microscopic finding (400×) for identification of cellular expression of the γ-H2AX (green color). (L) Analytical result of γ-H2AX+ cells, * vs. other groups with different symbols (†, ‡, §, ¶), $P < 0.0001$. Scale bars in right lower corner represent 20 μm. n = 6 for each group.

sions of γ-H2AX, an index of DNA-damaged marker, were the lowest in group 1, highest in group 2, and significantly and progressively reduced from groups to 5.

Additionally, the protein expression of NOX-1, NOX-2, and NOX-4, three indicators of oxidative stress, displayed an identical pattern to that of cleaved caspase 3 among the groups. These findings imply that combined melatonin and tofacitinib therapy is better than a single therapy for protecting the colon against SSD-induced damage by alleviating cell death and oxidative stress.

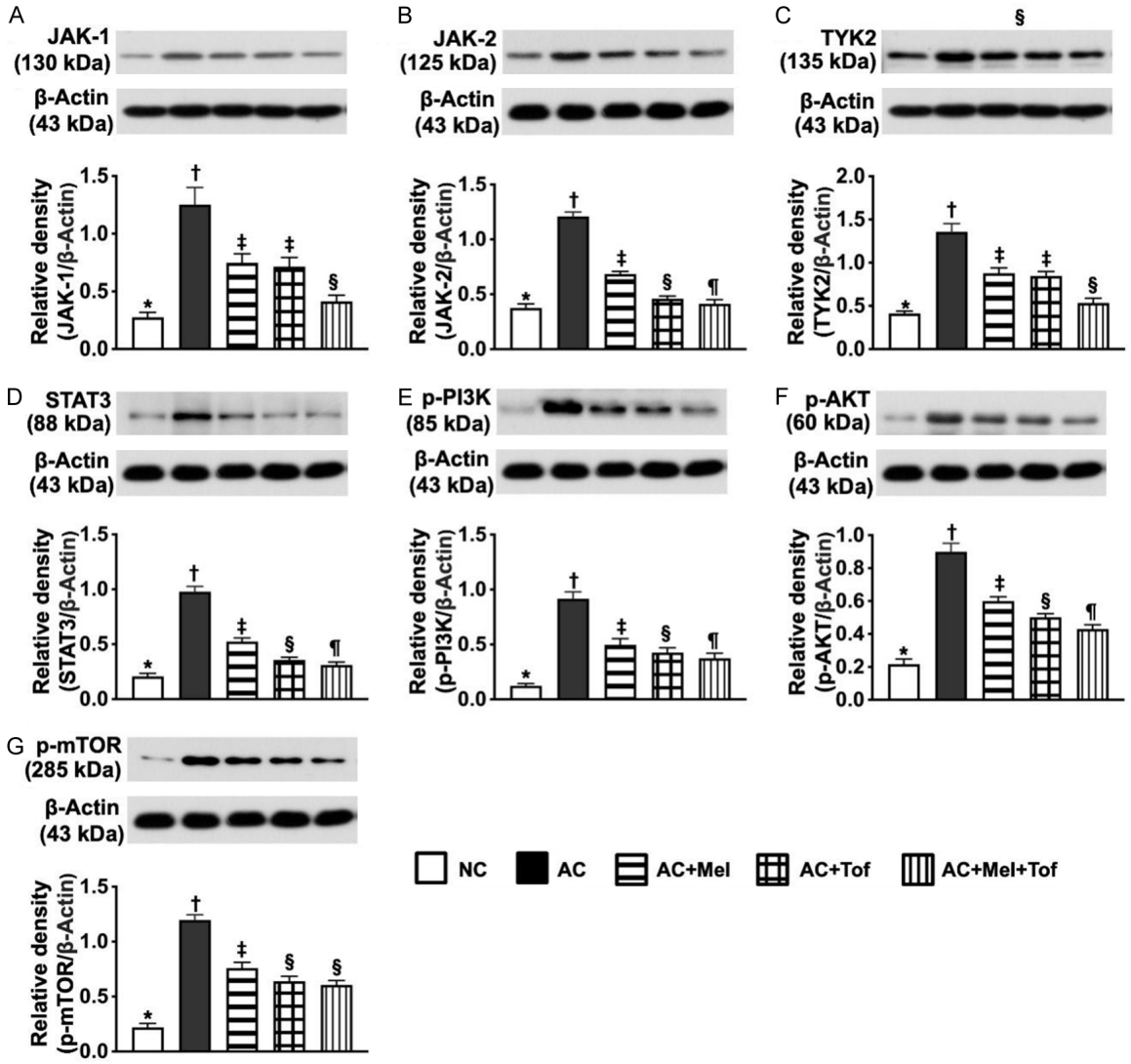


Figure 6. Impact of combined tofacitinib and melatonin therapy on regulating the cell-stress signaling and the components of Janus kinase by day 14 after AC induction by SSD. (A) Protein expression of JAK1, * vs. other groups with different symbols (†, ‡, §), $P < 0.0001$. (B) Protein expression of JAK2, * vs. other groups with different symbols (†, ‡, §, ¶), $P < 0.0001$. (C) Protein expression of Tyrosin Kinase 2 (TYK2), * vs. other groups with different symbols (†, ‡, §), $P < 0.0001$. (D-G) Protein expressions of STAT3 (D), phosphorylated (p)-PI3K (E), p-AKT (F) and p-mTOR (G), * vs. other groups with different symbols (†, ‡, §), $P < 0.0001$. n = 6 for each group.

*Impact of combined melatonin and tofacitinib therapy on ameliorating pro-inflammatory cytokines by day 14 after AC induction by SSD (**Figure 8**)*

To clarify whether a combined regimen of melatonin and tofacitinib would be better than a single regimen for inhibiting pro-inflammatory cytokine expression in colon tissues, western blot analysis was performed.

The results showed that the protein expression of NF-κB, IL-1β, TNF-α, and MMP-9, four indices of pro-inflammatory cytokines, was the lowest in group 1, highest in group 2, significantly lower in group 5 than in groups 3 and 4, and significantly lower in group 4 than in group 3.

Additionally, the expression of MMP-9, an index of inflammation at the cellular level, displayed an identical pattern of inflammatory protein levels among the groups.

It is well-recognized that IL-17 is a potent pro-inflammatory cytokine that participates in many inflammatory reactions. In the present study,

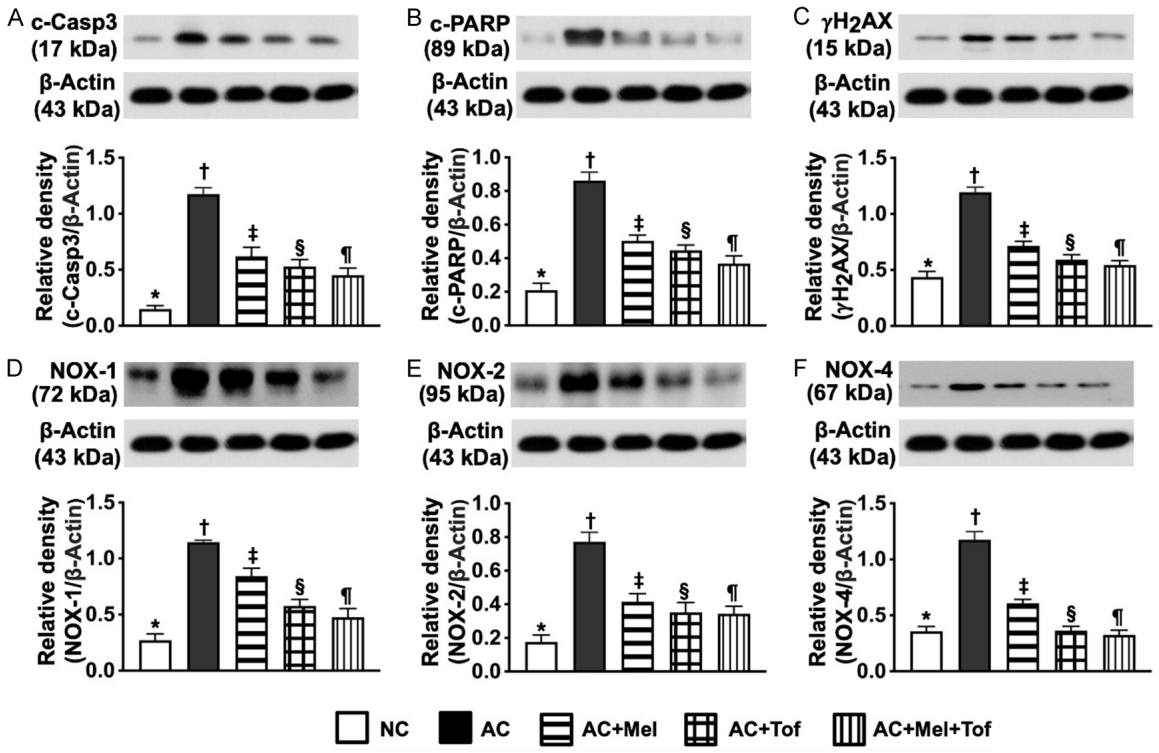


Figure 7. Impact of combined tofacitinib and melatonin therapy on attenuating the apoptosis, DNA damage and oxidative stress by day 14 after AC induction by SSD. (A-F) Represented the protein expression of cleaved caspase 3 (c-Casp3) (A), c-PARP (B), γ-H2AX (C), NOX-1 (D), NOX-2 (E) and NOX-4 (F), * vs. other groups with different symbols (†, ‡, §, ¶), $P < 0.0001$. n = 6 for each group.

we found that the protein expression of IL-17 displayed similar to that of the aforementioned pro-inflammatory cytokines among the groups.

Thus, our findings proved that the combined melatonin and tofacitinib therapy was superior to either therapy alone in suppressing the cellular and protein levels of inflammation.

*Impact of combined melatonin and tofacitinib therapy on suppressing immune cell infiltration by day 14 after AC induction by SSD (**Figure 9**)*

Finally, we want to delineate whether combined melatonin and tofacitinib therapy would be better than monotherapy on inhibiting the immune cell expression in the colon tissues, and the IHC microscopic finding was used in this study. The results showed that the expression of CD3 and CD4, two indicators of immune cells, was the lowest in group 1, highest in group 2, significantly lower in group 5 than in groups 3 and 4, and significantly different between groups 3 and 4.

Discussion

This study, which investigated the effect of a melatonin and tofacitinib combination on colon protection against SSD-induced AC, has several striking implications. We successfully created a reproducible animal model of SSD-induced AC to test the effects of different regimens on AC outcomes in rodent models. Second, the results of the in vitro study demonstrated that LPS-induced inflammation, cellular apoptosis, DNA damage, and autophagy were effectively suppressed by melatonin and tofacitinib treatment in the CCD-18Co cell culture model. Third, the results of the animal model study demonstrated that the combination of melatonin and tofacitinib was superior to a single therapy for protecting the colon against SSD-induced AC damage by regulating Janus kinase/STAT3 and cell proliferation signaling, as well as attenuating oxidative stress and inflammatory-immune reactions in the rodent colon.

An essential finding of the in vitro study was that LPS treatment markedly augmented the production of pro-inflammatory cytokines, au-

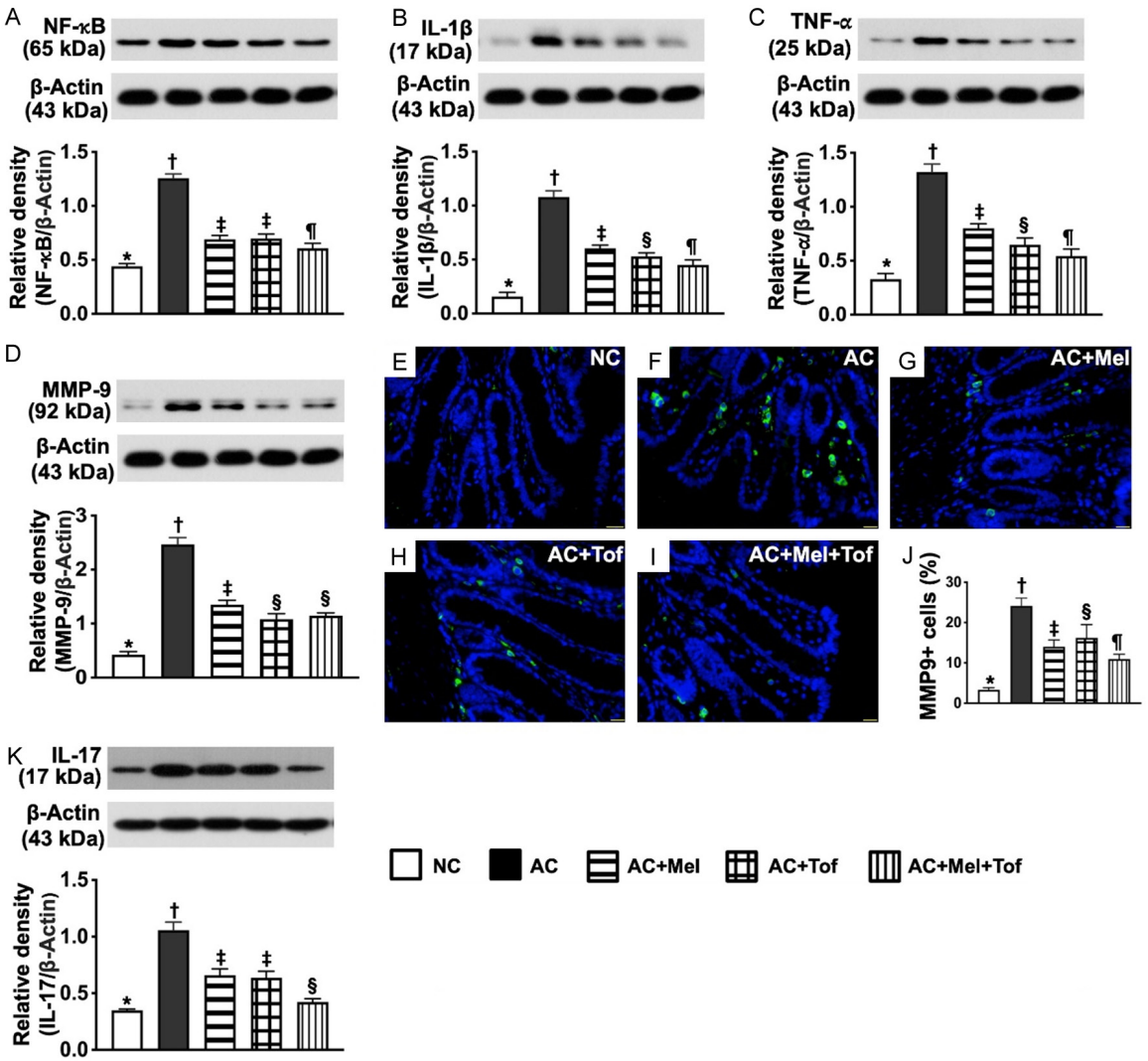
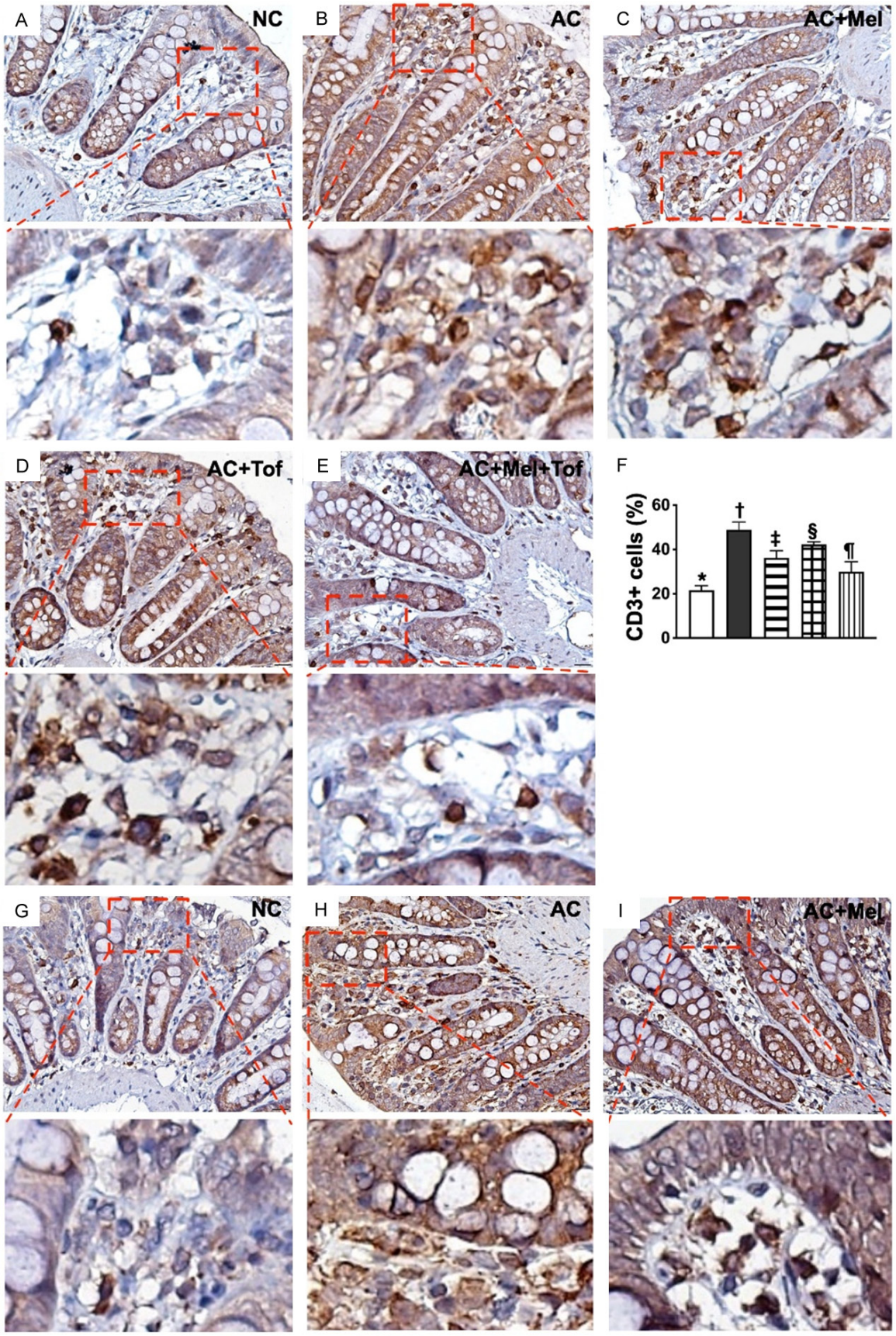


Figure 8. Impact of combined Tofacitinib and Melatonin therapy on ameliorating proinflammatory cytokines by day 14 after AC induction by SSD. (A-C) Represented the protein expression of nuclear factor (NF)-κB (A), interleukin (IL)-1β (B), and tumor necrosis factor (TNF)-α (C), * vs. other groups with different symbols (†, ‡, §, ¶), $P < 0.0001$. (D) Represented the protein expression of matrix metalloproteinase (MMP)-9, * vs. other groups with different symbols (†, ‡, §), $P < 0.0001$. (E-I) Illustrating the IF microscopic finding (400×) for identification of cellular expression of MMP-9 (green color). (J) Analytical result of number of MMP-9+ cells, * vs. other groups with different symbols (†, ‡, §), $P < 0.0001$. Scale bars in right lower corner represent 20 μm. (K) Protein expression of IL-17, * vs. other groups with different symbols (†, ‡, §, ¶), $P < 0.0001$. n = 6 for each group.

tophagy, and DNA damage in cultured CCD-18Co cells. Additionally, the in vitro study further demonstrated that LPS treatment markedly suppressed cell survival, suggesting that this could be due to LPS upregulation of proinflammatory cytokines and oxidative stress, which in turn caused cell apoptosis and death, as demonstrated by our flow cytometric studies. Intriguingly, these parameters were substantially reversed by melatonin or tofacitinib and substantially reversed by combined melatonin and tofacitinib treatment, suggesting that an optimal dosage of tofacitinib, especially when combined with melatonin therapy, could offer a great synergistic effect against endotoxin damage in colon cells.

Based on the promising results of our in vitro study, we developed an animal model of AC, specifically focusing on the therapeutic impact of a combined regimen of melatonin and tofacitinib in AC rodents.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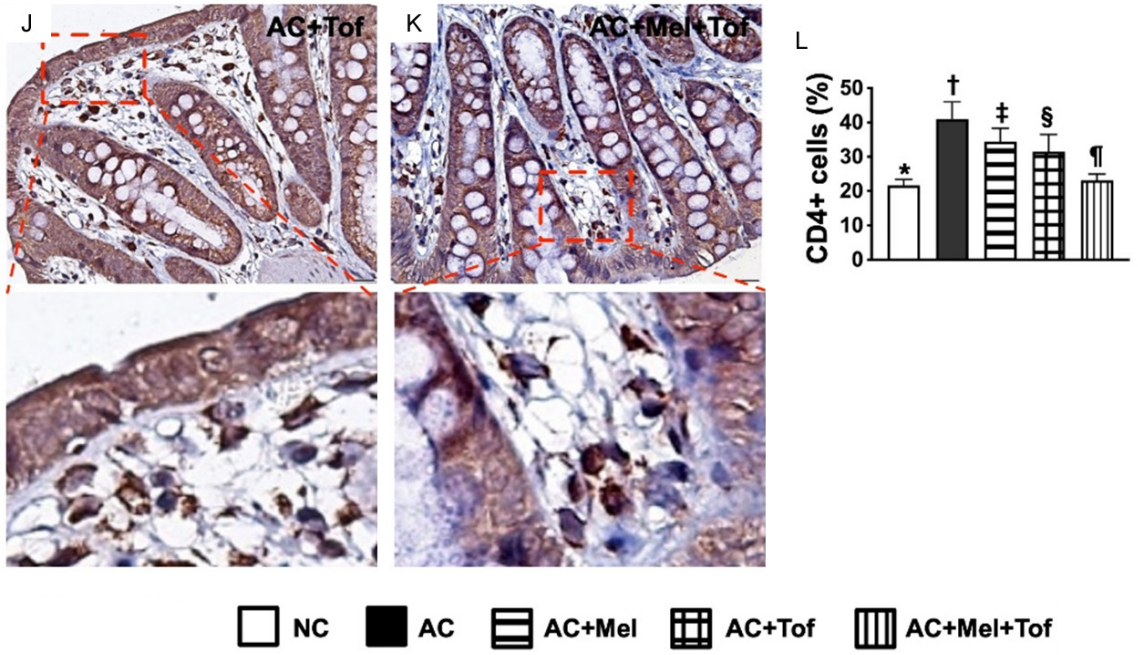

Figure 9. Impact of combined tofacitinib and melatonin therapy on suppressing immune cell infiltration by day 14 after AC induction by SSD. (A-E) Illustrating the microscopic finding (400×) of immunohistochemical stain (IHC) for identification of cellular expression of CD3 (gray color). (F) Analytical result of number of CD3+ cells, * vs. other groups with different symbols (†, ‡, §, ¶), $P < 0.0001$. (G-K) Illustrating the microscopic finding (400×) of IHC for identification of cellular expression of CD3 (gray color). (L) Analytical result of number of CD4+ cells, * vs. other groups with different symbols (†, ‡, §, ¶), $P < 0.0001$. n = 6 for each group.

The principal findings of the present in vivo study were that fecal stool (bloody stool assessment) and colon leakage/permeability (circulating FD4 fluorescein-dye intensity), two essential factors indicating colon mucosal layer damage, were substantially increased in AC animals without treatment compared with those of normal control animals, indicating that our experimental model of AC was successfully established. Another principal finding was that the colon injury score (i.e., histopathological assessment) was markedly higher in the AC group than in the normal control group. Importantly, these parameters (i.e., bloody stool, severity of colon leakage, and colon injury score) were strongly attenuated in AC animals treated with melatonin or tofacitinib and further strongly attenuated by the combination of these two regimens. Our previous studies have demonstrated that melatonin treatment effectively alleviates bloody stools, colon leakage/permeability, and colon damage [31, 33]. Additionally, clinical trials have shown that tofacitinib therapy was effective and safe for patients with UC [34, 35]. Therefore, our findings, in addition to strengthening the findings of previous studies [31, 33-35], highlight that this combination therapy, that is, melatonin + tofacitinib, has therapeutic potential for patients with UC, especially those who are refractory to conventional therapy.

The readers are interested in understanding the underlying mechanisms of our attractive and promising findings. We suggest that these two principles respond to readers' curiosity. First, it is well known that the JAK family of cytoplasmic protein tyrosine kinases comprises JAK1, JAK2, JAK3, and TYK2 [4]. Additionally, members of the JAK family bind to type I and II cytokine receptors and transmit extracellular cytokine signals to activate STATs [36-38]. Activated STATs translocate to the nucleus and modulate the transcription of effector genes [36-38]. Furthermore, basic research has shown that tofacitinib inhibits JAK family members [10, 12-14] in cell culture studies. Second, two cardinal findings from the in vivo study demonstrated that JAK/STAT and cell proliferation/cell-stress signaling (i.e., comprised of (PI3K/AKT/mTOR)) were markedly enhanced in AC compared with normal control animals.

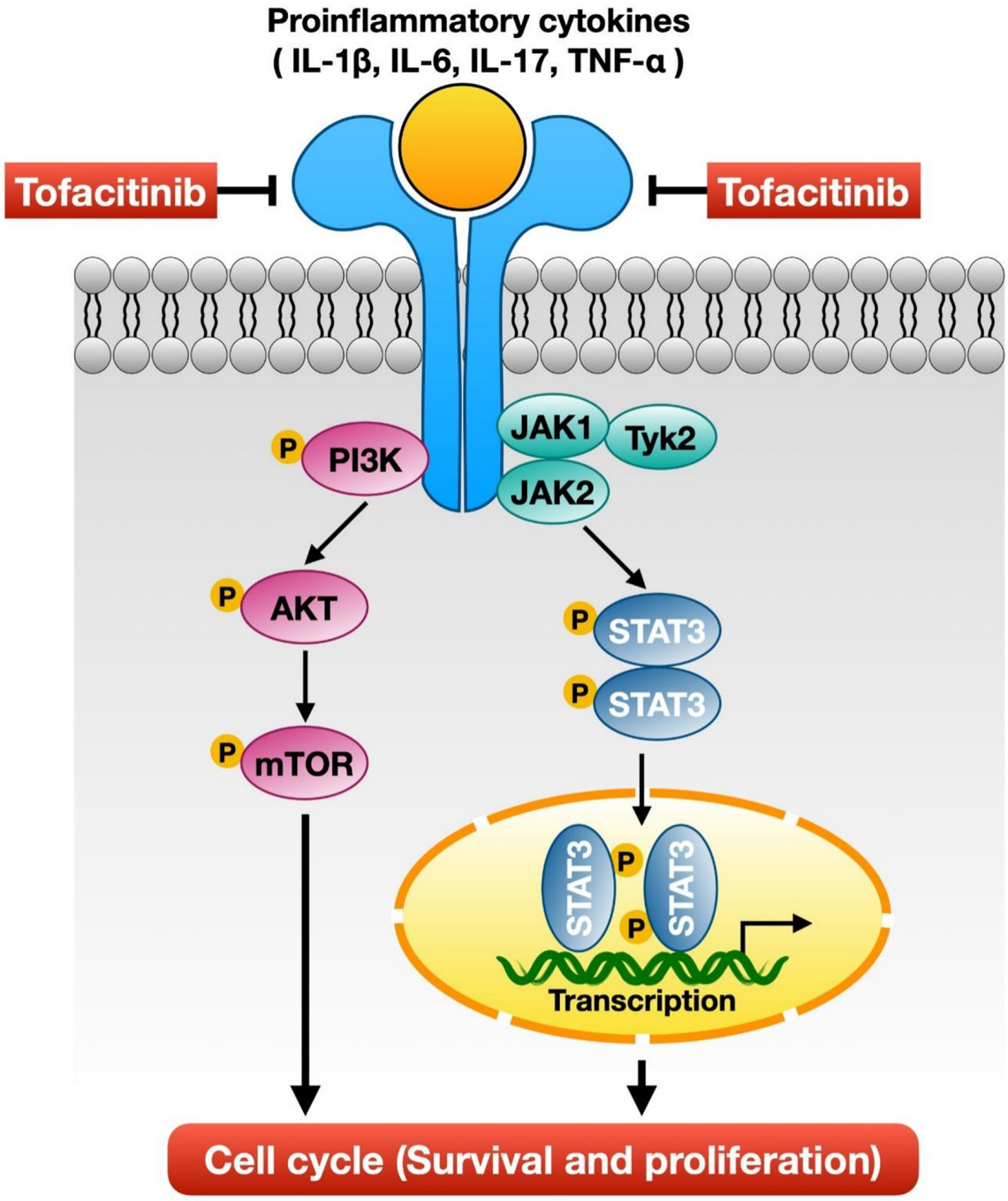

Figure 10. Schematically illustrated the underlying mechanisms of Janus kinase family/STAT and PI3K growth and survival pathways, and Tofacitinib treatment on suppressing the acute colitis. IL = interleukin; IFN = interferon.

Thus, our findings are supported by those of previous studies [37, 38]. A particularly important finding in the present in vivo study was that melatonin or tofacitinib therapy significantly suppressed and combined melatonin and tofacitinib therapy further significantly suppressed the activations of JAK/STAT and PI3K/AKT/mTOR signalings. In addition to corroborating the findings of previous studies [10, 12-14], our findings could, at least in part, explain why the molecular levels of inflammation, oxidative stress, apoptosis, cellular levels of DNA-

damaged biomarkers, and colon injury scores were considerably attenuated by melatonin-tofacitinib therapy.

One distinctive finding was that the protein levels of IL-17 and cellular expression of CD3 and CD4 were notably increased in the AC animals. IL-17, a potent pro-inflammatory cytokine, is involved in inflammatory-immune and cytotoxic cytokine interactions, resulting in organ damage [39-41]. These findings and those of previous studies [39-41] supported that IL-17 may participate in causing damage to the colon in the setting of AC through cytotoxic cytokine-immune interactions. We found that combined tofacitinib and melatonin therapy was superior to monotherapy in suppressing this parameter, resulting in attenuation of cytotoxic cytokine-immune interplay-induced colon damage.

Study limitations

Our study has several limitations. First, although the results were promising, the study period was relatively short, reflecting the acute response to the treatment. Accordingly, long-term outcomes are currently unclear. Second, stepwise increases in the concentrations of tofacitinib and melatonin were not performed to test their effects of tofacitinib and melatonin. Thus, we do not know the optimal concentrations of tofacitinib and melatonin used in this study. Finally, although extensive studies were performed in the present study, the exact underlying mechanism of action of melatonin-tofacitinib is currently not fully understood. Based on our results, we schematically illustrated the underlying mechanism of combined melatonin and tofacitinib therapy in protecting the rodent colon against AC-induced damage (**Figure 10**).

In conclusion, the results of the present study proved that the combination of tofacitinib and melatonin is safe and offers a synergistic effect in protecting the colon from AC-induced damage.

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Disclosure of conflict of interest

None.

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