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5-Fluorouracil Induced Intestinal Mucositis via Nuclear Factor-κB Activation by Transcriptomic Analysis and In Vivo Bioluminescence Imaging

--Manuscript Draft--

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Full Title:	5-Fluorouracil Induced Intestinal Mucositis via Nuclear Factor-κB Activation by Transcriptomic Analysis and In Vivo Bioluminescence Imaging
Short Title:	5-FU induced mucositis via NF-κB pathway
Corresponding Author:	Chien-Yun Hsiang China Medical University , TAIWAN
Keywords:	5-fluorouracil; intestinal mucositis; nuclear factor-κB; 5-aminosalicylic acid
Abstract:	5-Fluorouracil (5-FU) is a commonly used drug for the treatment of malignant cancers. However, approximately 80% of patients undergoing 5-FU treatment suffer from gastrointestinal mucositis. The aim of this report was to identify the drug target for the 5-FU-induced intestinal mucositis. 5-FU-induced intestinal mucositis was established by intraperitoneally administering mice with 100 mg/kg 5-FU. Network analysis of gene expression profile and bioluminescent imaging were applied to identify the critical molecule associated with 5-FU-induced mucositis. Our data showed that 5-FU induced inflammation in the small intestine, characterized by the increased intestinal wall thickness and crypt length, the decreased villus height, and the increased myeloperoxidase activity in tissues and proinflammatory cytokine production in sera. Network analysis of 5-FU-affected genes by transcriptomic tool showed that the expression of genes was regulated by nuclear factor-κB (NF-κB), and NF-κB was the central molecule in the 5-FU-regulated biological network. NF-κB activity was activated by 5-FU in the intestine, which was judged by in vivo bioluminescence imaging and immunohistochemical staining. However, 5-aminosalicylic acid (5-ASA) inhibited 5-FU-induced NF-κB activation and proinflammatory cytokine production. Moreover, 5-FU-induced histological changes were improved by 5-ASA. In conclusion, our findings suggested that NF-κB was the critical molecule associated with the pathogenesis of 5-FU-induced mucositis, and inhibition of NF-κB activity ameliorated the mucosal damage caused by 5-FU.
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Opposed Reviewers:	

Response to Reviewers:	Editor 1. indication in Fig 1 and Fig 6 which intestinal segments were evaluated The microscopic features of jejunum were evaluated in Figure 1 and Figure 6 (Figure 5 in the revised version). We have supplemented this information in the figure legends (p. 22-23). 2. presentation of the data of the gene expression analysis (Fig 2) also in a table, including the percentage of change for each gene The expression levels of genes in the network are shown in Table 1 (p. 25-27) 3. additional measurements as suggested by reviewer #2 should be considered We have revised our manuscript according to reviewer #2's comments. Our point-by-point reply to reviewer #2's comments is described as follows. Reviewer #1 This is an interesting well written paper that provides further information on pathobiology of mucositis. Thank you for your advices. By histological examination, DNA microarray, NF-κB bioluminescent imaging, immunohistochemical staining, MPO activity assay, and cytokine ELISA, we concluded that NF-κB was the critical molecule associated with the pathogenesis of 5-FU-induced mucositis, and inhibition of NF-κB activity ameliorated the mucosal damage caused by 5-FU. Reviewer #2 This paper provide evidence of an involvement of NF-κB in the intestinal mucositis caused by administration of 5-FU. While interesting, the results not support the conclusion that inhibition NF-κB suppressed the 5-FU-induced inflammation. The authors need show more experiments the aspects inflammatory of the mucositis and effect the 5-ASA administration. In relation the involvement of NF-κB in the intestinal mucositis induced by 5-FU the various manuscript showed the opposite theses results (Aota et al., Biochem Biophys Res Commun 273:1168-1174, 2000; Azuma et al., Biochem Biophys Res Commun 282: 292-296, 2001; Logan et al., Cancer Chemother Pharmacol 63:239-251, 2009), I think the authors need to do more experiments to confirm this objective. In this study, we found that NF-κB was the critical molecule that regulated the expression of 5-FU-affected genes, and NF-κB activity was induced by 5-FU in the intestine. In contrast, other studies indicated that 5-FU administration inhibits NF-κB activation in vitro. Aota et al. (Biochem Biophys Res Commun 273:1168-1174, 2000) and Azuma et al. (Biochem Biophys Res Commun 282: 292-296, 2001) reported that 5-FU suppresses NF-κB activity via the inhibition of IκB kinase activity and subsequently induces apoptosis in human salivary gland cancer cells. Contradictory effects of NF-κB activation on normal and cancer cells have been reported (Kaltschmidt et al., Eur J Biochem 267: 3828-3835, 2000). Activation of NF-κB can be either pro-apoptotic or anti-apoptotic, depending on the target cells. Therefore, it is possible that NF-κB activated by 5-FU results in apoptotic signals and proinflammatory cytokine production in normal mucosal tissue and sequentially contributed to the injury of gastrointestinal tract. We have supplemented this information in the Discussion section (p. 14, last paragraph; p. 15, 1st paragraph). Major revisions a) It is not clear why segments intestinal were evaluated in Fig 1 and Fig 6. The microscopic features of jejunum were evaluated in Figure 1 and Figure 6 (Figure 5 in the revised version). We have supplemented this information in the figure legends (p. 22-23). b) In my opinion the Fig 1 and Fig 6 could be only one. The order of figures in this manuscript is followed. 5-FU induces mucositis in the small intestine (Figure 1). The network center of 5-FU-affected genes is NF-κB (Figure 2). NF-κB in the small intestine is activated by 5-FU (Figure 3). 5-ASA suppresses 5-FU-induced mucositis via the inhibition of NF-κB activity (Figure 4 and Figure 5 (Figure 5 and Figure 6 in the last version)). We would like to maintain this order for a smooth layout.

c) In my opinion the Fig 3 and Fig 4 could be only one.
We have combined Figure 3 and Figure 4 into one figure.

d) The 5-ASA is specific inhibitor the NF-κB? In relation toxicity, is know high toxicity with use 5-ASA, in relation administration 5-ASA+5-FU was observation increased in toxicity? The 5-ASA treatment altered the up regulation the genes induced by 5-FU? The 5-ASA treatment was daily or not? Is need discussion more these question?
Oral administration of 5-ASA has been used for decades for the treatment of inflammatory bowel disease (Nikolaus et al., Hepatogastroenterology 47: 71-82, 2000). Activation of NF-κB in biopsies of ulcerative colitis is suppressed by 5-ASA, suggesting that 5-ASA is a potent inhibitor of NF-κB activation in vivo (Bantel et al., Am J Gastroenterol 95: 3452-3457, 2000).
Although 5-ASA often trigger undesirable side effects, we observed no visible toxicity during 5-ASA/5-FU treatment.
By NF-κB bioluminescent imaging, immunohistochemical staining, MPO activity assay, and cytokine ELISA, we found that 5-FU induced NF-κB activity and MPO activity in the jejunum and increased the levels of IL-1β and TNF-α in sera, while 5-ASA treatment decreased the 5-FU-induced NF-κB activity, MPO activity, and proinflammatory cytokine production in the samples. Therefore, these data suggested that 5-ASA altered the 5-FU-affected gene expression.
For 5-ASA treatment, mice were orally administered with 5-ASA (130 mM/kg) for two consecutive days before intraperitoneal administration of 5-FU and two consecutive days after 5-FU administration.
We have supplemented the action of 5-ASA in Results and Discussion sections (p. 11, 2nd paragraph; p. 15, 2nd paragraph), and 5-ASA treatment in Materials and Methods section (p. 6, 1st paragraph).

e) I suggest represent the data the gene expression (Fig 2) also in table include the percentage the regulation.
The expression levels of genes in the network are shown in Table 1 (p. 25-27)

f) The authors need to address the aspects inflammatory the mucositis induced by 5-FU experimentally with MPO activity measure, cytokines dosage (IL-1β and IL-6) at least, in addition, evaluated the 5-ASA effect.
We have performed immunohistochemical staining for detection of IL-1β and TNF-α in the intestine (Figure 4A). We also have performed MPO activity assay and cytokine ELISA for evaluation of MPO activity in the intestine and IL-1β and TNF-α production in sera (Figure 4B and C). These findings indicated that 5-FU induced intestinal inflammation, while 5-ASA suppressed 5-FU-induced inflammation in the intestine.
We have supplemented the MPO activity assay and cytokine ELISA in Materials and Methods section (p. 8-9). Experimental data have been supplemented in Results section (p. 12).

g) In experiments of the bioluminiescent, I suggest do the NF-κB measure of according Logan et al., Cancer Chemother Pharmacol 63:239-251, 2009 and evaluate the luciferase activity of according Wu et al., J Pharmacol Exp Ther 330:370-376, 2009;
Transgenic mice carrying the luciferase genes driven by NF-κB were constructed in our laboratory previously. Luciferase activity in whole body or organs was monitored and quantified as luminescent intensity by bioluminescent imaging (Figure 3). To correlate the luciferase activity with NF-κB activity in organs, we performed immunohistochemical staining for detection of p65 in the organs (Figure 4A). Additionally, previous study also has performed immunohistochemical staining to reveal the co-localization of luciferase protein-positive cells and NF-κB-positive cells in the tissue (Ho et al., Biomaterials 28: 4370-4377; 2007). Therefore, we concluded that the luciferase activity reflected the NF-κB trans-activity.
We have supplemented this information in Results section (p. 10, 3rd paragraph).

h) In addition immunohistochemical TNF-α, I suggest do quantitative analysis (immunomarcation scores) and the TNF-α measure by ELISA.
We have performed cytokine ELISA for detection of IL-1β and TNF-α production in sera (Figure 4C).
Cytokine ELISA has been supplemented in Materials and Methods section (p. 8).
Experimental data have been supplemented in Results section (p. 12).

	i) In "discussion" (pg 14, line 44-46), I think that the results not confirm suppression of the inflammation and the sequential amelioration of the mucositis in the intestine. It is necessary to evaluated parameters with: ratio villus/crypt, survival, diarrhea scores, MPO activity, and cytokines dosage for have amelioration in inflammation intestinal induced by 5-FU as showed by various manuscripts. I suggest change these paragraph. We have performed MPO activity assay and cytokine ELISA for evaluation of MPO activity in the intestine and IL-1β and TNF-α production in sera (Figure 4B and C). These findings indicated that 5-FU induced intestinal inflammation, while 5-ASA ameliorated 5-FU-induced inflammation in the intestine. We have supplemented the MPO activity assay and cytokine ELISA in Materials and Methods section (p. 8-9). Experimental data have been supplemented in Results section (p. 12). Minor revisions a) Pg 5, line 50. The correct is BALB/c. b) Pg 6, line 1. The concentration of the 5-FU is 100mg/kg or 150mg/kg. c) Pg 10, line 2. Delete "5". d) Pg 14, line 41. Change the expression "TNF-α production" to "immunomarcation for TNF-α". e) Pg 23, line 4. The correct is ** and ##. f) In Fig 3A, include the results the mock group. We have revised aforementioned errors according reviewer's advices.

Dear Dr. Zanger,

We would like to thank you for considering our article, as well as the referee for their critical reading and constructive remarks.

We have revised our manuscript (PONE-D-11-12010) according to editor's and reviewers' comments. Our point-by-point reply to editor's and reviewers' comments is described as follows.

We thank you for your consideration of this matter and hope that our manuscript will be acceptable for publication in PLoS ONE.

Yours sincerely,

Chien-Yun Hsiang

5-Fluorouracil Induced Intestinal Mucositis via Nuclear Factor-κB Activation by Transcriptomic Analysis and *In Vivo* Bioluminescence Imaging

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Short Title: 5-FU induced mucositis via NF-κB pathway

Abstract

5-Fluorouracil (5-FU) is a commonly used drug for the treatment of malignant cancers. However, approximately 80% of patients undergoing 5-FU treatment suffer from gastrointestinal mucositis. The aim of this report was to identify the drug target for the 5-FU-induced intestinal mucositis. 5-FU-induced intestinal mucositis was established by intraperitoneally administering mice with 100 mg/kg 5-FU. Network analysis of gene expression profile and bioluminescent imaging were applied to identify the critical molecule associated with 5-FU-induced mucositis. Our data showed that 5-FU induced inflammation in the small intestine, characterized by the increased intestinal wall thickness and crypt length, the decreased villus height, and the increased myeloperoxidase activity in tissues and proinflammatory cytokine production in sera. Network analysis of 5-FU-affected genes by transcriptomic tool showed that the expression of genes was regulated by nuclear factor-κB (NF-κB), and NF-κB was the central molecule in the 5-FU-regulated biological network. NF-κB activity was activated by 5-FU in the intestine, which was judged by *in vivo* bioluminescence imaging and immunohistochemical staining. However, 5-aminosalicylic acid (5-ASA) inhibited 5-FU-induced NF-κB activation and proinflammatory cytokine production. Moreover, 5-FU-induced histological changes were improved by 5-ASA. In conclusion, our findings suggested that NF-κB was the critical molecule associated with the pathogenesis of 5-FU-induced mucositis, and inhibition of NF-κB activity ameliorated the mucosal damage caused by 5-FU.

Keywords: 5-fluorouracil; intestinal mucositis; nuclear factor-κB; 5-aminosalicylic acid

Introduction

5-Fluorouracil (5-FU) is the most commonly used chemotherapy drug in the clinical oncologic practice. It is widely used for the treatment of various cancers, including gastrointestinal cancer, breast cancer, and head and neck cancer [1,2]. Clinical evidence from patients undergoing 5-FU therapy indicates that personal response to 5-FU is different. Some people display slight side effects, while others suffer from severe adverse effects that lead to the discontinuance of cancer therapy. The commonly side effects of 5-FU include myelosuppression, dermatitis, cardiac toxicity, diarrhea, and mucositis [1,3]. Among these adverse effects, gastrointestinal mucositis is a major complication that occurs in approximately 80% of patients receiving 5-FU and results in abdominal bloating as well as vomiting and diarrhea [4].

Mucositis usually appears along the entire gastrointestinal tract from mouth to anus and causes general debility. Mucositis of the intestine is characterized by increased crypt apoptosis and villus atrophy, leaving the mucosal tissue open to ulceration and infection [5-7]. Several factors or genes contributing to the 5-FU-induced mucositis have been studied. For examples, increased apoptosis and decreased cellularity by 5-FU cause the histological change in the intestine [8]. The formation of reactive oxygen species (ROS) and the production of proinflammatory cytokines, such as interleukin-1β (IL-1β), IL-6 and tumor necrosis factor-α (TNF-α), lead to the mucosal damage [9-11]. Additionally, the production of platelet-activating factor (PAF) participates in the pathogenesis of mucositis [12]. Although several genes have been suggested to be involved in the 5-FU-induced intestinal mucositis, the key molecules, especially the upstream transcription factors that regulate the downstream genes associated with the pathogenesis of mucositis are still uncertain. Moreover, better compounds targeting to the mechanism of mucosal injury remain to

be developed for the treatment of mucositis.

Nuclear factor-κB (NF-κB) is an inducible transcription factor that consists of heterodimers of RelA (p65), c-Rel, RelB, p50/NF-κB1, and p52/ NF-κB2. NF-κB is a central coordinator of innate and adaptive immune responses. It is also involved in the regulation of inflammatory cytokine production and inflammation [13,14]. When cells are exposed to the inflammatory and stress stimulators, NF-κB activates the downstream genes, including cytokine, cytokine receptor and cyclooxygenase genes, resulting in the inflammatory process [15,16]. Accordingly, controlling NF-κB activation may be a potent strategy for the treatment of inflammation.

In this study, we analyzed the mechanism of 5-FU-induced intestinal damage by transcriptomic analysis and bioluminescent imaging. Our findings demonstrated that NF-κB was the likely key molecule involved in the 5-FU-induced mucositis and inhibition of NF-κB activity by 5-aminosalicylic acid (5-ASA) improved the mucosal damage caused by 5-FU.

Materials and Methods

Animal experiments

Mouse experiments were conducted under ethics approval from China Medical University Animal Ethics Committee (Permit Number: 97-28-N). Transgenic mice carrying the NF-κB-driven luciferase gene were constructed as described previously [17]. BALB/c mice were purchased from National Laboratory Animal Center (Taipei, Taiwan).

The 5-FU-induced mucositis model was established as described previously [7]. Male mice (6 to 8 weeks old) were injected intraperitoneally with phosphate-buffered saline (PBS) (137 mM NaCl, 1.4 mM KH_2PO_4, 4.3 mM Na_2HPO_4, 2.7 mM KCl, pH

7.2) or 5-FU (100 mg/kg) (Sigma, St Louis, MO, USA). For 5-ASA treatment, mice were orally administered with 5-ASA (130 mM/kg) for two consecutive days before intraperitoneal administration of 5-FU and two consecutive days after 5-Fu administration. Mice were imaged for the luciferase activity or sacrificed for histological and immunohistochemical evaluations at indicated periods.

Histological analysis

Mice intestines were fixed in 10% phosphate-buffered formalin for 2 d and dehydrated in a series of graded alcohols (50%, 70%, and 95%) for 30 min each. Samples were then embedded in paraffin, cut into 5-μm sections, stained with hematoxylin and eosin (H&E), and subjected to blinded histological assessment. Villus height was measured from the baseline to the villus tip. Crypt length was measured from the baseline to the submucosa. The thickness of intestinal wall was measured from the submucosa to the serosa. Three independent measurements from 3 different longitudinal sections per mouse were made.

Microarray analysis

Total RNA was extracted from jejunum using RNeasy Mini kit (Qiagen, Valencia, CA, USA). Total RNA was quantified and evaluated as described previously [18]. Microarray analysis was performed as described previously [18-20]. Briefly, fluorescent-labeled RNA targets were prepared from 5 μg of total RNA using MessageAmp^{TM} aRNA kit (Ambion, Austin, TX, USA) and Cy5 dye (Amersham Pharmacia, Piscataway, NJ, USA). Fluorescent targets were hybridized to the Mouse Whole Genome OneArray^{TM} (Phalanx Biotech Group, Hsinchu, Taiwan) and scanned by an Axon 4000 scanner (Molecular Devices, Sunnyvale, CA, USA). Three

replicates from three independent mice were performed. The Cy5 fluorescent intensity of each spot was analyzed by genepix 4.1 software (Molecular Devices). The signal intensity of each spot was corrected by subtracting background signals in the surroundings. We filtered out spots that signal-to-noise ratio was less than 0 or control probes. Spots that passed these criteria were normalized by R program [21]. The fold changes of genes were calculated by dividing the normalized signal intensities of genes in 5-FU-treated mice by those in PBS-treated mice. Genes with fold changes ≥2 or ≤-2 were selected and used as the input genes for the generation of biological network using Transcription Regulation algorithm in MetaCore™ Analytical suite (GeneGo Inc., St. Joseph, MI, USA). All microarray data are MIAME compliant and the raw data have been deposited in a MIAME compliant database (Gene Expression Omnibus, accession number GSE28873).

In vivo and *ex vivo* imaging of luciferase activity

For *in vivo* imaging, mice were anesthetized with isoflurane and injected intraperitoneally with 150 mg/kg D-luciferin. Five minutes later, mice were placed facing up in the chamber and imaged for 1 min with the camera set at the highest sensitivity by IVIS Imaging System® 200 Series (Xenogen, Hopkinton, MA, USA). Photons emitted from the whole bodies were quantified using Living Image® software (Xenogen). Signal intensity was quantified as the sum of all detected photon counts from the whole body and presented as photon/sec. For *ex vivo* imaging, mice were anesthetized and injected with luciferin intraperitoneally. Five minutes later, mice were sacrificed and tissues were rapidly removed. Tissues were placed in the IVIS system and imaged with the same setting used for *in vivo* studies. Signal intensity was quantified as the sum of all detected photon counts per second within the region of

interest after subtracting the background luminescence and presented as photon/sec/cm^2/steradian (photon/sec/cm^2/sr).

Immunohistochemical staining

Sections of 5 μm were deparaffinized in xylene and rehydrated in graded alcohol. Endogenous peroxidase was quenched with 3% hydrogen peroxide in methanol for 15 min and the nonspecific binding was blocked with 1% bovine serum albumin at room temperature for 1 h. Sections were incubated with mouse monoclonal antibody against NF-κB p65 subunit (Chemicon, Temecula, CA, USA) or rabbit polyclonal antibody against IL-1β (Santa Cruz, CA, USA) or TNF-α (Abcam®, Cambridge, UK) at 1:50 dilution overnight at 4°C and then incubated with biotinylated secondary antibody (Zymed Laboratories, Carlsbad, CA, USA) at room temperature for 20 min. Finally, slides were incubated with avidin-biotin complex reagent and stained with 3,3'-diaminobenzidine according to manufacturer's protocol (Histostains®-Plus, Zymed Laboratories).

Cytokine enzyme-linked immunosorbent assay (ELISA)

IL-1β and TNF-α in sera were quantified by ELISA with Quantikine® mouse immunoassay kits (R&D Systems, Minneapolis, MN, USA). Briefly, sera were added to wells, which were coated with monoclonal antibody against IL-1β or TNF-α. After five washes, the biotinylated antibody against IL-1β or TNF-α, the peroxidase-conjugated avidin, and the chromogenic substrates were sequentially added to each well. The absorbance was read at 450 nm in an ELISA reader.

Myeloperoxidase (MPO) assay

MPO activities in the jejunum were quantified with MPO colorimetric activity assay kit (BioVision, Mountain View, CA, USA). Briefly, the frozen tissues were homogenized and centrifuged to remove insoluble materials. Supernatants were collected, mixed with MPO assay buffer and MPO substrate, incubated at room temperature for 1 h, and then mixed with tetramethylbenzidine probe. The absorbance was read at 412 nm in an ELISA reader.

Statistical analysis

Data were presented as mean ± standard error. Student's *t*-test was used for comparisons between two experiments. A value of $p<0.05$ was considered statistically significant.

Results

5-FU induced intestinal mucositis

Chemotherapy-induced diarrhea occurs in approximately 80% of patients treated with 5-FU. Previous studies have shown that 5-FU kills progenitor cells in the crypts of Lieberkühn and the bases of villi, leading to the breakdown of mucosal barrier [22]. Moreover, 5-FU administration results in increased apoptosis and decreased cellularity in the small intestine [8]. We therefore intraperitoneally administered mice with 5-FU and the histological changes in the small intestine were evaluated 2 days later. In comparison with mock, 5-FU caused mucosal damage in the small intestine (Figure 1). 5-FU decreased the height of villi and caused the blunting and fusion of villi. Moreover, 5-FU led to the intestinal inflammation, characterized by the infiltration of immune cells and the accumulation of fluid, and subsequently increased

the length of crypts. 5-FU also increased the thickness of intestinal wall. These findings indicated that intraperitoneal administration of 5-FU caused the mucosal damage and inflammation in the small intestine.

NF-κB was the central molecule in the 5-FU-affected gene expression network

We further elucidated the mechanism of 5-FU-induced intestinal mucositis by transcriptomic analysis. In a total of 29,922 genes, 1,614 genes were upregulated and 1,574 genes were downregulated by 2 fold by 5-FU. These genes were selected for the generation of biological network using Transcription Regulation algorithm in MetaCore. As shown in Figure 2, 5-FU-affected genes were directly connected to the NF-κB, suggesting that expressions of 5-FU-affected genes were regulated by NF-κB. The expression levels of genes in the network are shown in Table 1. Furthermore, NF-κB seemed to be the central molecule of the network. These findings suggested that NF-κB was the likely key molecule involved in the 5-FU-induced intestinal mucositis.

5-FU evoked the NF-κB activity judged by *in vivo* and *ex vivo* imaging

Transcriptomic analysis showed that NF-κB was the central molecule in the 5-FU-affected gene expression network. We therefore performed *in vivo* and *ex vivo* imaging to elucidate the NF-κB activity in mice following 5-FU administration. Transgenic mice carrying the luciferase gene driven by a promoter with five NF-κB responsive elements were used here. The luciferase activity reflected the NF-κB *trans*-activity.

Transgenic mice were intraperitoneally given with PBS or 5-FU, and the bioluminescent imaging was performed on 0, 1, 2, 5, 7, and 14 d. Figure 3A shows

that a maximal induction of NF-κB activity was observed on 2 d following 5-FU administration and *ex vivo* imaging was therefore performed on 2 d. As shown in Figure 3B and Figure 3C, 5-FU slightly affected the NF-κB activities in lung, liver, spleen, and stomach, while 5-FU significantly activated the NF-κB activity in the small intestine by 2.2-fold. These findings indicated that 5-FU evoked the whole body NF-κB activity on 2 d and induced the NF-κB activation in the small intestine. Moreover, 5-FU-induced intestinal mucositis could be assessed by NF-κB bioluminescent imaging.

5-FU-induced NF-κB activity was inhibited by 5-ASA

5-ASA is an anti-inflammatory drug that has been used for the treatment of ulcerative colitis for decades [23]. Activation of NF-κB in biopsies of ulcerative colitis is suppressed by 5-ASA, suggesting that 5-ASA is a potent inhibitor of NF-κB activation *in vivo* [23]. We therefore evaluated whether 5-ASA inhibited 5-FU-induced NF-κB activation and subsequently ameliorated the 5-FU-caused mucositis. Transgenic mice were administered with 5-FU and/or 5-ASA and imaged 2 days later. 5-FU induced the NF-κB activity in the small intestine, which was in agreement with aforementioned findings (Figure 3D). However, 5-ASA significantly reduced the 5-FU-induced NF-κB activity, with a 42% reduction of bioluminescent intensity. Immunohistochemical staining with antibody against NF-κB p65 subunit revealed that, in comparison with mock, there were many brown p65-reactive cells in the crypts and villi of 5-FU-treated intestine (Figure 4A). However, 5-ASA reduced the number of brown p65-reactive cells in the intestine. These findings indicated that 5-FU evoked the NF-κB activity, while 5-ASA inhibited 5-FU-induced NF-κB activity in the intestine.

Inhibition of NF-κB activity ameliorated the 5-FU-induced mucositis in the small intestine

We further tested whether the inhibition of 5-FU-induced NF-κB activity by 5-ASA improved the 5-FU-caused intestinal mucositis. Histological examination of the small intestine following 5-FU and/or 5-ASA treatment showed that 5-FU increased the thickness of intestinal wall and the length of crypt, while 5-ASA significantly decreased 5-FU-caused histological changes (Figure 5). 5-FU also decreased the height of villus, while 5-ASA slightly increased it. In addition to the histological changes, MPO activities in the intestine were induced by 5-FU and suppressed by 5-ASA, also indicating that 5-FU induced intestinal inflammation, while 5-ASA suppressed 5-FU-induced inflammation (Figure 4B). The levels of IL-1β and TNF-α in the tissues and sera were evaluated by immunohistochemical staining and cytokine ELISA, respectively. As shown in Figures 4A and 4C, 5-FU induced the immunomarcation for IL-1β and TNF-α in the tissues and increased the levels of IL-1β and TNF-α in sera, while 5-ASA suppressed 5-FU-induced IL-1β and TNF-α production in the tissues and sera. These findings suggested that 5-FU induced intestinal mucositis via NF-κB activity. Moreover, inhibition of NF-κB activity decreased the 5-FU-induced TNF-α production and subsequently improved the 5-FU-caused mucosal damage in the small intestine.

Discussion

5-FU is a commonly used chemotherapy drug for the treatment of malignant tumors. It kills tumor cells through interfering DNA synthesis and affecting protein

synthesis [2]. Approximately 80% of patients undergoing 5-FU therapy suffer from a range of symptoms, including mucositis and diarrhea. Gastrointestinal mucositis is frequently associated with pain and increased risk of infection. It leads to impaired quality of life in patients. Moreover, patients may no longer be able to continue cancer therapy in cases of severe mucositis [24]. Therefore, developing better therapeutic drug targeting to the mechanisms of mucosal damage is awaited.

Mechanisms involved in the pathogenesis of mucositis are very complex. Apoptosis, hypoproliferation, and inflammation contribute to the mucosal injury [9]. It has been reported that the expression of proinflammatory cytokines, such as IL-6 and TNF-α, in the small intestine and colon of rodents after chemotherapy is significantly increased [7,25]. IL-1 and IL-1 receptor antagonist are produced locally in the intestinal mucosa, and their expressions are increased in inflammatory mucosa [26,27]. Moreover, IL-1β plays a critical role in the genesis and development of intestinal mucositis after chemotherapy, and this type of effect is caused by inducing crypt cell apoptosis [11]. In addition to the proinflammatory cytokines, ROS generated by inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) lead to the mucosal injury. Increased iNOS and COX-2 activity in the 5-FU- and radiation-induced mucositis, suggesting the important role of ROS in the pathogenesis of oral mucositis [9,10]. Recently, the role of PAF in 5-FU-induced intestinal mucositis has been suggested using knockout animals and an antagonist of PAF receptor [12]. Because the expressions of proinflammatory cytokines, iNOS, COX-2, and PAF are regulated by various transcription factors, we applied transcriptomic analysis to find the upstream transcription factors that regulate the downstream gene expression and lead to mucosal injury.

Transcriptomic analysis by DNA microarray tool is a popular research and

screening tool for differentially expressed genes. Microarray-based gene expression patterns have been used to predict the clinical outcome and prognosis of patients undergoing 5-FU therapy [28-30]. It has also been applied to predict the therapeutic efficacy of 5-FU and to identify the biomarkers in various cancers [31,32]. We used microarray tool for the first time to identify the key molecule involved in the 5-FU-caused intestinal injury in this study. The expression levels of IL-6, TNF-α, and IL-1β were increased, with fold changes of 2.28, 3.37, and 6.77, respectively (data not shown). These data were in agreement with previous reports. Further network analysis using Transcription Regulation algorithm indicated that the expression of 5-FU-affected genes was regulated by NF-κB, and NF-κB was the central molecule in the biological network. These findings suggested that NF-κB was the upstream key molecule that regulated the expression of downstream genes and led to the mucositis of intestine.

NF-κB is a central coordinator of innate and adaptive immune responses. NF-κB has also been linked to the control of cell growth, apoptosis, and cell cycle [33]. Previous reports have implicated the NF-κB in the pathogenesis of several inflammatory diseases, such as local joint inflammation, glomerulonephritis, and inflammatory bowel diseases [34-36]. NF-κB activation is also found in biopsy tissues in cancer patients treated with radiation and several chemotherapeutic drugs, except 5-FU [6,37]. As a consequence of the gene upregulation by the initial activation of NF-κB, a broad range of biological active proteins accumulate and target to the submucosa tissue in the gastrointestinal tract. NF-κB activation induced by anti-neoplastic agents and radiation is therefore though to elicit the inflammatory and apoptotic responses that lead to the mucosal injury. In this study, we found that NF-κB was the critical molecule that regulated the expression of 5-FU-affected genes,

and NF-κB activity was induced by 5-FU in the intestine. In contrast, other studies indicated that 5-FU administration inhibits NF-κB activation *in vitro*. Aota et al [38] and Azuma et al [39] reported that 5-FU suppresses NF-κB activity via the inhibition of IκB kinase activity and subsequently induces apoptosis in human salivary gland cancer cells. Contradictory effects of NF-κB activation on normal and cancer cells have been reported [40]. Activation of NF-κB can be either pro-apoptotic or anti-apoptotic, depending on the target cells. Therefore, it is possible that NF-κB activated by 5-FU results in apoptotic signals and proinflammatory cytokine production in normal mucosal tissue and sequentially contributed to the injury of gastrointestinal tract.

Bioluminescent imaging was applied to evaluate the NF-κB activity after 5-FU administration. Transgenic mice carrying the luciferase gene under the control of NF-κB-responsive element were constructed previously, and the bioluminescent signal correlated with NF-κB activity indicated that bioluminescent intensity represents NF-κB activity *in vivo* [17,36]. Oral administration of 5-ASA has been used for decades for the treatment of inflammatory bowel disease [23]. 5-ASA is an anti-inflammatory drug that inhibits NF-κB activation and suppressed the inflammatory response [23]. In this study, we also found that 5-ASA decreased 5-FU-induced NF-κB activity and immunomarcation for IL-1β and TNF-α in the intestine. The histological changes of mucositis have also been improved. These findings suggested that inhibition of NF-κB activity might result in the suppression of inflammation and the sequential amelioration of mucositis in the intestine.

In conclusion, our findings suggested that NF-κB was the critical molecule involved in the 5-FU-caused mucosal injury, while inhibition of NF-κB activity suppressed the 5-FU-induced inflammation and sequentially improved the

5-FU-induced mucosal damage. These findings suggested that NF-κB was the potent target for the development of drugs for the treatment of 5-FU-induced mucositis.

Author Contributions

Conceived and designed the experiments: CTC TYH CYH. Performed the experiments: HL JAL HCH CCL HYL SLW YFH. Analyzed the data: CCL HYL CYH. Wrote the paper: CTC TYH CYH.

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Figure Legends

Figure 1. Histological examination of the small intestine following 5-FU administration. BALB/c mice were intraperitoneally administered with PBS (mock) or 5-FU and sacrificed 2 days later. (A) Microscopic features of the jejunum. Sections were stained with H&E and observed using light microscopy. Magnification 100×. Photos are representative images. (B) Intestinal morphometry of intestinal wall thickness, crypt length, and villus height. Six mice in each group were sacrificed for the morphometry analysis. Three intestinal walls, crypts, and villi in 3 longitudinal sections per mouse were counted. Results are expressed as relative length, which is presented as a comparison with the length or thickness relative to mock. Values are mean ± standard error. $^{**}p<0.01$, $^{***}p<0.001$, compared with mock.

Figure 2. Network analysis of 5-FU-affected genes in the small intestine. Upregulated genes are marked with red circles/disks. Downregulated genes are marked with blue circles/disks. Cyan lines indicate the fragments of canonical pathways.

Figure 3. NF-κB-dependent bioluminescence in living mice and individual organs following 5-FU administration. (A) *In vivo* imaging. Transgenic mice were injected intraperitoneally with PBS or 5-FU and imaged at indicated periods. Results are expressed as relative intensity, which is presented as the comparison with the NF-κB-dependent bioluminescent signal relative to mock. Values are mean ± standard error (n=6 per group). $^{*}p<0.05$, compared with mock. (B) *Ex vivo* imaging. Transgenic mice were injected intraperitoneally with PBS (mock) or 5-FU. Two days later, mice were sacrificed and organs were subjected to image. The color overlay on the image represents the photon/sec emitted from the organs, as indicated by the color

scale. Photos are representative images (n=6 per group). (C) Quantification of photon emission from the organs. Values are mean ± standard error (n=6 per group). **p<0.01, compared with mock. (D) NF-κB-dependent bioluminescence in the intestine following 5-FU and/or 5-ASA administration. Transgenic mice were administered with 5-FU and/or 5-ASA and imaged 2 days later. The color overlay on the image represents the photon/sec emitted from the intestine, as indicated by the color scale. Photos are representative images (n=6 per group). Quantification of photon emission from the intestine was shown on the top. Values are mean ± standard error. *p<0.05, compared with mock. $^{\#}p$<0.05, compared with 5-FU treatment.

Figure 4. Immunohistochemical staining and MPO activity of jejunum and cytokine ELISA of sera following 5-FU and/or 5-ASA administration. Transgenic mice were administered with 5-FU and/or 5-ASA and sacrificed 2 days later. (A) Sections were stained by immunohistochemistry using antibody against NF-κB, IL-1β, or TNF-α. Magnification 100×. Photos are representative images (n=6 per group). (B) MPO activity assay. Frozen jejunum was homogenized and MPO activity in the tissue was analyzed. Values are mean ± standard error. *p<0.05, compared with mock. (C) Cytokine ELISA. The levels of IL-1β and TNF-α were analyzed by cytokine ELISA. Values are mean ± standard error. *p<0.05, ***p<0.001, compared with mock. $^{\#\#\#}p$<0.001, compared with 5-FU treatment.

Figure 5. Histological examination of the small intestine following 5-FU and/or 5-ASA administration. Transgenic mice were administered with 5-FU and/or 5-ASA and sacrificed 2 days later. (A) Microscopic features of the jejunum. Sections were stained with H&E and observed using light microscopy. Magnification 40×. Photos

are representative images (n=6 per group). (B) Intestinal morphometry of intestinal wall thickness, crypt length, and villus height. Six mice in each group were sacrificed for the morphometry analysis. Three intestinal walls, crypts, and villi in 3 longitudinal sections per mouse were counted. Results are expressed as relative length, which is presented as a comparison with the length or thickness relative to mock. Values are mean ± standard error. $^{**}p<0.01$, compared with mock. $^{\#\#}p<0.01$, compared to 5-FU treatment.

Table 1 Expression levels of genes in the network in 5-FU-induced mucositis.

Gene_symbol	Gene_description	Fold change[a]	*p* value[b]
Ccl11	Small chemokine (C-C motif) ligand 11 (Eotaxin)	5.39±0.79	4.2×10^{-5}
Chrna6	Cholinergic receptor, nicotinic, alpha polypeptide 6	4.84±5.74	0.18105
Il25	Interleukin 25	4.66±5.72	0.24311
Rhob	Ras homolog gene family, member B	4.61±6.28	0.38070
Pfkfb3	6-Phosphofructo-2-kinase/fructose-2,6-biphosphatase 3	4.30±2.90	0.11444
Akr1c21	Aldo-keto reductase family 1, member C21	4.23±2.93	0.02762
Adipoq	Adipocyte-specific protein AdipoQ	3.79±2.65	0.15414
Tpm1	Tropomyosin 1, alpha	3.77±1.74	0.01850
Ly6e	Lymphocyte antigen 6 complex, locus E	3.59±0.45	5.4×10^{-5}
Kcnj2	Potassium inwardly-rectifying channel, subfamily J, member 2	3.55±3.46	0.14500
Tnip2	TNFAIP3 interacting protein 2	3.44±3.35	0.15565
Scyl1	SCY1-like 1 (*S. cerevisiae*)	3.41±1.32	0.01068
Wbp5	WW domain binding protein 5	3.39±1.33	0.00927
Tnfrsf1b	Tumor necrosis factor receptor superfamily, member 1b	3.37±3.94	0.32369
Pax2	Paired box gene 2	3.12±3.34	0.26449
Irf4	Interferon regulatory factor 4	3.10±2.07	0.15196
P2rx2	Purinergic receptor P2X, ligand-gated ion channel, 2	3.07±1.17	0.01485
Sqstm1	Sequestosome 1	3.04±3.33	0.31461
Top2a	Topoisomerase (DNA) II alpha	3.03±1.96	0.04543
Cbs	Cystathionine beta-synthase	3.03±2.83	0.20677
Enah	Enabled homolog (*Drosophila*)	2.95±2.75	0.49872
Grin1	Glutamate receptor, ionotropic, NMDA1 (zeta 1) (NR1)	2.85±2.19	0.11010
Lxn	Latexin	2.79±3.07	0.36349
Bace1	Beta-site APP cleaving enzyme 1	2.71±1.37	0.08244
Ltf	Lactotransferrin	2.64±3.11	0.48107
Ccl4	Chemokine (C-C motif) ligand 4 (MIP-1-beta)	2.52±2.40	0.29653
Hc	Hemolytic complement (C5)	2.51±1.32	0.10525
Ssb	Sjogren syndrome antigen B	2.49±2.39	0.29861
Cdh16	Cadherin 16	2.42±1.04	0.02407
Cyp1b1	Cytochrome P450, family 1, subfamily b, polypeptide 1	2.37±1.02	0.02609
Grap2	GRB2-related adaptor protein 2	2.35±1.06	0.03017
Ccl5	Chemokine (C-C motif) ligand 5	2.34±1.05	0.03075
Cxcl10	Chemokine (C-X-C motif) ligand 10 (ip10)	2.34±1.05	0.03076
Il6	Interleukin 6	2.28±1.38	0.14487
Rac1	RAS-related C3 botulinum substrate 1	2.27±2.06	0.61901
Mmp24	Matrix metallopeptidase 24	2.23±0.57	0.00509
Slc1a2	Solute carrier family 1 (glial high affinity glutamate transporter), member 2	2.21±0.91	0.04296
Muc4	Mucin 4	2.21±1.23	0.08205
Ppp2r4	Protein phosphatase 2A, regulatory subunit B (PR 53)	2.18±1.59	0.23068
Zdhhc17	Zinc finger, DHHC domain containing 17	2.17±1.20	0.11078
Ahsg	Alpha-2-HS-glycoprotein	2.14±1.18	0.11679
Ccbp2	Chemokine binding protein 2	2.14±1.31	0.16119
Chrm3	Cholinergic receptor, muscarinic 3, cardiac	2.13±0.86	0.03724
Pla2g4a	Phospholipase A2, group IVA (cytosolic, calcium-dependent)	2.10±1.37	0.18116
Sele	Selectin, endothelial cell	2.07±0.82	0.04074
Slc12a3	Solute carrier family 12, member 3	2.05±1.34	0.19739
St6gal2	Beta galactoside alpha 2,6 sialyltransferase 2	2.04±0.98	0.11112
Slc7a5	Solute carrier family 7 (cationic amino acid transporter, y+ system), member 5	2.04±0.81	0.04360
Mefv	Mediterranean fever	2.03±1.53	0.25031
Il12b	Interleukin 12b	2.02±1.54	0.26543

	App	Amyloid beta (A4) precursor protein	-2.00±0.59	0.01319
1	Sdc4	Syndecan 4	-2.01±0.15	8.8×10^{-5}
2	Tfrc	Transferrin receptor (TfR1)	-2.03±0.82	0.02629
3	Pcdh7	Protocadherin 7	-2.06±0.08	7.2×10^{-6}
4	Grb2	Growth factor receptor bound protein 2	-2.06±0.15	8.1×10^{-5}
5	G6pc	Glucose-6-phosphatase, catalytic	-2.07±0.10	1.7×10^{-5}
6	Slc12a2	Solute carrier family 12, member 2	-2.10±0.40	0.00223
7	Eif4ebp1	Eukaryotic translation initiation factor 4E binding protein 1	-2.11±0.22	0.00024
8	Dpyd	Dihydropyrimidine dehydrogenase	-2.13±0.31	0.00099
9	Gch1	GTP cyclohydrolase 1	-2.16±0.16	6.8×10^{-5}
10	Birc5	Baculoviral IAP repeat-containing 5	-2.16±0.16	6.1×10^{-5}
11	St6gal1	Beta galactoside alpha 2,6 sialyltransferase 1 (SIAT1)	-2.17±0.26	0.00045
12	Bst2	Bone marrow stromal cell antigen 2	-2.23±0.12	1.3×10^{-5}
13	Ilk	Integrin linked kinase	-2.23±0.39	0.00122
14	Zfpm1	Zinc finger protein, multitype 1	-2.24±0.49	0.00329
15	Itga3	Integrin alpha 3	-2.35±0.10	4.2×10^{-6}
16	Fcgrt	Fc receptor, IgG, alpha chain transporter	-2.36±0.15	2.2×10^{-5}
17	Gpx4	Glutathione peroxidase 4	-2.36±1.85	0.06871
18	Ldlr	Low density lipoprotein receptor	-2.37±0.33	0.00046
19	Pak1	P21 (CDKN1A)-activated kinase 1	-2.40±0.34	0.00038
20	Cdkn1a	Cyclin-dependent kinase inhibitor 1A (P21)	-2.45±0.04	4.5×10^{-8}
21	Comt	Catechol-O-methyltransferase	-2.45±0.26	0.000146
22	Pigr	Polymeric immunoglobulin receptor	-2.49±2.08	0.06463
23	Sod2	Superoxide dismutase, mitochondrial precursor	-2.53±0.44	0.00091
24	Ctnna1	Catenin alpha-1	-2.54±0.57	0.00209
25	Acadvl	Acyl-Coenzyme A dehydrogenase, very long chain	-2.56±0.34	0.00024
26	Ptbp1	Polypyrimidine tract binding protein 1	-2.56±0.14	7.3×10^{-6}
27	Lgals3	Lectin, galactose binding, soluble 3	-2.57±1.20	0.03328
28	Ndrg2	N-myc downstream regulated gene 2	-2.64±0.14	3.9×10^{-6}
29	Mlycd	Malonyl-CoA decarboxylase	-2.70±0.11	2.3×10^{-6}
30	Psmb9	Proteosome subunit, beta type 9	-2.81±0.36	0.00016
31	Il10rb	Interleukin 10 receptor, beta	-3.06±0.25	1.9×10^{-5}
32	Prkcd	Protein kinase C, delta	-3.09±0.23	1.3×10^{-5}
33	Slc6a4	Solute carrier family 6 (neurotransmitter transporter, serotonin), member 4	-3.11±0.24	1.6×10^{-5}
34	Bst1	Bone marrow stromal cell antigen 1	-3.14±0.54	0.00043
35	Cyp4b1	Cytochrome P450, family 4, subfamily b, polypeptide 1	-3.21±0.12	8.4×10^{-7}
36	Pglyrp1	Peptidoglycan recognition protein 1	-3.43±0.33	2.4×10^{-5}
37	H1fx	H1 histone family, member X	-3.57±0.46	6.9×10^{-5}
38	Txnip	Thioredoxin interacting protein	-3.60±0.65	0.00024
39	Tbk1	TANK-binding kinase 1	-3.60±0.44	5.7×10^{-5}
40	Tnfaip1	Tumor necrosis factor, alpha-induced protein 1 (endothelial)	-3.66±0.39	3.2×10^{-5}
41	Eif4a1	Eukaryotic translation initiation factor 4A1	-3.77±0.33	1.3×10^{-5}
42	Muc3	Mucin 3, intestinal	-3.83±0.45	4.5×10^{-5}
43	Pdha1	Pyruvate dehydrogenase E1 alpha 1	-3.87±0.43	3.1×10^{-5}
44	Pafah1b3	Platelet-activating factor acetylhydrolase, isoform 1b, alpha1 subunit	-3.98±0.38	1.5×10^{-5}
45	Aco2	Aconitase 2, mitochondrial	-4.22±2.20	0.00547
46	Sod1	Superoxide dismutase 1, soluble	-4.23±2.70	0.01145
47	Pgk1	Phosphoglycerate kinase 1	-4.36±1.49	0.00188
48	Gnb1	Guanine nucleotide binding protein, beta 1	-4.42±1.75	0.00198
49	Gstm1	Glutathione S-transferase, mu 1	-4.65±1.88	0.00239
50	Cyp4f14	Cytochrome P450, family 4, subfamily f, polypeptide 14	-4.77±3.12	0.03197
51	Lima1	LIM domain and actin-binding protein 1	-4.83±0.47	1.1×10^{-5}
52	Glo1	Glyoxalase 1	-5.24±0.61	1.7×10^{-5}
53	Cyp4f13	Cytochrome P450, family 4, subfamily f, polypeptide 13	-5.29±0.63	1.6×10^{-5}
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	Apoc3	Apolipoprotein C-III	-5.77±2.10	0.00109
1	B2m	Beta-2 microglobulin	-5.78±6.05	0.02204
2	Hes6	Hairy and enhancer of split 6 (*Drosophila*)	-6.11±0.52	3.4×10^{-6}
3	S100a10	S100 calcium binding protein A10 (calpactin)	-6.53±1.28	9.9×10^{-5}
4	Abcg5	ATP-binding cassette, sub-family G (WHITE), member 5	-6.99±0.61	2.8×10^{-6}
5	Cyp3a11	Cytochrome P450, family 3, subfamily a, polypeptide 11	-7.35±0.48	7.8×10^{-7}
6	Krt8	Keratin, type II cytoskeletal 8	-7.95±2.47	0.00031
7	Irf1	Interferon regulatory factor 1	-8.15±1.27	2.2×10^{-5}
8	Prdx5	Peroxiredoxin 5	-8.29±1.71	5.0×10^{-5}
9	Abcd3	ATP-binding cassette, sub-family D (ALD), member 3 (PMP70)	-8.51±1.32	1.6×10^{-5}
10	Gstp1	Glutathione S-transferase, pi 1	-9.88±4.95	0.00150
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[a] Values are mean ± standard error (n=3).

[b] p values were calculated by the geneSetTest function implemented in the limma package.

Figure 1
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Figure 1

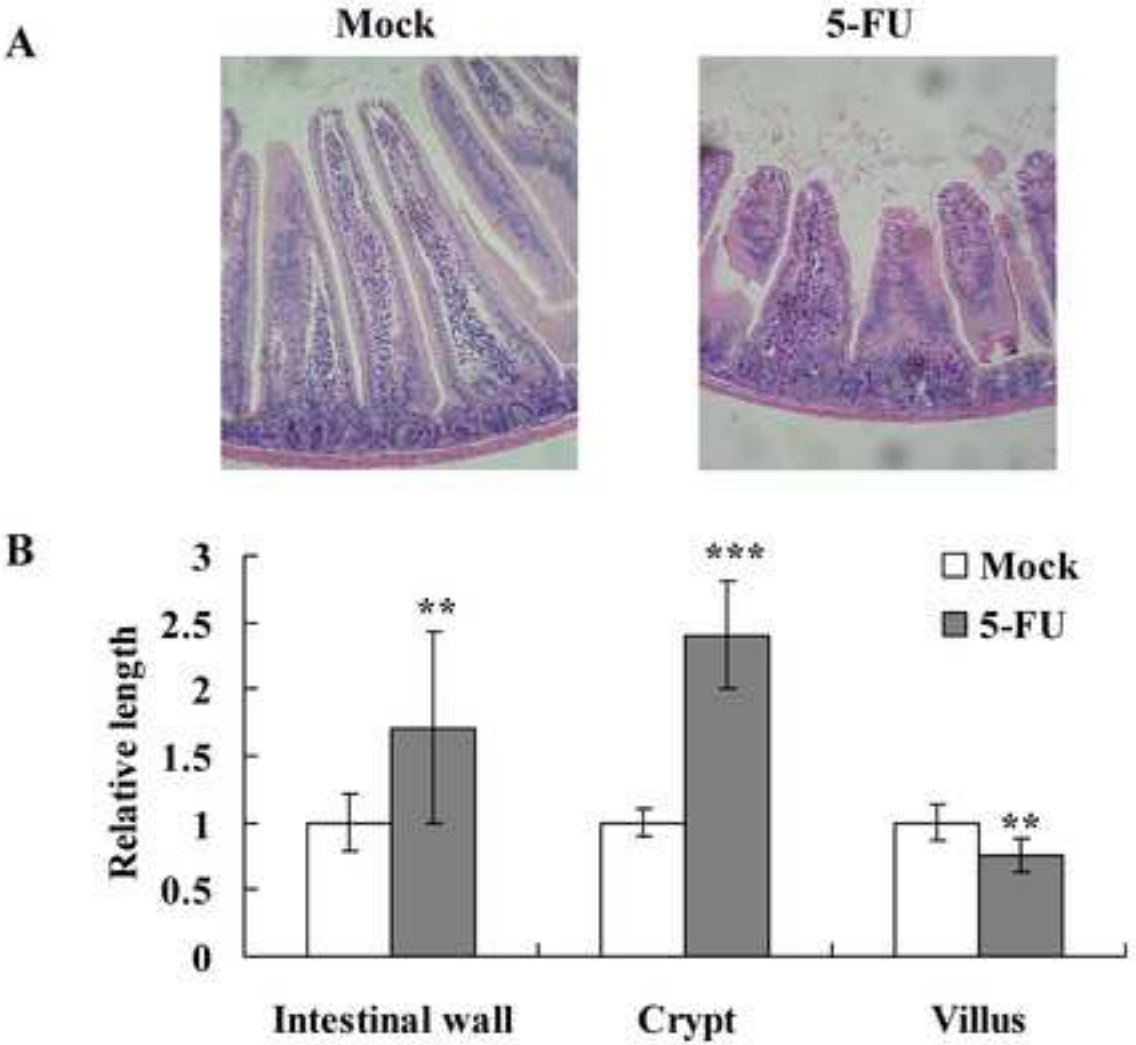

Figure 2

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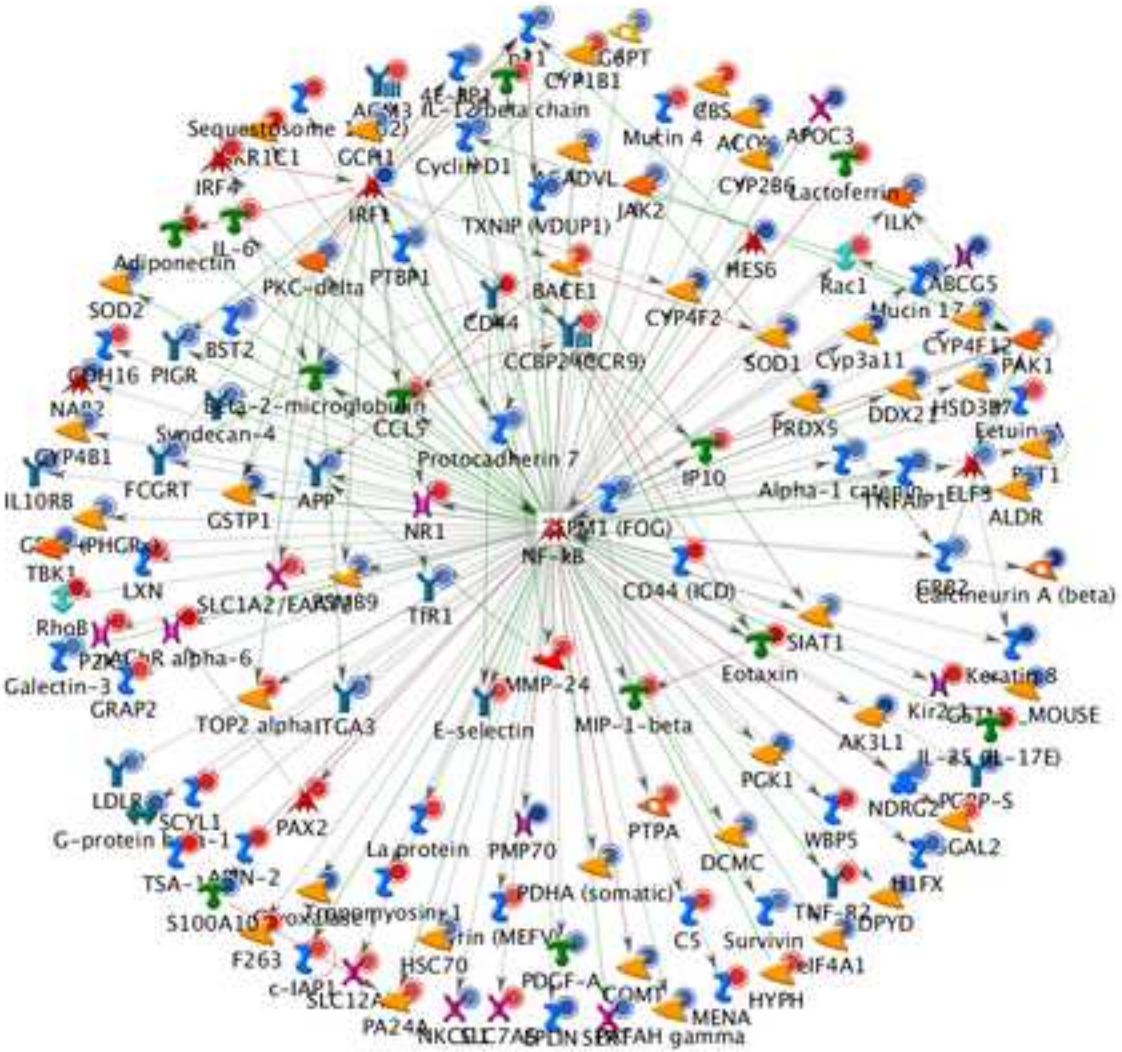
Figure 2



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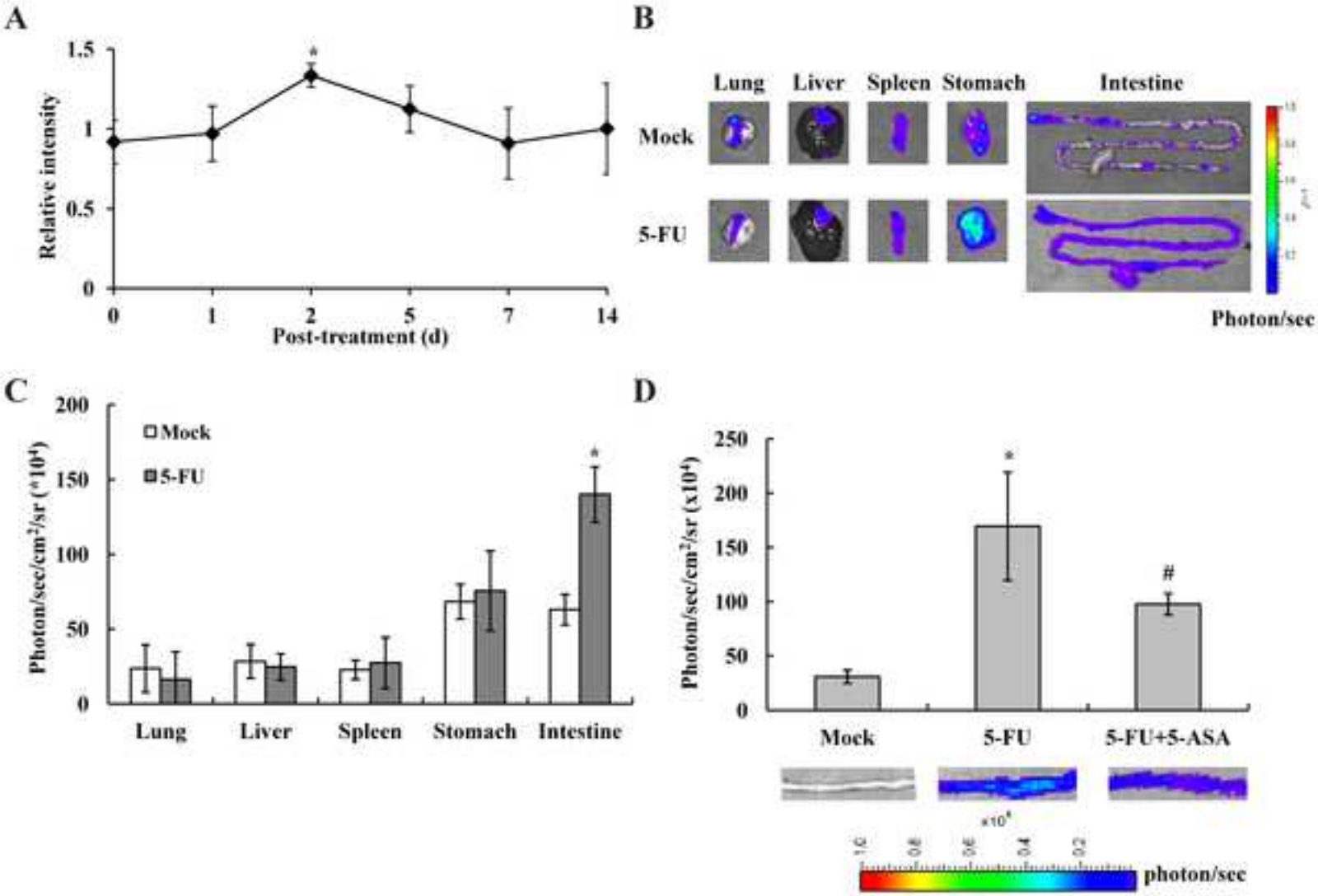

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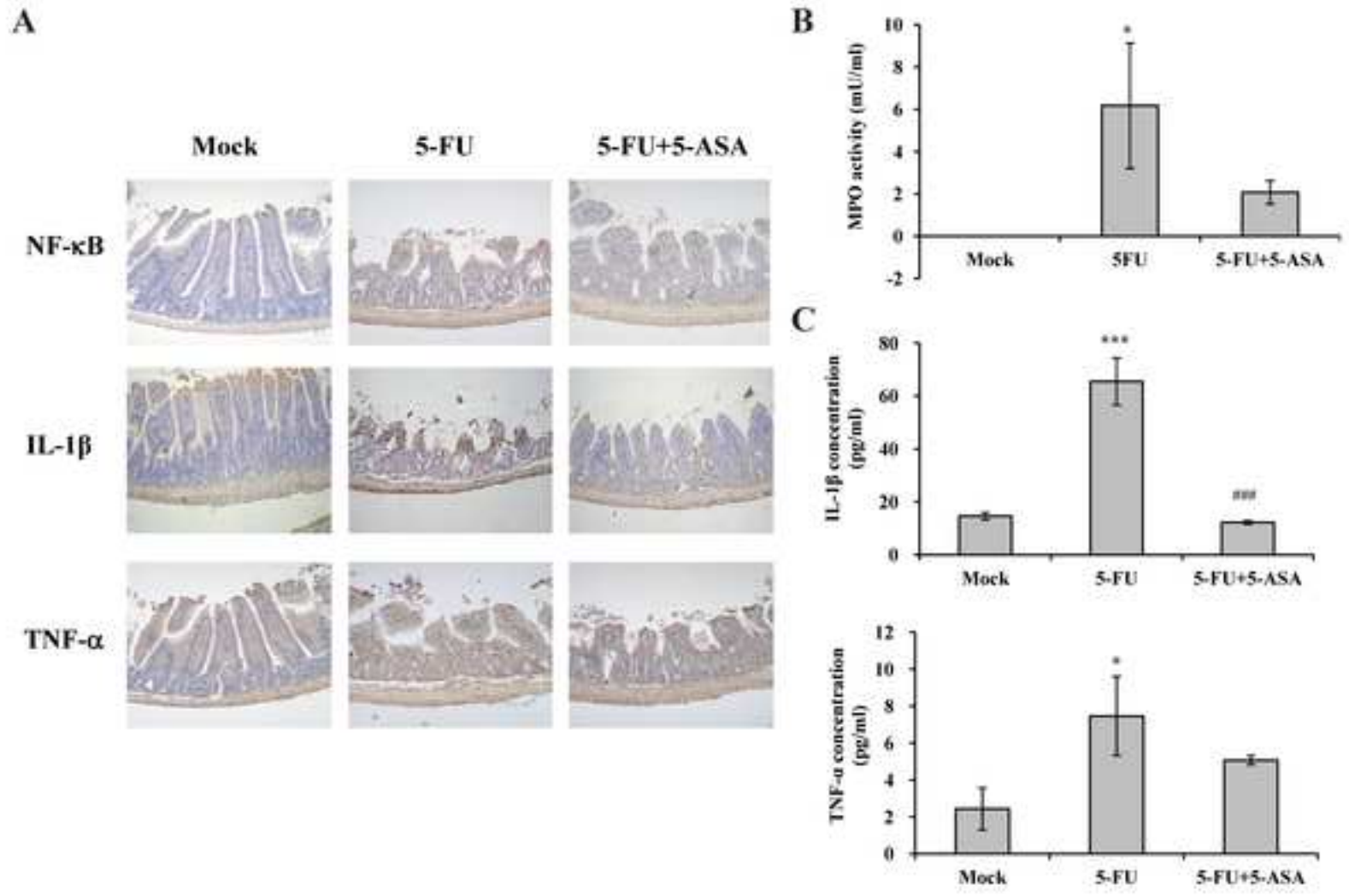

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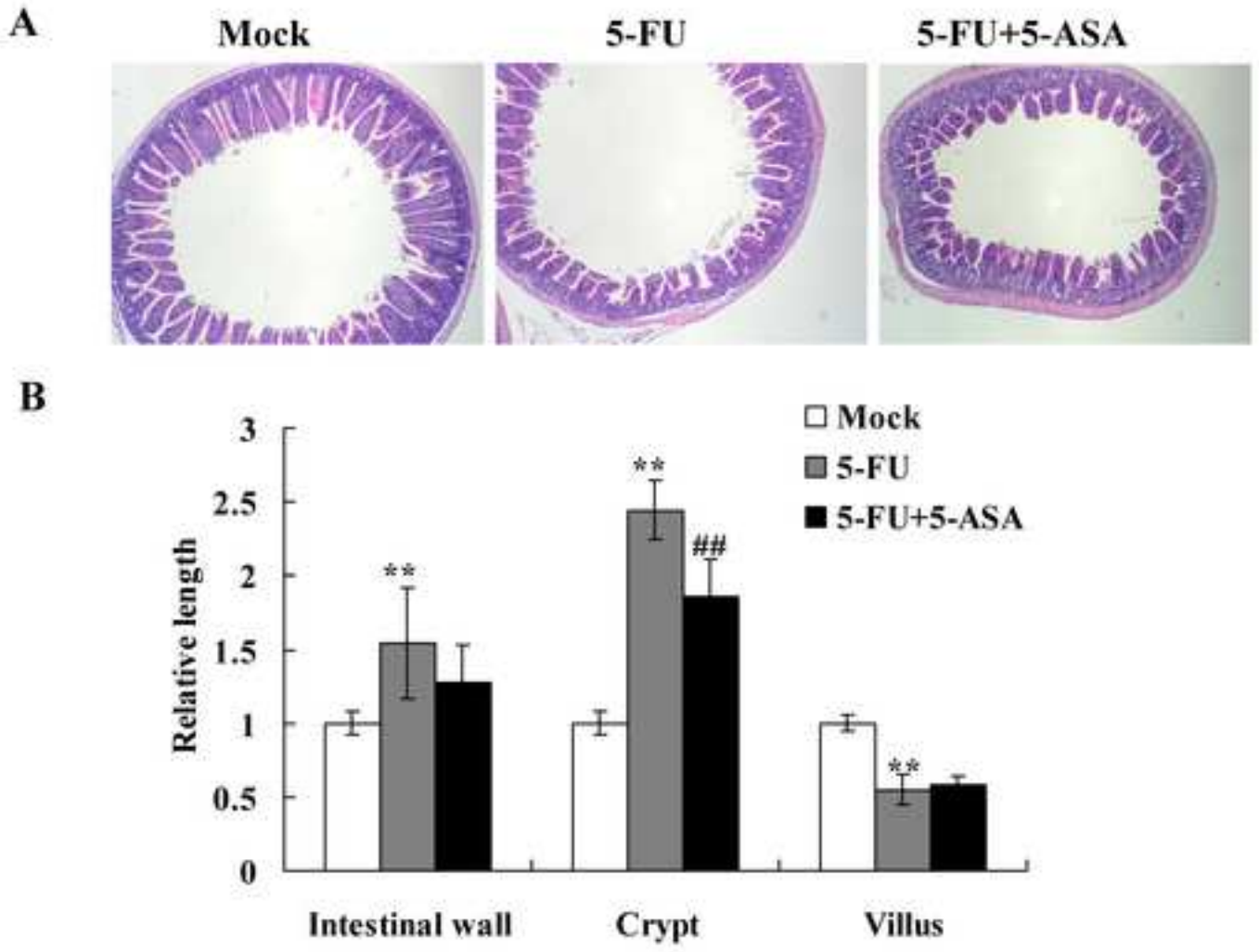

5-Fluorouracil Induced Intestinal Mucositis via Nuclear Factor-κB Activation by Transcriptomic Analysis and *In Vivo* Bioluminescence Imaging

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Short Title: 5-FU induced mucositis via NF-κB pathway

Abstract

5-Fluorouracil (5-FU) is a commonly used drug for the treatment of malignant cancers. However, approximately 80% of patients undergoing 5-FU treatment suffer from gastrointestinal mucositis. The aim of this report was to identify the drug target for the 5-FU-induced intestinal mucositis. 5-FU-induced intestinal mucositis was established by intraperitoneally administering mice with 100 mg/kg 5-FU. Network analysis of gene expression profile and bioluminescent imaging were applied to identify the critical molecule associated with 5-FU-induced mucositis. Our data showed that 5-FU induced inflammation in the small intestine, characterized by the increased intestinal wall thickness and crypt length, the decreased villus height, and the increased myeloperoxidase activity in tissues and proinflammatory cytokine production in sera. Network analysis of 5-FU-affected genes by transcriptomic tool showed that the expression of genes was regulated by nuclear factor-κB (NF-κB), and NF-κB was the central molecule in the 5-FU-regulated biological network. NF-κB activity was activated by 5-FU in the intestine, which was judged by *in vivo* bioluminescence imaging and immunohistochemical staining. However, 5-aminosalicylic acid (5-ASA) inhibited 5-FU-induced NF-κB activation and proinflammatory cytokine production. Moreover, 5-FU-induced histological changes were improved by 5-ASA. In conclusion, our findings suggested that NF-κB was the critical molecule associated with the pathogenesis of 5-FU-induced mucositis, and inhibition of NF-κB activity ameliorated the mucosal damage caused by 5-FU.

Keywords: 5-fluorouracil; intestinal mucositis; nuclear factor-κB; 5-aminosalicylic acid

Introduction

5-Fluorouracil (5-FU) is the most commonly used chemotherapy drug in the clinical oncologic practice. It is widely used for the treatment of various cancers, including gastrointestinal cancer, breast cancer, and head and neck cancer [1,2]. Clinical evidence from patients undergoing 5-FU therapy indicates that personal response to 5-FU is different. Some people display slight side effects, while others suffer from severe adverse effects that lead to the discontinuance of cancer therapy. The commonly side effects of 5-FU include myelosuppression, dermatitis, cardiac toxicity, diarrhea, and mucositis [1,3]. Among these adverse effects, gastrointestinal mucositis is a major complication that occurs in approximately 80% of patients receiving 5-FU and results in abdominal bloating as well as vomiting and diarrhea [4].

Mucositis usually appears along the entire gastrointestinal tract from mouth to anus and causes general debility. Mucositis of the intestine is characterized by increased crypt apoptosis and villus atrophy, leaving the mucosal tissue open to ulceration and infection [5-7]. Several factors or genes contributing to the 5-FU-induced mucositis have been studied. For examples, increased apoptosis and decreased cellularity by 5-FU cause the histological change in the intestine [8]. The formation of reactive oxygen species (ROS) and the production of proinflammatory cytokines, such as interleukin-1β (IL-1β), IL-6 and tumor necrosis factor-α (TNF-α), lead to the mucosal damage [9-11]. Additionally, the production of platelet-activating factor (PAF) participates in the pathogenesis of mucositis [12]. Although several genes have been suggested to be involved in the 5-FU-induced intestinal mucositis, the key molecules, especially the upstream transcription factors that regulate the downstream genes associated with the pathogenesis of mucositis are still uncertain. Moreover, better compounds targeting to the mechanism of mucosal injury remain to

be developed for the treatment of mucositis.

Nuclear factor-κB (NF-κB) is an inducible transcription factor that consists of heterodimers of RelA (p65), c-Rel, RelB, p50/NF-κB1, and p52/ NF-κB2. NF-κB is a central coordinator of innate and adaptive immune responses. It is also involved in the regulation of inflammatory cytokine production and inflammation [13,14]. When cells are exposed to the inflammatory and stress stimulators, NF-κB activates the downstream genes, including cytokine, cytokine receptor and cyclooxygenase genes, resulting in the inflammatory process [15,16]. Accordingly, controlling NF-κB activation may be a potent strategy for the treatment of inflammation.

In this study, we analyzed the mechanism of 5-FU-induced intestinal damage by transcriptomic analysis and bioluminescent imaging. Our findings demonstrated that NF-κB was the likely key molecule involved in the 5-FU-induced mucositis and inhibition of NF-κB activity by 5-aminosalicylic acid (5-ASA) improved the mucosal damage caused by 5-FU.

Materials and Methods

Animal experiments

Mouse experiments were conducted under ethics approval from China Medical University Animal Ethics Committee (Permit Number: 97-28-N). Transgenic mice carrying the NF-κB-driven luciferase gene were constructed as described previously [17]. BALB/c mice were purchased from National Laboratory Animal Center (Taipei, Taiwan).

The 5-FU-induced mucositis model was established as described previously [7]. Male mice (6 to 8 weeks old) were injected intraperitoneally with phosphate-buffered saline (PBS) (137 mM NaCl, 1.4 mM KH_2PO_4, 4.3 mM Na_2HPO_4, 2.7 mM KCl, pH

7.2) or 5-FU (100 mg/kg) (Sigma, St Louis, MO, USA). For 5-ASA treatment, mice were orally administered with 5-ASA (130 mM/kg) for two consecutive days before intraperitoneal administration of 5-FU and two consecutive days after 5-Fu administration. Mice were imaged for the luciferase activity or sacrificed for histological and immunohistochemical evaluations at indicated periods.

Histological analysis

Mice intestines were fixed in 10% phosphate-buffered formalin for 2 d and dehydrated in a series of graded alcohols (50%, 70%, and 95%) for 30 min each. Samples were then embedded in paraffin, cut into 5-μm sections, stained with hematoxylin and eosin (H&E), and subjected to blinded histological assessment. Villus height was measured from the baseline to the villus tip. Crypt length was measured from the baseline to the submucosa. The thickness of intestinal wall was measured from the submucosa to the serosa. Three independent measurements from 3 different longitudinal sections per mouse were made.

Microarray analysis

Total RNA was extracted from jejunum using RNeasy Mini kit (Qiagen, Valencia, CA, USA). Total RNA was quantified and evaluated as described previously [18]. Microarray analysis was performed as described previously [18-20]. Briefly, fluorescent-labeled RNA targets were prepared from 5 μg of total RNA using MessageAmp^{TM} aRNA kit (Ambion, Austin, TX, USA) and Cy5 dye (Amersham Pharmacia, Piscataway, NJ, USA). Fluorescent targets were hybridized to the Mouse Whole Genome OneArray^{TM} (Phalanx Biotech Group, Hsinchu, Taiwan) and scanned by an Axon 4000 scanner (Molecular Devices, Sunnyvale, CA, USA). Three

replicates from three independent mice were performed. The Cy5 fluorescent intensity of each spot was analyzed by genepix 4.1 software (Molecular Devices). The signal intensity of each spot was corrected by subtracting background signals in the surroundings. We filtered out spots that signal-to-noise ratio was less than 0 or control probes. Spots that passed these criteria were normalized by R program [21]. The fold changes of genes were calculated by dividing the normalized signal intensities of genes in 5-FU-treated mice by those in PBS-treated mice. Genes with fold changes ≥2 or ≤-2 were selected and used as the input genes for the generation of biological network using Transcription Regulation algorithm in MetaCore™ Analytical suite (GeneGo Inc., St. Joseph, MI, USA). All microarray data are MIAME compliant and the raw data have been deposited in a MIAME compliant database (Gene Expression Omnibus, accession number GSE28873).

In vivo and *ex vivo* imaging of luciferase activity

For *in vivo* imaging, mice were anesthetized with isoflurane and injected intraperitoneally with 150 mg/kg D-luciferin. Five minutes later, mice were placed facing up in the chamber and imaged for 1 min with the camera set at the highest sensitivity by IVIS Imaging System® 200 Series (Xenogen, Hopkinton, MA, USA). Photons emitted from the whole bodies were quantified using Living Image® software (Xenogen). Signal intensity was quantified as the sum of all detected photon counts from the whole body and presented as photon/sec. For *ex vivo* imaging, mice were anesthetized and injected with luciferin intraperitoneally. Five minutes later, mice were sacrificed and tissues were rapidly removed. Tissues were placed in the IVIS system and imaged with the same setting used for *in vivo* studies. Signal intensity was quantified as the sum of all detected photon counts per second within the region of

interest after subtracting the background luminescence and presented as photon/sec/cm^2/steradian (photon/sec/cm^2/sr).

Immunohistochemical staining

Sections of 5 μm were deparaffinized in xylene and rehydrated in graded alcohol. Endogenous peroxidase was quenched with 3% hydrogen peroxide in methanol for 15 min and the nonspecific binding was blocked with 1% bovine serum albumin at room temperature for 1 h. Sections were incubated with mouse monoclonal antibody against NF-κB p65 subunit (Chemicon, Temecula, CA, USA) or rabbit polyclonal antibody against IL-1β (Santa Cruz, CA, USA) or TNF-α (Abcam®, Cambridge, UK) at 1:50 dilution overnight at 4°C and then incubated with biotinylated secondary antibody (Zymed Laboratories, Carlsbad, CA, USA) at room temperature for 20 min. Finally, slides were incubated with avidin-biotin complex reagent and stained with 3,3'-diaminobenzidine according to manufacturer's protocol (Histostains®-Plus, Zymed Laboratories).

Cytokine enzyme-linked immunosorbent assay (ELISA)

IL-1β and TNF-α in sera were quantified by ELISA with Quantikine® mouse immunoassay kits (R&D Systems, Minneapolis, MN, USA). Briefly, sera were added to wells, which were coated with monoclonal antibody against IL-1β or TNF-α. After five washes, the biotinylated antibody against IL-1β or TNF-α, the peroxidase-conjugated avidin, and the chromogenic substrates were sequentially added to each well. The absorbance was read at 450 nm in an ELISA reader.

Myeloperoxidase (MPO) assay

MPO activities in the jejunum were quantified with MPO colorimetric activity assay kit (BioVision, Mountain View, CA, USA). Briefly, the frozen tissues were homogenized and centrifuged to remove insoluble materials. Supernatants were collected, mixed with MPO assay buffer and MPO substrate, incubated at room temperature for 1 h, and then mixed with tetramethylbenzidine probe. The absorbance was read at 412 nm in an ELISA reader.

Statistical analysis

Data were presented as mean ± standard error. Student's *t*-test was used for comparisons between two experiments. A value of $p<0.05$ was considered statistically significant.

Results

5-FU induced intestinal mucositis

Chemotherapy-induced diarrhea occurs in approximately 80% of patients treated with 5-FU. Previous studies have shown that 5-FU kills progenitor cells in the crypts of Lieberkühn and the bases of villi, leading to the breakdown of mucosal barrier [22]. Moreover, 5-FU administration results in increased apoptosis and decreased cellularity in the small intestine [8]. We therefore intraperitoneally administered mice with 5-FU and the histological changes in the small intestine were evaluated 2 days later. In comparison with mock, 5-FU caused mucosal damage in the small intestine (Figure 1). 5-FU decreased the height of villi and caused the blunting and fusion of villi. Moreover, 5-FU led to the intestinal inflammation, characterized by the infiltration of immune cells and the accumulation of fluid, and subsequently increased

the length of crypts. 5-FU also increased the thickness of intestinal wall. These findings indicated that intraperitoneal administration of 5-FU caused the mucosal damage and inflammation in the small intestine.

NF-κB was the central molecule in the 5-FU-affected gene expression network

We further elucidated the mechanism of 5-FU-induced intestinal mucositis by transcriptomic analysis. In a total of 29,922 genes, 1,614 genes were upregulated and 1,574 genes were downregulated by 2 fold by 5-FU. These genes were selected for the generation of biological network using Transcription Regulation algorithm in MetaCore. As shown in Figure 2, 5-FU-affected genes were directly connected to the NF-κB, suggesting that expressions of 5-FU-affected genes were regulated by NF-κB. The expression levels of genes in the network are shown in Table 1. Furthermore, NF-κB seemed to be the central molecule of the network. These findings suggested that NF-κB was the likely key molecule involved in the 5-FU-induced intestinal mucositis.

5-FU evoked the NF-κB activity judged by *in vivo* and *ex vivo* imaging

Transcriptomic analysis showed that NF-κB was the central molecule in the 5-FU-affected gene expression network. We therefore performed *in vivo* and *ex vivo* imaging to elucidate the NF-κB activity in mice following 5-FU administration. Transgenic mice carrying the luciferase gene driven by a promoter with five NF-κB responsive elements were used here. The luciferase activity reflected the NF-κB *trans*-activity.

Transgenic mice were intraperitoneally given with PBS or 5-FU, and the bioluminescent imaging was performed on 0, 1, 2, 5, 7, and 14 d. Figure 3A shows

that a maximal induction of NF-κB activity was observed on 2 d following 5-FU administration and *ex vivo* imaging was therefore performed on 2 d. As shown in Figure 3B and Figure 3C, 5-FU slightly affected the NF-κB activities in lung, liver, spleen, and stomach, while 5-FU significantly activated the NF-κB activity in the small intestine by 2.2-fold. These findings indicated that 5-FU evoked the whole body NF-κB activity on 2 d and induced the NF-κB activation in the small intestine. Moreover, 5-FU-induced intestinal mucositis could be assessed by NF-κB bioluminescent imaging.

5-FU-induced NF-κB activity was inhibited by 5-ASA

5-ASA is an anti-inflammatory drug that has been used for the treatment of ulcerative colitis for decades [23]. Activation of NF-κB in biopsies of ulcerative colitis is suppressed by 5-ASA, suggesting that 5-ASA is a potent inhibitor of NF-κB activation *in vivo* [23]. We therefore evaluated whether 5-ASA inhibited 5-FU-induced NF-κB activation and subsequently ameliorated the 5-FU-caused mucositis. Transgenic mice were administered with 5-FU and/or 5-ASA and imaged 2 days later. 5-FU induced the NF-κB activity in the small intestine, which was in agreement with aforementioned findings (Figure 3D). However, 5-ASA significantly reduced the 5-FU-induced NF-κB activity, with a 42% reduction of bioluminescent intensity. Immunohistochemical staining with antibody against NF-κB p65 subunit revealed that, in comparison with mock, there were many brown p65-reactive cells in the crypts and villi of 5-FU-treated intestine (Figure 4A). However, 5-ASA reduced the number of brown p65-reactive cells in the intestine. These findings indicated that 5-FU evoked the NF-κB activity, while 5-ASA inhibited 5-FU-induced NF-κB activity in the intestine.

Inhibition of NF-κB activity ameliorated the 5-FU-induced mucositis in the small intestine

We further tested whether the inhibition of 5-FU-induced NF-κB activity by 5-ASA improved the 5-FU-caused intestinal mucositis. Histological examination of the small intestine following 5-FU and/or 5-ASA treatment showed that 5-FU increased the thickness of intestinal wall and the length of crypt, while 5-ASA significantly decreased 5-FU-caused histological changes (Figure 5). 5-FU also decreased the height of villus, while 5-ASA slightly increased it. In addition to the histological changes, MPO activities in the intestine were induced by 5-FU and suppressed by 5-ASA, also indicating that 5-FU induced intestinal inflammation, while 5-ASA suppressed 5-FU-induced inflammation (Figure 4B). The levels of IL-1β and TNF-α in the tissues and sera were evaluated by immunohistochemical staining and cytokine ELISA, respectively. As shown in Figures 4A and 4C, 5-FU induced the immunomarcation for IL-1β and TNF-α in the tissues and increased the levels of IL-1β and TNF-α in sera, while 5-ASA suppressed 5-FU-induced IL-1β and TNF-α production in the tissues and sera. These findings suggested that 5-FU induced intestinal mucositis via NF-κB activity. Moreover, inhibition of NF-κB activity decreased the 5-FU-induced TNF-α production and subsequently improved the 5-FU-caused mucosal damage in the small intestine.

Discussion

5-FU is a commonly used chemotherapy drug for the treatment of malignant tumors. It kills tumor cells through interfering DNA synthesis and affecting protein

synthesis [2]. Approximately 80% of patients undergoing 5-FU therapy suffer from a range of symptoms, including mucositis and diarrhea. Gastrointestinal mucositis is frequently associated with pain and increased risk of infection. It leads to impaired quality of life in patients. Moreover, patients may no longer be able to continue cancer therapy in cases of severe mucositis [24]. Therefore, developing better therapeutic drug targeting to the mechanisms of mucosal damage is awaited.

Mechanisms involved in the pathogenesis of mucositis are very complex. Apoptosis, hypoproliferation, and inflammation contribute to the mucosal injury [9]. It has been reported that the expression of proinflammatory cytokines, such as IL-6 and TNF-α, in the small intestine and colon of rodents after chemotherapy is significantly increased [7,25]. IL-1 and IL-1 receptor antagonist are produced locally in the intestinal mucosa, and their expressions are increased in inflammatory mucosa [26,27]. Moreover, IL-1β plays a critical role in the genesis and development of intestinal mucositis after chemotherapy, and this type of effect is caused by inducing crypt cell apoptosis [11]. In addition to the proinflammatory cytokines, ROS generated by inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) lead to the mucosal injury. Increased iNOS and COX-2 activity in the 5-FU- and radiation-induced mucositis, suggesting the important role of ROS in the pathogenesis of oral mucositis [9,10]. Recently, the role of PAF in 5-FU-induced intestinal mucositis has been suggested using knockout animals and an antagonist of PAF receptor [12]. Because the expressions of proinflammatory cytokines, iNOS, COX-2, and PAF are regulated by various transcription factors, we applied transcriptomic analysis to find the upstream transcription factors that regulate the downstream gene expression and lead to mucosal injury.

Transcriptomic analysis by DNA microarray tool is a popular research and

screening tool for differentially expressed genes. Microarray-based gene expression patterns have been used to predict the clinical outcome and prognosis of patients undergoing 5-FU therapy [28-30]. It has also been applied to predict the therapeutic efficacy of 5-FU and to identify the biomarkers in various cancers [31,32]. We used microarray tool for the first time to identify the key molecule involved in the 5-FU-caused intestinal injury in this study. The expression levels of IL-6, TNF-α, and IL-1β were increased, with fold changes of 2.28, 3.37, and 6.77, respectively (data not shown). These data were in agreement with previous reports. Further network analysis using Transcription Regulation algorithm indicated that the expression of 5-FU-affected genes was regulated by NF-κB, and NF-κB was the central molecule in the biological network. These findings suggested that NF-κB was the upstream key molecule that regulated the expression of downstream genes and led to the mucositis of intestine.

NF-κB is a central coordinator of innate and adaptive immune responses. NF-κB has also been linked to the control of cell growth, apoptosis, and cell cycle [33]. Previous reports have implicated the NF-κB in the pathogenesis of several inflammatory diseases, such as local joint inflammation, glomerulonephritis, and inflammatory bowel diseases [34-36]. NF-κB activation is also found in biopsy tissues in cancer patients treated with radiation and several chemotherapeutic drugs, except 5-FU [6,37]. As a consequence of the gene upregulation by the initial activation of NF-κB, a broad range of biological active proteins accumulate and target to the submucosa tissue in the gastrointestinal tract. NF-κB activation induced by anti-neoplastic agents and radiation is therefore though to elicit the inflammatory and apoptotic responses that lead to the mucosal injury. In this study, we found that NF-κB was the critical molecule that regulated the expression of 5-FU-affected genes,

and NF-κB activity was induced by 5-FU in the intestine. In contrast, other studies indicated that 5-FU administration inhibits NF-κB activation *in vitro*. Aota et al [38] and Azuma et al [39] reported that 5-FU suppresses NF-κB activity via the inhibition of IκB kinase activity and subsequently induces apoptosis in human salivary gland cancer cells. Contradictory effects of NF-κB activation on normal and cancer cells have been reported [40]. Activation of NF-κB can be either pro-apoptotic or anti-apoptotic, depending on the target cells. Therefore, it is possible that NF-κB activated by 5-FU results in apoptotic signals and proinflammatory cytokine production in normal mucosal tissue and sequentially contributed to the injury of gastrointestinal tract.

Bioluminescent imaging was applied to evaluate the NF-κB activity after 5-FU administration. Transgenic mice carrying the luciferase gene under the control of NF-κB-responsive element were constructed previously, and the bioluminescent signal correlated with NF-κB activity indicated that bioluminescent intensity represents NF-κB activity *in vivo* [17,36]. Oral administration of 5-ASA has been used for decades for the treatment of inflammatory bowel disease [23]. 5-ASA is an anti-inflammatory drug that inhibits NF-κB activation and suppressed the inflammatory response [23]. In this study, we also found that 5-ASA decreased 5-FU-induced NF-κB activity and immunomarcation for IL-1β and TNF-α in the intestine. The histological changes of mucositis have also been improved. These findings suggested that inhibition of NF-κB activity might result in the suppression of inflammation and the sequential amelioration of mucositis in the intestine.

In conclusion, our findings suggested that NF-κB was the critical molecule involved in the 5-FU-caused mucosal injury, while inhibition of NF-κB activity suppressed the 5-FU-induced inflammation and sequentially improved the

5-FU-induced mucosal damage. These findings suggested that NF-κB was the potent target for the development of drugs for the treatment of 5-FU-induced mucositis.

Author Contributions

Conceived and designed the experiments: CTC TYH CYH. Performed the experiments: HL JAL HCH CCL HYL SLW YFH. Analyzed the data: CCL HYL CYH. Wrote the paper: CTC TYH CYH.

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Figure Legends

Figure 1. Histological examination of the small intestine following 5-FU administration. BALB/c mice were intraperitoneally administered with PBS (mock) or 5-FU and sacrificed 2 days later. (A) Microscopic features of the jejunum. Sections were stained with H&E and observed using light microscopy. Magnification 100×. Photos are representative images. (B) Intestinal morphometry of intestinal wall thickness, crypt length, and villus height. Six mice in each group were sacrificed for the morphometry analysis. Three intestinal walls, crypts, and villi in 3 longitudinal sections per mouse were counted. Results are expressed as relative length, which is presented as a comparison with the length or thickness relative to mock. Values are mean ± standard error. $^{**}p<0.01$, $^{***}p<0.001$, compared with mock.

Figure 2. Network analysis of 5-FU-affected genes in the small intestine. Upregulated genes are marked with red circles/disks. Downregulated genes are marked with blue circles/disks. Cyan lines indicate the fragments of canonical pathways.

Figure 3. NF-κB-dependent bioluminescence in living mice and individual organs following 5-FU administration. (A) *In vivo* imaging. Transgenic mice were injected intraperitoneally with PBS or 5-FU and imaged at indicated periods. Results are expressed as relative intensity, which is presented as the comparison with the NF-κB-dependent bioluminescent signal relative to mock. Values are mean ± standard error (n=6 per group). $^{*}p<0.05$, compared with mock. (B) *Ex vivo* imaging. Transgenic mice were injected intraperitoneally with PBS (mock) or 5-FU. Two days later, mice were sacrificed and organs were subjected to image. The color overlay on the image represents the photon/sec emitted from the organs, as indicated by the color

scale. Photos are representative images (n=6 per group). (C) Quantification of photon emission from the organs. Values are mean ± standard error (n=6 per group). **p<0.01, compared with mock. (D) NF-κB-dependent bioluminescence in the intestine following 5-FU and/or 5-ASA administration. Transgenic mice were administered with 5-FU and/or 5-ASA and imaged 2 days later. The color overlay on the image represents the photon/sec emitted from the intestine, as indicated by the color scale. Photos are representative images (n=6 per group). Quantification of photon emission from the intestine was shown on the top. Values are mean ± standard error. *p<0.05, compared with mock. $^{\#}p$<0.05, compared with 5-FU treatment.

Figure 4. Immunohistochemical staining and MPO activity of jejunum and cytokine ELISA of sera following 5-FU and/or 5-ASA administration. Transgenic mice were administered with 5-FU and/or 5-ASA and sacrificed 2 days later. (A) Sections were stained by immunohistochemistry using antibody against NF-κB, IL-1β, or TNF-α. Magnification 100×. Photos are representative images (n=6 per group). (B) MPO activity assay. Frozen jejunum was homogenized and MPO activity in the tissue was analyzed. Values are mean ± standard error. *p<0.05, compared with mock. (C) Cytokine ELISA. The levels of IL-1β and TNF-α were analyzed by cytokine ELISA. Values are mean ± standard error. *p<0.05, ***p<0.001, compared with mock. $^{\#\#\#}p$<0.001, compared with 5-FU treatment.

Figure 5. Histological examination of the small intestine following 5-FU and/or 5-ASA administration. Transgenic mice were administered with 5-FU and/or 5-ASA and sacrificed 2 days later. (A) Microscopic features of the jejunum. Sections were stained with H&E and observed using light microscopy. Magnification 40×. Photos

are representative images (n=6 per group). (B) Intestinal morphometry of intestinal wall thickness, crypt length, and villus height. Six mice in each group were sacrificed for the morphometry analysis. Three intestinal walls, crypts, and villi in 3 longitudinal sections per mouse were counted. Results are expressed as relative length, which is presented as a comparison with the length or thickness relative to mock. Values are mean ± standard error. $^{**}p<0.01$, compared with mock. $^{\#\#}p<0.01$, compared to 5-FU treatment.

Table 1 Expression levels of genes in the network in 5-FU-induced mucositis.

Gene_symbol	Gene_description	Fold change[a]	*p* value[b]
Ccl11	Small chemokine (C-C motif) ligand 11 (Eotaxin)	5.39±0.79	4.2×10^{-5}
Chrna6	Cholinergic receptor, nicotinic, alpha polypeptide 6	4.84±5.74	0.18105
Il25	Interleukin 25	4.66±5.72	0.24311
Rhob	Ras homolog gene family, member B	4.61±6.28	0.38070
Pfkfb3	6-Phosphofructo-2-kinase/fructose-2,6-biphosphatase 3	4.30±2.90	0.11444
Akr1c21	Aldo-keto reductase family 1, member C21	4.23±2.93	0.02762
Adipoq	Adipocyte-specific protein AdipoQ	3.79±2.65	0.15414
Tpm1	Tropomyosin 1, alpha	3.77±1.74	0.01850
Ly6e	Lymphocyte antigen 6 complex, locus E	3.59±0.45	5.4×10^{-5}
Kcnj2	Potassium inwardly-rectifying channel, subfamily J, member 2	3.55±3.46	0.14500
Tnip2	TNFAIP3 interacting protein 2	3.44±3.35	0.15565
Scyl1	SCY1-like 1 (*S. cerevisiae*)	3.41±1.32	0.01068
Wbp5	WW domain binding protein 5	3.39±1.33	0.00927
Tnfrsf1b	Tumor necrosis factor receptor superfamily, member 1b	3.37±3.94	0.32369
Pax2	Paired box gene 2	3.12±3.34	0.26449
Irf4	Interferon regulatory factor 4	3.10±2.07	0.15196
P2rx2	Purinergic receptor P2X, ligand-gated ion channel, 2	3.07±1.17	0.01485
Sqstm1	Sequestosome 1	3.04±3.33	0.31461
Top2a	Topoisomerase (DNA) II alpha	3.03±1.96	0.04543
Cbs	Cystathionine beta-synthase	3.03±2.83	0.20677
Enah	Enabled homolog (*Drosophila*)	2.95±2.75	0.49872
Grin1	Glutamate receptor, ionotropic, NMDA1 (zeta 1) (NR1)	2.85±2.19	0.11010
Lxn	Latexin	2.79±3.07	0.36349
Bace1	Beta-site APP cleaving enzyme 1	2.71±1.37	0.08244
Ltf	Lactotransferrin	2.64±3.11	0.48107
Ccl4	Chemokine (C-C motif) ligand 4 (MIP-1-beta)	2.52±2.40	0.29653
Hc	Hemolytic complement (C5)	2.51±1.32	0.10525
Ssb	Sjogren syndrome antigen B	2.49±2.39	0.29861
Cdh16	Cadherin 16	2.42±1.04	0.02407
Cyp1b1	Cytochrome P450, family 1, subfamily b, polypeptide 1	2.37±1.02	0.02609
Grap2	GRB2-related adaptor protein 2	2.35±1.06	0.03017
Ccl5	Chemokine (C-C motif) ligand 5	2.34±1.05	0.03075
Cxcl10	Chemokine (C-X-C motif) ligand 10 (ip10)	2.34±1.05	0.03076
Il6	Interleukin 6	2.28±1.38	0.14487
Rac1	RAS-related C3 botulinum substrate 1	2.27±2.06	0.61901
Mmp24	Matrix metallopeptidase 24	2.23±0.57	0.00509
Slc1a2	Solute carrier family 1 (glial high affinity glutamate transporter), member 2	2.21±0.91	0.04296
Muc4	Mucin 4	2.21±1.23	0.08205
Ppp2r4	Protein phosphatase 2A, regulatory subunit B (PR 53)	2.18±1.59	0.23068
Zdhhc17	Zinc finger, DHHC domain containing 17	2.17±1.20	0.11078
Ahsg	Alpha-2-HS-glycoprotein	2.14±1.18	0.11679
Ccbp2	Chemokine binding protein 2	2.14±1.31	0.16119
Chrm3	Cholinergic receptor, muscarinic 3, cardiac	2.13±0.86	0.03724
Pla2g4a	Phospholipase A2, group IVA (cytosolic, calcium-dependent)	2.10±1.37	0.18116
Sele	Selectin, endothelial cell	2.07±0.82	0.04074
Slc12a3	Solute carrier family 12, member 3	2.05±1.34	0.19739
St6gal2	Beta galactoside alpha 2,6 sialyltransferase 2	2.04±0.98	0.11112
Slc7a5	Solute carrier family 7 (cationic amino acid transporter, y+ system), member 5	2.04±0.81	0.04360
Mefv	Mediterranean fever	2.03±1.53	0.25031
Il12b	Interleukin 12b	2.02±1.54	0.26543

App	Amyloid beta (A4) precursor protein	-2.00±0.59	0.01319
Sdc4	Syndecan 4	-2.01±0.15	8.8×10^{-5}
Tfrc	Transferrin receptor (TfR1)	-2.03±0.82	0.02629
Pcdh7	Protocadherin 7	-2.06±0.08	7.2×10^{-6}
Grb2	Growth factor receptor bound protein 2	-2.06±0.15	8.1×10^{-5}
G6pc	Glucose-6-phosphatase, catalytic	-2.07±0.10	1.7×10^{-5}
Slc12a2	Solute carrier family 12, member 2	-2.10±0.40	0.00223
Eif4ebp1	Eukaryotic translation initiation factor 4E binding protein 1	-2.11±0.22	0.00024
Dpyd	Dihydropyrimidine dehydrogenase	-2.13±0.31	0.00099
Gch1	GTP cyclohydrolase 1	-2.16±0.16	6.8×10^{-5}
Birc5	Baculoviral IAP repeat-containing 5	-2.16±0.16	6.1×10^{-5}
St6gal1	Beta galactoside alpha 2,6 sialyltransferase 1 (SIAT1)	-2.17±0.26	0.00045
Bst2	Bone marrow stromal cell antigen 2	-2.23±0.12	1.3×10^{-5}
Ilk	Integrin linked kinase	-2.23±0.39	0.00122
Zfpm1	Zinc finger protein, multitype 1	-2.24±0.49	0.00329
Itga3	Integrin alpha 3	-2.35±0.10	4.2×10^{-6}
Fcgrt	Fc receptor, IgG, alpha chain transporter	-2.36±0.15	2.2×10^{-5}
Gpx4	Glutathione peroxidase 4	-2.36±1.85	0.06871
Ldlr	Low density lipoprotein receptor	-2.37±0.33	0.00046
Pak1	P21 (CDKN1A)-activated kinase 1	-2.40±0.34	0.00038
Cdkn1a	Cyclin-dependent kinase inhibitor 1A (P21)	-2.45±0.04	4.5×10^{-8}
Comt	Catechol-O-methyltransferase	-2.45±0.26	0.000146
Pigr	Polymeric immunoglobulin receptor	-2.49±2.08	0.06463
Sod2	Superoxide dismutase, mitochondrial precursor	-2.53±0.44	0.00091
Ctnna1	Catenin alpha-1	-2.54±0.57	0.00209
Acadvl	Acyl-Coenzyme A dehydrogenase, very long chain	-2.56±0.34	0.00024
Ptbp1	Polypyrimidine tract binding protein 1	-2.56±0.14	7.3×10^{-6}
Lgals3	Lectin, galactose binding, soluble 3	-2.57±1.20	0.03328
Ndrg2	N-myc downstream regulated gene 2	-2.64±0.14	3.9×10^{-6}
Mlycd	Malonyl-CoA decarboxylase	-2.70±0.11	2.3×10^{-6}
Psmb9	Proteosome subunit, beta type 9	-2.81±0.36	0.00016
Il10rb	Interleukin 10 receptor, beta	-3.06±0.25	1.9×10^{-5}
Prkcd	Protein kinase C, delta	-3.09±0.23	1.3×10^{-5}
Slc6a4	Solute carrier family 6 (neurotransmitter transporter, serotonin), member 4	-3.11±0.24	1.6×10^{-5}
Bst1	Bone marrow stromal cell antigen 1	-3.14±0.54	0.00043
Cyp4b1	Cytochrome P450, family 4, subfamily b, polypeptide 1	-3.21±0.12	8.4×10^{-7}
Pglyrp1	Peptidoglycan recognition protein 1	-3.43±0.33	2.4×10^{-5}
H1fx	H1 histone family, member X	-3.57±0.46	6.9×10^{-5}
Txnip	Thioredoxin interacting protein	-3.60±0.65	0.00024
Tbk1	TANK-binding kinase 1	-3.60±0.44	5.7×10^{-5}
Tnfaip1	Tumor necrosis factor, alpha-induced protein 1 (endothelial)	-3.66±0.39	3.2×10^{-5}
Eif4a1	Eukaryotic translation initiation factor 4A1	-3.77±0.33	1.3×10^{-5}
Muc3	Mucin 3, intestinal	-3.83±0.45	4.5×10^{-5}
Pdha1	Pyruvate dehydrogenase E1 alpha 1	-3.87±0.43	3.1×10^{-5}
Pafah1b3	Platelet-activating factor acetylhydrolase, isoform 1b, alpha1 subunit	-3.98±0.38	1.5×10^{-5}
Aco2	Aconitase 2, mitochondrial	-4.22±2.20	0.00547
Sod1	Superoxide dismutase 1, soluble	-4.23±2.70	0.01145
Pgk1	Phosphoglycerate kinase 1	-4.36±1.49	0.00188
Gnb1	Guanine nucleotide binding protein, beta 1	-4.42±1.75	0.00198
Gstm1	Glutathione S-transferase, mu 1	-4.65±1.88	0.00239
Cyp4f14	Cytochrome P450, family 4, subfamily f, polypeptide 14	-4.77±3.12	0.03197
Lima1	LIM domain and actin-binding protein 1	-4.83±0.47	1.1×10^{-5}
Glo1	Glyoxalase 1	-5.24±0.61	1.7×10^{-5}
Cyp4f13	Cytochrome P450, family 4, subfamily f, polypeptide 13	-5.29±0.63	1.6×10^{-5}

Apoc3	Apolipoprotein C-III	-5.77±2.10	0.00109
B2m	Beta-2 microglobulin	-5.78±6.05	0.02204
Hes6	Hairy and enhancer of split 6 (*Drosophila*)	-6.11±0.52	3.4×10^{-6}
S100a10	S100 calcium binding protein A10 (calpactin)	-6.53±1.28	9.9×10^{-5}
Abcg5	ATP-binding cassette, sub-family G (WHITE), member 5	-6.99±0.61	2.8×10^{-6}
Cyp3a11	Cytochrome P450, family 3, subfamily a, polypeptide 11	-7.35±0.48	7.8×10^{-7}
Krt8	Keratin, type II cytoskeletal 8	-7.95±2.47	0.00031
Irf1	Interferon regulatory factor 1	-8.15±1.27	2.2×10^{-5}
Prdx5	Peroxiredoxin 5	-8.29±1.71	5.0×10^{-5}
Abcd3	ATP-binding cassette, sub-family D (ALD), member 3 (PMP70)	-8.51±1.32	1.6×10^{-5}
Gstp1	Glutathione S-transferase, pi 1	-9.88±4.95	0.00150

[a] Values are mean ± standard error (n=3).

[b] p values were calculated by the geneSetTest function implemented in the limma package.

Editor

1. indication in Fig 1 and Fig 6 which intestinal segments were evaluated

   The microscopic features of jejunum were evaluated in Figure 1 and Figure 6 (Figure 5 in the revised version). We have supplemented this information in the figure legends (p. 22-23).

2. presentation of the data of the gene expression analysis (Fig 2) also in a table, including the percentage of change for each gene

   The expression levels of genes in the network are shown in Table 1 (p. 25-27)

3. additional measurements as suggested by reviewer #2 should be considered

   We have revised our manuscript according to reviewer #2's comments. Our point-by-point reply to reviewer #2's comments is described as follows.

Reviewer #1

This is an interesting well written paper that provides further information on pathobiology of mucositis.

Thank you for your advices. By histological examination, DNA microarray, NF-κB bioluminescent imaging, immunohistochemical staining, MPO activity assay, and cytokine ELISA, we concluded that NF-κB was the critical molecule associated with the pathogenesis of 5-FU-induced mucositis, and inhibition of NF-κB activity ameliorated the mucosal damage caused by 5-FU.

Reviewer #2

This paper provide evidence of an involvement of NF-<kappa>B in the intestinal mucositis caused by administration of 5-FU. While interesting, the results not support the conclusion that inhibition NF-<kappa>B suppressed the 5-FU-induced inflammation. The authors need show more experiments the aspects inflammatory of the mucositis and effect the 5-ASA administration. In relation the involvement of NF-<kappa>B in the intestinal mucositis induced by 5-FU the various manuscript showed the opposite theses results (Aota et al., Biochem Biophys Res Commun 273:1168-1174, 2000; Azuma et al., Biochem Biophys Res Commun 282: 292-296, 2001; Logan et al., Cancer Chemother Pharmacol 63:239-251, 2009), I think the authors need to do more experiments to confirm this objective.

In this study, we found that NF-κB was the critical molecule that regulated the expression of 5-FU-affected genes, and NF-κB activity was induced by 5-FU in the intestine. In contrast, other studies indicated that 5-FU administration inhibits NF-κB activation *in vitro*. Aota et al. (Biochem Biophys Res Commun 273:1168-1174, 2000) and Azuma et al. (Biochem Biophys Res Commun 282: 292-296, 2001) reported that 5-FU suppresses NF-κB activity via the inhibition of IκB kinase activity and subsequently induces apoptosis in human salivary gland cancer cells. Contradictory effects of NF-κB activation on normal and cancer cells have been reported (Kaltschmidt et al., Eur J Biochem 267: 3828-3835, 2000). Activation of NF-κB can be either pro-apoptotic or anti-apoptotic, depending on the target cells. Therefore, it is possible that NF-κB activated by 5-FU results in apoptotic signals and proinflammatory cytokine production in normal mucosal tissue and sequentially contributed to the injury of gastrointestinal tract.

We have supplemented this information in the Discussion section (p. 14, last paragraph; p. 15, 1st paragraph).

Major revisions

a) It is not clear why segments intestinal were evaluated in Fig 1 and Fig 6.

The microscopic features of jejunum were evaluated in Figure 1 and Figure 6 (Figure 5 in the revised version). We have supplemented this information in the figure legends (p. 22-23).

b) In my opinion the Fig 1 and Fig 6 could be only one.

The order of figures in this manuscript is followed. 5-FU induces mucositis in the small intestine (Figure 1). The network center of 5-FU-affected genes is NF-κB (Figure 2). NF-κB in the small intestine is activated by 5-FU (Figure 3). 5-ASA suppresses 5-FU-induced mucositis via the inhibition of NF-κB activity (Figure 4 and Figure 5 (Figure 5 and Figure 6 in the last version)). We would like to maintain this order for a smooth layout.

c) In my opinion the Fig 3 and Fig 4 could be only one.

We have combined Figure 3 and Figure 4 into one figure.

d) The 5-ASA is specific inhibitor the NF-κB? In relation toxicity, is know high toxicity with use 5-ASA, in relation administration 5-ASA+5-FU was observation increased in toxicity? The 5-ASA treatment altered the up regulation the genes induced by 5-FU? The 5-ASA treatment was daily or not? Is need discussion more these question?

Oral administration of 5-ASA has been used for decades for the treatment of inflammatory bowel disease (Nikolaus et al., Hepatogastroenterology 47: 71-82, 2000). Activation of NF-κB in biopsies of ulcerative colitis is suppressed by 5-ASA, suggesting that 5-ASA is a potent inhibitor of NF-κB activation *in vivo* (Bantel et al., Am J Gastroenterol 95: 3452-3457, 2000).

Although 5-ASA often trigger undesirable side effects, we observed no visible toxicity during 5-ASA/5-FU treatment.

By NF-κB bioluminescent imaging, immunohistochemical staining, MPO activity assay, and cytokine ELISA, we found that 5-FU induced NF-κB activity and MPO activity in the jejunum and increased the levels of IL-1β and TNF-α in sera, while 5-ASA treatment decreased the 5-FU-induced NF-κB activity, MPO activity, and proinflammatory cytokine production in the samples. Therefore, these data suggested that 5-ASA altered the 5-FU-affected gene expression.

For 5-ASA treatment, mice were orally administered with 5-ASA (130 mM/kg) for two consecutive days before intraperitoneal administration of 5-FU and two consecutive days after 5-FU administration.

We have supplemented the action of 5-ASA in Results and Discussion sections (p. 11, 2nd paragraph; p. 15, 2nd paragraph), and 5-ASA treatment in Materials and Methods section (p. 6, 1st paragraph).

e) I suggest represent the data the gene expression (Fig 2) also in table include the percentage the regulation.

The expression levels of genes in the network are shown in Table 1 (p. 25-27)

f) The authors need to address the aspects inflammatory the mucositis induced by 5-FU experimentally with MPO activity measure, cytokines dosage (IL-1β and IL-6) at least, in addition, evaluated the 5-ASA effect.

We have performed immunohistochemical staining for detection of IL-1β and TNF-α in the intestine (Figure 4A). We also have performed MPO activity assay and cytokine ELISA for evaluation of MPO activity in the intestine and IL-1β and TNF-α production in sera (Figure 4B and C). These findings indicated that 5-FU induced intestinal inflammation, while 5-ASA suppressed 5-FU-induced inflammation in the intestine.

We have supplemented the MPO activity assay and cytokine ELISA in Materials and Methods section (p. 8-9). Experimental data have been supplemented in Results section (p. 12).

g) In experiments of the bioluminiescent, I suggest do the NF-κB measure of according Logan et al., Cancer Chemother Pharmacol 63:239-251, 2009 and evaluate the luciferase activity of according Wu et al., J Pharmacol Exp Ther 330:370-376, 2009;

Transgenic mice carrying the luciferase genes driven by NF-κB were constructed in our laboratory previously. Luciferase activity in whole body or organs was monitored and quantified as luminescent intensity by bioluminescent imaging (Figure 3). To correlate the luciferase activity with NF-κB activity in organs, we performed immunohistochemical staining for detection of p65 in the organs (Figure 4A). Additionally, previous study also has performed immunohistochemical staining to reveal the co-localization of luciferase protein-positive cells and NF-κB-positive cells in the tissue (Ho et al., Biomaterials 28: 4370-4377; 2007). Therefore, we concluded that the luciferase activity reflected the NF-κB *trans*-activity.

We have supplemented this information in Results section (p. 10, 3rd paragraph).

h) In addition immunohistochemical TNF-α, I suggest do quantitative analysis (immunomarcation scores) and the TNF-α measure by ELISA.

We have performed cytokine ELISA for detection of IL-1β and TNF-α production in sera (Figure 4C).

Cytokine ELISA has been supplemented in Materials and Methods section (p. 8). Experimental data have been supplemented in Results section (p. 12).

i) In "discussion" (pg 14, line 44-46), I think that the results not confirm suppression of the inflammation and the sequential amelioration of the mucositis in the intestine. It is necessary to evaluated parameters with: ratio villus/crypt, survival, diarrhea scores, MPO activity, and cytokines dosage for have amelioration in inflammation intestinal induced by 5-FU as showed by various manuscripts. I suggest change these paragraph.

We have performed MPO activity assay and cytokine ELISA for evaluation of MPO activity in the intestine and IL-1β and TNF-α production in sera (Figure 4B and C). These findings indicated that 5-FU induced intestinal inflammation, while 5-ASA ameliorated 5-FU-induced inflammation in the intestine.

We have supplemented the MPO activity assay and cytokine ELISA in Materials and Methods section (p. 8-9). Experimental data have been supplemented in Results section (p. 12).

Minor revisions

a) Pg 5, line 50. The correct is BALB/c.

b) Pg 6, line 1. The concentration of the 5-FU is 100mg/kg or 150mg/kg.

c) Pg 10, line 2. Delete "5".

d) Pg 14, line 41. Change the expression "TNF-<alpha> production" to "immunomarcation for TNF-<alpha>".

e) Pg 23, line 4. The correct is ** and ##.

f) In Fig 3A, include the results the mock group.

We have revised aforementioned errors according to reviewer's advices.