JPET Fast Forward. Published on May 7, 2009 as DOI: 10.1124/jpet.109.152835

JPET # 152835

Title page

Vanillin Improves and Prevents Trinitrobenzene Sulfonic Acid-Induced Colitis in Mice

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Running title page

Running title: Vanillin improves IBD in mice

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Number of text pages: 28

Number of tables: 0

Number of figures: 7

Number of references: 40

Number of words in the Abstract: 176

Number of words in the Introduction: 590

Number of words in the Discussion: 1084

ABBREVIATIONS: 5-ASA, 5-aminosalicylic acid; CD, Crohn's disease; EMSA, electrophoretic mobility shift assay; IBD, inflammatory bowel disease; IFN-γ, interferon-γ; IL, interleukin; IKK, IκB kinase; NF-κB, nuclear factor-κB; RT-PCR, reverse transcription-polymerase chain reaction; TNBS, trinitrobenzene sulfonic acid; TNF-α, tumor necrosis factor-α.

Recommended section: Gastrointestinal, Hepatic, Pulmonary, and Renal

Abstract

Inflammatory bowel diseases (IBD) are chronic inflammatory and relapsing diseases of the gut. It has been known that activation of nuclear factor-κB (NF-κB) and production of proinflammatory cytokines play important roles in the pathogenesis of IBD. In this study, the effect of vanillin, a potent NF-κB inhibitor, was evaluated in mice with trinitrobenzene sulfonic acid (TNBS)-induced colitis. Oral administration of vanillin improved macroscopic and histological features of TNBS-induced colitis in a dose-dependent manner. Vanillin not only prevented TNBS-induced colitis but also ameliorated the established colitis. By *in vivo* NF-κB bioluminescence imaging, electrophoretic mobility shift assay, and Western blot, we found that vanillin suppressed *in vivo* NF-κB activities through the inhibition of p65 translocation, IκB-α phosphorylation, and IκB kinase activation. Furthermore, vanillin reduced the expressions of proinflammatory cytokines (IL-1β, IL-6, IFN-γ, TNF-α) and stimulated the expression of anti-inflammatory cytokine (IL-4) in colonic tissues. In conclusion, this work identified vanillin as an anti-inflammatory compound with the capacity to prevent and ameliorate TNBS-induced colitis. Due to its safety, vanillin could be a potent candidate for the treatment of IBD.

Introduction

A sharp rise in the incidence of inflammatory bowel disease (IBD) has been observed in the Western world since the early 1950s. At present, IBD is fairly common in Northern countries, such as the United States and United Kingdom, affecting 0.5-1.0% of the population during their lifetime (Russel, 2000). Although IBD is more common in the developed countries, there are indications that more cases are being seen lately in low-incidence areas, such as Southern Europe, Middle East, Eastern Asia, the Indian subcontinent, Latin America, and Eastern Europe (Economou and Pappas, 2008).

IBDs, including Crohn's disease (CD) and ulcerative colitis in clinic, are chronic intestinal inflammatory diseases characterized by frequently relapsing with clinical manifestations, such as diarrhea, blood in the stool, abdominal pain, and weight loss. The etiology of IBD is still unknown so far, but the imbalance of mucosal homeostasis plays an important role in the pathogenesis of IBD (Cho, 2008). Several murine models resembling the main symptoms and molecular mechanisms of CD have been established and been used to explore new therapeutics for the diseases (Strober et al., 2002). The murine model frequently used is based on the intrarectal administration of trinitrobenzene sulfonic acid (TNBS), which randomly haptenates the proteins in the colonic mucosa and triggers an inflammatory response similar to that in CD (Neurath et al., 1995). In the TNBS-induced colitis model, increases of mucosal proinflammatory cytokines, such as interleukin-1β (IL-1β), IL-6, IL-12, tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ), have been shown to play important roles in sustained inflammatory responses (Neurath et al., 1995). It has been shown that the expressions of these proinflammatory cytokine genes are mainly regulated by the transcription factor, nuclear factor-κB (NF-κB)

(Barnes and Karin, 1997). Additionally, activation of NF-κB plays a central role in initiating the inflammatory process in IBD (Schreiber et al., 1998). Thus, inhibition of NF-κB activation may be a promising target for the treatment of patients with IBD.

At present, the mainstay of therapy for IBD is anti-inflammatory drugs, such as 5-aminosalicylic acid (5-ASA), and immunosuppressants, such as glucocorticoids, azathioprine, and 6-mercaptopurine. These drugs have been shown to inhibit NF-κB activities; however, these drugs often trigger undesirable side effects, such as pneumonitis, pancreatitis, hepatitis, bone marrow suppression, and risk of infection (Bantel et al., 2000). Therefore, developments of natural anti-inflammatory medicines with high efficacy, low toxicity, and cheap are desired for the treatment of patients with IBD.

Vanillin (4-hydroxy-3-methoxybenzaldehyde) is a widely used flavor compound in food and cosmetics, with an estimated annual worldwide consumption of over 2000 tons (Walton et al., 2003). Vanillin has been reported to inhibit mutagenesis induced by chemical and physical mutagens, and to suppress the invasion and migration of cancer cells (Lirdprapamongkol et al., 2005; Cheng et al., 2007; Liang et al., 2009). It also displays chemopreventive effects in multiorgan carcinogenesis and hepatocarcinogenesis models in rats (Akagi et al., 1995). Moreover, vanillin displays antimicrobial and antioxidative properties, and is used as a food preservative and for medicinal purposes (Fitzgerald et al., 2004; Santosh et al., 2004). Recently, vanillin has been shown to inhibit lipopolysaccharide (LPS)-stimulated NF-κB activation and cyclooxygenase-2 gene expression in murine macrophages (Murakami et al., 2007). The *in vitro* inhibitory ability of vanillin on the NF-κB activation implied that vanillin might be effective in mice with

colitis. Therefore, we administered TNBS-induced mice with vanillin to evaluate the beneficial effects of vanillin on intestinal inflammation. Our data showed that oral administration of vanillin prevented colitis and ameliorated established colitis via NF-κB signaling pathway. These findings suggested that vanillin exhibited therapeutic value for patients with IBD.

Methods

Induction of Colitis and Vanillin Treatment. Female BALB/c mice (6-8-week old, 21±2 g weight) were obtained from the National Laboratory Animal Center (Taipei, Taiwan). Mouse experiments were conducted under ethics approval from the China Medical University Animal Ethics Committee.

To induce colitis, 5 mg of TNBS (Sigma, St. Louis, MO) in 0.1 ml of 50% ethanol was slowly administered into the colon of the lightly anesthetized mouse via a thin catheter (PE-50, BD Biosciences, Heidelberg, Germany) attached to a 0.5-ml syringe. The catheter tip was inserted 3 cm proximal to the anal verge, and the mouse was held in a vertical position for 30 sec after instillation. Control mice received the same volume of 50% ethanol alone. If the mouse excluded this solution quickly (<10 min), it was rejected for the further analysis.

To investigate the dosage effect of vanillin, various amounts of vanillin (Sigma, St. Louis, MO) and 5 mg of TNBS in 0.1 ml of 50% ethanol were administered into the colon. To examine the preventive effect of vanillin, vanillin (50 mM, equivalent to 10 mg/kg body weight) was administered orally for 3 consecutive days before TNBS administration. To study the therapeutic effect of vanillin, vanillin (50 mM, equivalent to 10 mg/kg body weight) was orally administered for 7 consecutive days starting 1 day after TNBS administration. Animals were monitored daily for appearance of diarrhea, loss of body weight, and survival. All the mice were sacrificed 7 days after administration of TNBS.

Macroscopic and Microscopic (Histological) Assessment. Colonic weight and length were measured as a gross indicator of colitis. Colons were also examined under a

dissecting microscope and graded for macroscopic lesions. Macroscopic assessment of the colitis severity was scored according to a previously established scoring system as follows: 0, no ulcer and no inflammation; 1, local hyperemia without ulceration; 2, ulceration without hyperemia; 3, ulceration and inflammation at one site only; 4, two or more sites of ulceration and inflammation; 5, ulceration extending more than 2 cm (Wallace and Keenan, 1990). For histological analysis, colons were fixed, sectioned, and stained with hematoxylin/eosin. Histological changes were graded semi-quantitatively from 0 to 4 according to previously described criteria as follows in a blinded fashion: 0, no sign of inflammation; 1, very low level of leukocyte infiltration; 2, low level of leukocyte infiltration; 3, high level of leukocyte infiltration, high vascular density, and thickening of the colon wall; 4, transmural infiltration, loss of goblet cells, high vascular density, and thickening of the colon wall (Neurath et al., 1995). The macroscopic and microscopic scores were assessed by three experts.

***In Vivo* and *Ex Vivo* Imaging of NF-κB Activity.** Transgenic mice, which carried the luciferase gene driven by NF-κB responsive element, were constructed as described previously (Ho et al., 2007). Transgenic mice were orally administered 50 mM vanillin, equivalent to 10 mg vanillin/kg body weight, for 7 consecutive days after TNBS administration. One week later, mice were anesthetized with isoflurane, injected intraperitoneally with 150 mg D-luciferin/kg body weight, placed face up in the chamber, and imaged for 5 min with the camera set at the highest sensitivity by IVIS Imaging System® 100 Series (Xenogen, Alameda, CA). Photons emitted from tissues were quantified using Living Image® software (Xenogen, Alameda, CA,). For *ex vivo* imaging, mice were sacrificed and entire colons were rapidly removed. Colons were imaged with

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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 of background luminescence and presented as photons/second/cm^2/steradian (photons/sec/cm^2/sr).

Luciferase Activity Assay in Organ Homogenates. Colons were removed, homogenized in 1 ml of Triton lysis buffer (50 mM Tris-HCl, pH 7.8, 1% Triton X-100, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride), centrifuged at 12,000 rpm for 15 min at 4^0C, and stored at -20^0C. Protein concentration was quantified with a Bradford method (Bio-Rad, Hercules, CA). Luciferase activity was measured as relative light units (RLU) as described previously (Hsiang et al., 2005) and normalized for total protein content.

Nuclear Extraction and Biotinylated Electrophoretic Mobility Shift Assay (EMSA). The excised colons were homogenized in ice-cold lysis buffer (10 mM HEPES-KOH, pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.1% NP-40, 0.5 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml pepstatin). The homogenates were then incubated on ice for 10 min and centrifuged at 15,000 rpm for 10 min at 4^0C. The supernatants were stored at -70^0C as the cytosolic extracts and the pellets were incubated on ice for 1 h with nuclear extraction buffer (20 mM HEPES-KOH, pH 7.9, 420 mM NaCl, 1.5 mM $MgCl_2$, 25% glycerol, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 0.2% NP-40, 1 μg/ml leupeptin, 1 μg/ml pepstatin). The resultant homogenates were centrifuged at 12,000 rpm for 20 min at 4^0C, and the supernatants were collected and stored at -70^0C as nuclear extracts. Protein concentration was quantified with a Bradford method (Bio-Rad, Hercules, CA). EMSA was performed

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as described previously (Lee et al., 2008)

Western Blot Assay. The cytosolic or nuclear proteins (10 μg) were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and the protein bands were then transferred electrophoretically to nitrocellulose membranes. Membranes were blocked in blocking buffer (20 mM Tris-HCl, pH 7.6, 140 mM NaCl, 0.1% Tween-20, 5% skim milk powder) and probed with polyclonal antibodies against IκB kinase (IKK), phosphorylated IKK, IκB-α, phosphorylated IκB-α, and p65 (Cell Signaling Technology, Beverly, MA). The bound antibody was detected with peroxidase-conjugated anti-rabbit antibody followed by chemiluminescence (ECL system, Amersham, Buckinghamshire, UK) and exposed by autoradiography.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR). Total RNAs were extracted from colonic tissues using RNeasy Mini kit (Qiagen, Valencia, CA). One microgram of total RNA was reverse transcribed using oligo$(dT)_{15}$ primer and SuperScript^{TM} III (Invitrogen, Carlsbad, CA) in a total volume of 20 μl. Two microliters of reverse transcription mixture were subjected to PCR to measure the mRNAs of cytokines and β-actin. PCR amplification was performed with *Taq* polymerase (Promega, Madison, WI) for 26 cycles at 92^0C for 45 sec, 55^0C for 45 sec, and 72^0C for 2 min. PCR primers for cytokines and β-actin were as follows: IL-1β sense, 5'-CATCCTCTGTGACTCATGGG-3'; IL-1β antisense, 5'-CTTCTTCTTGGGTATTGCTTG-3'; IL-4 sense, 5'-TCGGCATTTTGAACGAGGTC-3'; IL-4 antisense, 5'-GAAAAGCCCGAAAGAGTCTC-3'; IL-6 sense, 5'-TATGAAGTTCCTCTCTGCAA-3'; IL-6 antisense,

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5'-CTTTGTATCTCTGGAAGTTTCAG-3'; IFN-γ sense,

5'-GCTCTGAGACAATGAACGCT-3'; IFN-γ antisense,

5'-AAAGAGATAATCTGGCTCTGC-3'; TNF-α sense,

5'-TAGCCCACGTCGTAGCAAAC-3'; TNF-α antisense,

5'-CACCCATTCCCTTCACAGAG-3'; β-actin sense,

5'-GGAGAAGATCTGGCACCACACC-3'; β-actin antisense,

5'-CCTGCTTGCTGATCCACATCTGCTGG- 3'.

Statistics Analysis. Data were presented as mean ± S.E.M. Student's *t*-test was used for a comparison between two experiments. A value of $P<0.05$ was considered statistically significant.

Results

Vanillin Suppressed TNBS-Induced Colitis in Mice. Previous studies showed that intrarectal administration of TNBS induced colitis resembling human IBD (Neurath et al., 1995). Therefore, we first tested the dosage effect of TNBS in mice. Colitis severity was evaluated by macroscopic and histological features. In control mice, no sign or a very low level of injury in the colon was observed (Fig. 1). TNBS significantly induced the macroscopic and microscopic damages in colons in a dose-dependent manner. The entire colonic wall became thick due to edema. The major injury of colitis was observed in the distal half of the colon, and focal ulcers were detected in approximately 63% of colonic tissues treated with 5 mg TNBS.

Next, we evaluated the effect of vanillin on the TNBS-induced colitis. Various amounts of vanillin and 5 mg of TNBS were co-administered into the colon. As shown in Fig. 2, vanillin improved the macroscopic and histological features of TNBS-induced colitis in a dose-dependent manner. Macroscopic examination of colons after TNBS induction showed inflammation compared with controls (Fig. 2A). In contrasts, colons from vanillin-treated mice showed mild inflammation. Histological examination of colons from TNBS-induced mice showed mucosal thickening and increase of lymphoid follicle sizes (Fig. 2B). In contrasts, vanillin significantly ameliorated the signs of colitis compared with TNBS-induced mice. Macroscopic score, colonic weight/length ratio, and microscopic score were significantly lower in the group treated with 50 mM vanillin. These findings suggested the therapeutic potential of vanillin in mice with TNBS-induced colitis.

Vanillin Prevented Colitis and Ameliorated Established Colitis. We further tested

whether vanillin was able to prevent TNBS-induced colitis or ameliorate established colitis. Vanillin (50 mM, equivalent to 10 mg/kg) was orally administered for 3 consecutive days before TNBS treatment. 5-ASA has been used over decades for the treatment of IBD (Hanauer and Meyers, 1997). Therefore, 5-ASA was used as a positive control for the comparison. As shown in Fig. 3, pretreatment of 5-ASA slightly decreased the macroscopic damages and histological features of colons in mice with TNBS-induced colitis. However, pretreatment of vanillin significantly reduced the weight/length ratio and improved the macroscopic and histological damages of TNBS-induced colitis. These data suggested that preventive treatment with vanillin was capable of protecting colonic injuries of mice after the administration of TNBS.

Therapeutic effect of vanillin was then assessed by oral administration of vanillin for 7 consecutive days after TNBS treatment. As expected, oral administration of 5-ASA significantly reduced the colonic weight/length ratio (Fig. 4). Vanillin also significantly reduced the weight/length ratio. Additionally, vanillin significantly improved the distortion of crypts, loss of goblet cells, and infiltration of mononuclear cells in mice with TNBS-induced colitis. These findings suggested that vanillin was capable of ameliorating the established colitis in mice.

Vanillin Suppressed NF-κB Activities of Colons in Mice with TNBS-Induced Colitis. NF-κB is a critical molecule involved in the regulation of inflammation (Barnes and Karin, 1997). We wondered whether vanillin improved TNBS-induced colitis via the suppression of NF-κB activity. Therefore, the *in vivo* bioluminescence was used to monitor the NF-κB activity in NF-κB transgenic mice. As shown in Fig. 5A, a strong luminescence was observed in the lower abdominal region of TNBS-treated mice. *Ex*

vivo imaging showed that bioluminescent signal was detected in colon. These data suggested that the lower abdominal luminescence originated from the colons. Oral administration of vanillin in mice with established colitis led to a decrease in bioluminescent signals in the lower abdominal region and colonic tissues, suggesting that vanillin inhibited NF-κB activities of colons in mice with colitis.

To demonstrate a correlation between imaging, actual luciferase enzyme activity, and NF-κB activity, we excised the colons, prepared the organ extracts, and performed the luciferase activity assay and biotinylated EMSA. Luminescence imaged with the camera correlated highly with luciferase enzyme activity (Fig. 5B). Luciferase activity assay showed that TNBS induced luciferase activity in the colon, whereas vanillin suppressed TNBS-induced luciferase activity. Biotinylated EMSA showed that TNBS increased the DNA-binding ability of NF-κB in the colon, whereas vanillin reduced the TNBS-induced DNA-binding ability of NF-κB (Fig. 5C). These findings suggested that vanillin improved TNBS-induced colitis through the inhibition of NF-κB activity.

Vanillin Inhibited NF-κB Signal Transduction Pathways of Colons in Mice with TNBS-Induced Colitis. The activation of NF-κB is preceded by translocation of NF-κB to the nucleus following phosphorylation and degradation of IκB-α (Karin and Ben-Neriah, 2000). Western blot was therefore performed to analyze the signaling pathway involved in the regulation of NF-κB activity by vanillin. As shown in Fig. 6, administration of TNBS elevated the translocation of p65 to the nucleus, stimulated the phosphorylation of IκB-α, and induced the phosphorylation of IKK. However, vanillin inhibited the p65 translocation and suppressed the levels of phosphorylated IκB-α and IKK in the colon. These data suggested that vanillin inhibited TNBS-induced NF-κB

activation in colons via the inhibition of IκB-α phosphorylation and IKK activation.

Vanillin Reduced the Expressions of Cytokine Genes of Colonic Tissues in Mice with TNBS-Induced Colitis. We further evaluated the effect of vanillin on the productions of inflammatory cytokines that are linked to TNBS-induced colitis. As shown in Fig. 7, TNBS induced mRNA expressions of proinflammatory cytokines, such as IL-1β, IL-6, IFN-γ and TNF-α, in colonic mucosa, while vanillin suppressed the mRNA expressions of these cytokines. The expression of anti-inflammatory cytokine IL-4 was down-regulated in TNBS-treated colonic tissues, whereas vanillin stimulated the expression of IL-4 gene. These findings suggested that oral administration of vanillin suppressed proinflammatory cytokine productions and stimulated anti-inflammatory cytokine production in mice with TNBS-induced colitis.

Discussion

TNBS-induced colitis is widely used to evaluate the effects of drugs because of its similarity to CD and the availability of a quantitative scoring system (Neurath et al., 1995). Our data showed that vanillin was capable of decreasing the colonic weight/length ratio and improving macroscopic and microscopic damages in a concentration manner. Oral administration of vanillin not only prevented TNBS-induced colitis but also ameliorated established colitis. Therefore, these findings suggested that vanillin could be a potent therapeutic agent for the treatment of patients with IBD.

NF-κB is the key transcription factor for pro-inflammatory responses in IBD. It is thought to be important in the initiation and progression of human IBD and animal models of colitis (Schreiber et al., 1998). Disease activity in mice with TNBS-induced colitis is inhibited by antisense oligonucleotides for p65 subunit of NF-κB, also suggesting the critical role of NF-κB in mediating inflammatory responses of IBD (Neurath et al., 1996). NF-κB bioluminescence imaging was used to monitor the *in vivo* NF-κB activities of transgenic mice with TNBS-induced colitis in this study. NF-κB transgenic mice model has been used to monitor the chronic inflammation induced by ultraviolet light, TNF-α, IL-1α, or LPS (Carlsen et al., 2002; Carlsen et al., 2004). Our data showed that TNBS induced a strong NF-κB-driven luminescent signal in the lower abdominal region and colonic tissues of mice with colitis. Moreover, macroscopic and histological analysis indicated that inflammation was evoked in the same region. Therefore, the correlation between the NF-κB bioluminescence imaging and histological changes enforced the critical role of NF-κB in TNBS-induced colitis. Oral administration of vanillin led to a markedly decrease of bioluminescent signals in the lower abdominal

region and colonic tissues of mice with established colitis. Furthermore, biotinylated EMSA and Western blotting showed that vanillin inhibited NF-κB activity via the inhibition of IκB-α phosphorylation and IKK activation. Therefore, results presented here first demonstrated that noninvasive NF-κB imaging system can be used to assess the inflammation induced by TNBS in living animals. Additionally, our data demonstrated the feasibility of NF-κB bioluminescence imaging for the discovery of agents against TNBS-induced colitis.

Homeostasis of T helper 1 (Th1)/Th2 cytokines is important in intestinal mucosal immunity (Strober et al., 2002). T helper cells are a subgroup of lymphocytes that activate and direct other immune cells through release of cytokines. An imbalance between Th1 and Th2 cytokines has been implicated in the pathogenesis of IBD, particularly CD (Holland et al., 2008). Moreover, increase of proinflammatory cytokines, mainly Th1 cytokines, is observed in patients with CD, also suggesting that CD is a Th1-mediated disease (Neurath et al., 1997). TNBS-induced colitis has been reported to be associated with Th1 cell responses, requiring T cell activation as the central initiating event that subsequently leads to macrophage recruitment and activation (Elson et al., 1996). The bias toward Th1 cytokines, such as IL-1β, IL-6, TNF-α and IFN-γ, is critical in the establishment of chronic inflammation (Cho, 2008). On the other hand, administration of anti-inflammatory cytokine IL-4, the principal effector molecule of Th2 cells, has led to the therapeutic potentials in TNBS-induced colitis (Hogaboam et al., 1997). These results suggested the benefit effects of Th2 cytokines in the treatment of IBD. Our data showed that vanillin down-regulated the expression of Th1 proinflammatory cytokines in mice with TNBS-induced colitis. In contrast to Th1

cytokines, vanillin increased the mRNA expression of anti-inflammatory cytokine *in vivo*. Vanillin inhibiting a Th1 response and favoring a Th2 response might explain why vanillin was effective in preventing and ameliorating the Th1-mediated disease, TNBS-induced colitis.

Increased levels of inflammatory cytokines were secreted in the colons of IBD patients, leading to the production of other inflammatory mediators, such as nitric oxide and reactive oxygen species (Reinecker et al., 1993). In patients with IBD, nitric oxide overproduction results in mucosal injury (Ischiropoulos et al., 1992). The massive infiltration of leukocytes in IBD is thought to produce large amounts of reactive oxygen species that would participate in intestinal damage (Kruidenier et al., 2003). These findings clearly indicate that these proinflammatory mediators are involved in the pathogenesis of IBD. Vanillin is a potent antioxidant. It inhibits protein oxidation and lipid peroxidation by quenching singlet oxygen (Kamat et al., 2000). It scavenges free radicals. It also protects DNA and mitochondrial membrane against oxidative stress *in vitro* (Santosh et al., 2004). These findings implied that, in addition to the regulation of Th1 cytokines, the antioxidative potential of vanillin might also contribute to its therapeutic effect on TNBS-induced colitis. Additionally, it has been known that antioxidant properties, such as free radical scavenging activity, of 5-ASA may be the potential mechanism for its protective effect in IBD (Joshi et al., 2005). Vanillin was more effective than 5-ASA in prevention or therapy of TNBS-induced colitis in this study, suggesting that the antioxidant activity of 5-ASA might be less than that of vanillin.

Oral or colonic administration of 5-ASA has been used over decades for the treatment of IBD. However, patients administered with 5-ASA or its derivatives suffer several

serious adverse conditions, such as anorexia, dyspepsia, nausea/vomiting, hemolysis, neutropenia, agranulocytocis, folate malabsorption, reversible, male infertility, and neuropathy (Sands, 2000). Vanillin is generally regarded as safe by the Flavor and Extract Manufacturers Association, and is recognized as suitable for food use by the Food and Drug Administration (Opdyke, 1977). Vanillin displays no genetic toxic effect in *Salmonella* mutagenicity test and human lymphocytes (Mortelmans et al., 1986; Jansson and Zech, 1987). Additionally, oral chronic toxicity studies with rats fed with diets containing 1000 mg/kg/day for 2 years show no effect on growth or hematology, and no macroscopic or microscopic changes in the tissues (Hagan et al., 1967). Moreover, no adverse effect is observed when the diet contains 150 mg vanillin/kg body weight (Kirwin and Galvin 1993). In our study, vanillin at 50 mM (equivalent to 10 mg/kg) was sufficient to prevent and improve IBD in mice. Therefore, due to its safety, vanillin seems to be a potent drug candidate for the treatment of IBD.

In conclusion, our work identified vanillin as an anti-inflammatory compound with the capacity to prevent and ameliorate TNBS-induced colitis. Vanillin inhibited *in vivo* NF-κB activity via the inhibition of IκB-α phosphorylation and IKK activation, reduced the productions of Th1 cytokines, and in turn, suppressed TNBS-induced colitis in mice. In addition to the inhibition of NF-κB activity, vanillin is able to scavenge free radicals and quench singlet oxygen, the important risk factors of CD. Therefore, these results suggested that vanillin may be a potent therapeutic agent for IBD with a broad spectrum of therapeutic mechanisms.

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Footnotes

This work was supported by the National Science Council (NSC 97-2320-B-039-012-MY3), Committee on Chinese Medicine and Pharmacy at Department of Health (CCMP 96-RD-201 and CCMP 97-RD-201), and China Medical University (CMU97-CMC-004).

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Legends for Figures

Fig. 1. Dose-dependent effects of TNBS on the colonic tissues. Mice were administered with various amounts of TNBS in 0.1 ml of 50% ethanol by an intrarectal route. Mice administered with 50% ethanol were used as controls. All mice were sacrificed on day 7 after TNBS treatment. (A) Macroscopic changes of the colons. (B) Microscopic features of the colons. Magnification, 400x. (C) Macroscopic and microscopic scores of TNBS-induced colitis. Values are mean ± S.E.M. of three independent experiments (n=9). $^{**}P<0.01$, compared with mock.

Fig. 2. Dose-dependent effects of vanillin in mice with TNBS-induced colitis. Mice were administered with various amounts of vanillin and 5 mg of TNBS in 0.1 ml of 50% ethanol by an intracolonic route. Mice administered with 50% ethanol were used as controls. All mice were sacrificed on day 7 after TNBS treatment. (A) Macroscopic changes (top) and microscopic features (bottom) of the colons. Magnification, 400x. (B) Colonic weight/length ratio, macroscopic score, and microscopic score of TNBS-induced colitis. Values are mean ± S.E.M. of three independent experiments (n=9). $^{**}P<0.01$, compared with TNBS-treated mice.

Fig. 3. Preventive effect of vanillin in mice with TNBS-induced colitis. Mice were orally administered with 50 mM vanillin or 130 mM 5-ASA for 3 consecutive days before TNBS induction. Mice administered with 50% ethanol were used as controls. All mice were sacrificed on day 7 after TNBS treatment. (A) Macroscopic changes (top) and microscopic features (bottom) of the colons. Magnification, 400x. (B) Colonic

weight/length ratio, macroscopic score, and microscopic score of TNBS-induced colitis. Values are mean ± S.E.M. of three independent experiments (n=9). $^{*}P<0.05$, compared with TNBS-treated mice.

Fig. 4. Therapeutic effect of vanillin in mice with TNBS-induced colitis. Mice were orally administered with 50 mM vanillin or 130 mM 5-ASA for 7 consecutive days after TNBS induction. Mice administered with 50% ethanol were used as controls. All mice were sacrificed on day 7 after TNBS treatment. (A) Macroscopic changes (top) and microscopic features (bottom) of the colons. Magnification, 400x. (B) Colonic weight/length ratio, macroscopic score, and microscopic score of TNBS-induced colitis. Values are mean ± S.E.M. of three independent experiments (n=9). $^{*}P<0.05$, $^{**}P<0.01$, compared with TNBS-treated mice.

Fig. 5. Effect of vanillin on the NF-κB activity of the colon in mice with TNBS-induced colitis. Transgenic mice were orally administered with 50 mM vanillin for 7 consecutive days after TNBS induction. All mice were imaged and sacrificed on day 7 after the initial TNBS administration. (A) *In vivo* and *ex vivo* imaging. Transgenic mice were injected intraperitoneally with D-luciferin and imaged *in vivo* for 5 min after luciferin injection (left panel). Transgenic mice were then sacrificed, and organs were excised rapidly and subjected to image (right panel). The color overlay on the image represents the photons/sec emitted from the animal, as indicated by the color scales. The quantified photon signal is shown below each image. (B) Luciferase activity assay. Results are expressed as RLU/μg protein. (C) Biotinylated EMSA. The nuclear extracts of colons

were incubated with biotin-labeled double-stranded oligonucleotides corresponding to NF-κB responsive elements. The arrowhead points to the location of NF-κB/DNA complex. Values are mean ± S.E.M. of three independent experiments (n=9). $^{**}P<0.01$, compared with TNBS-treated mice.

Fig. 6. Signal transduction pathways contributing to the suppression of vanillin on TNBS-induced NF-κB activity. Mice were orally administered with 50 mM vanillin for 7 consecutive days after TNBS induction. All mice were sacrificed on day 7 after the initial TNBS administration. The phosphorylated (phospho-IκB-α, phospho-IKK-α/β) and non-phosphorylated proteins (IκB-α, IKK-α, IKK-β) in colonic extracts were detected by Western blot. The levels of p65 in cytoplasm (cyto) and nucleus (nu) were also determined by Western blot. Similar results were obtained in three independent experiments (n=9).

Fig. 7. Effect of vanillin on the expressions of cytokine genes in mice with TNBS-induced colitis. Mice were orally administered with 50 mM vanillin for 7 consecutive days after TNBS induction. All mice were sacrificed on day 7 after the initial TNBS administration. Total RNAs were extracted from colonic tissues and 1 μg of total RNA was reverse transcribed. The resulting cDNAs were then amplified by PCR using primers for cytokines or β-actin. PCR products were resolved in 1% agarose gels and visualized with ethidium bromide. Similar results were obtained in three independent experiments (n=9).

Figure 1

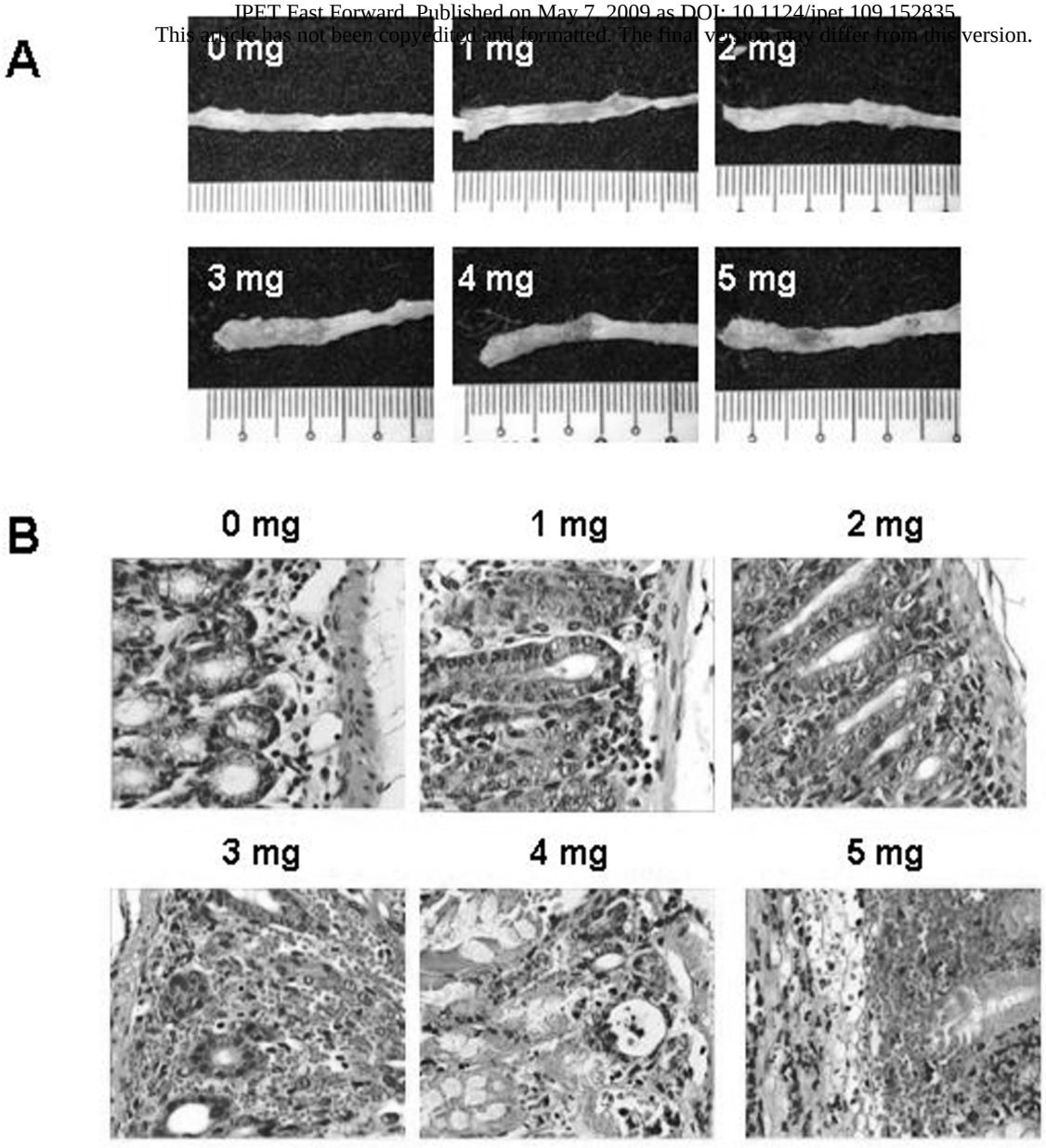

Figure 2

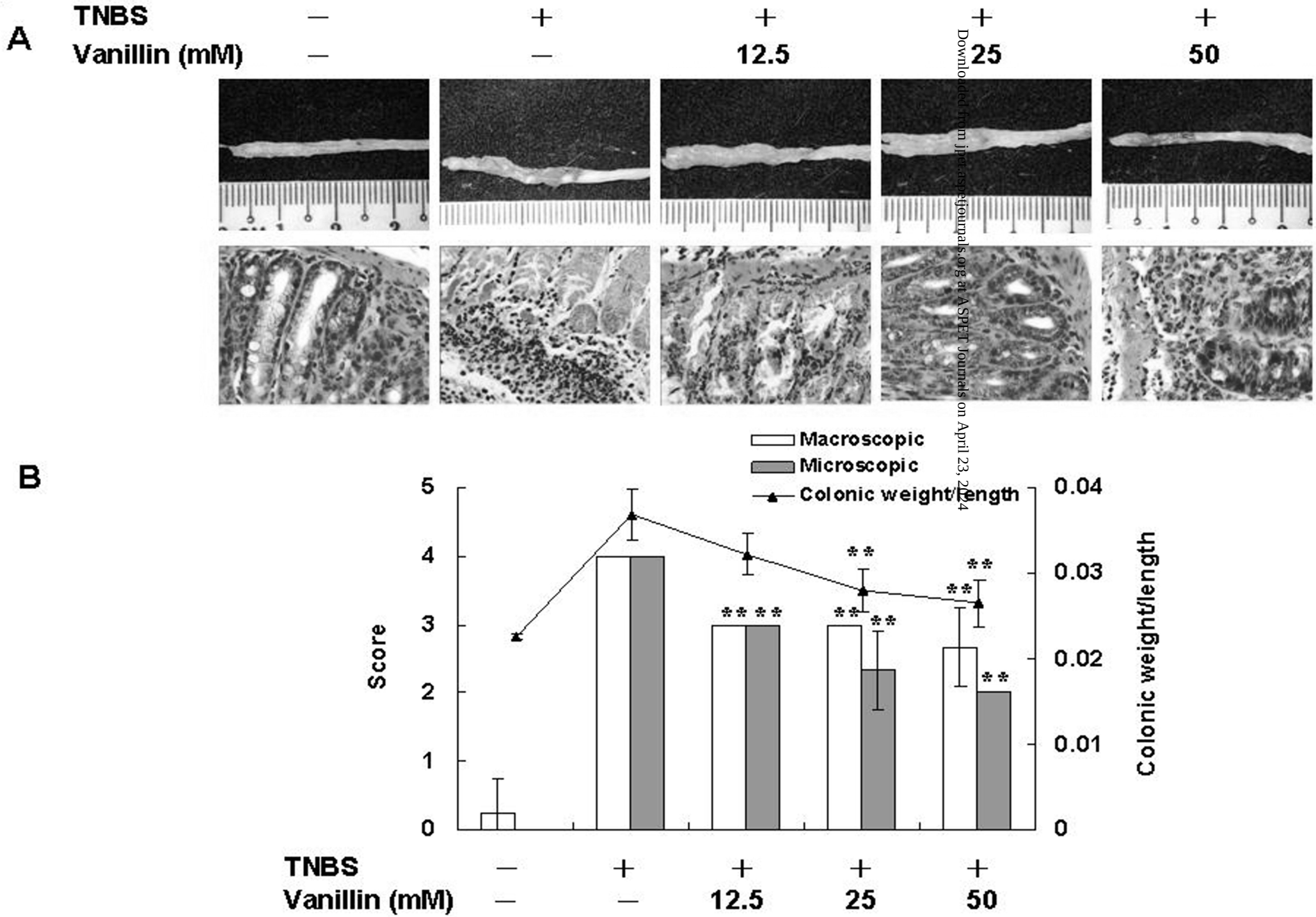

Figure 3

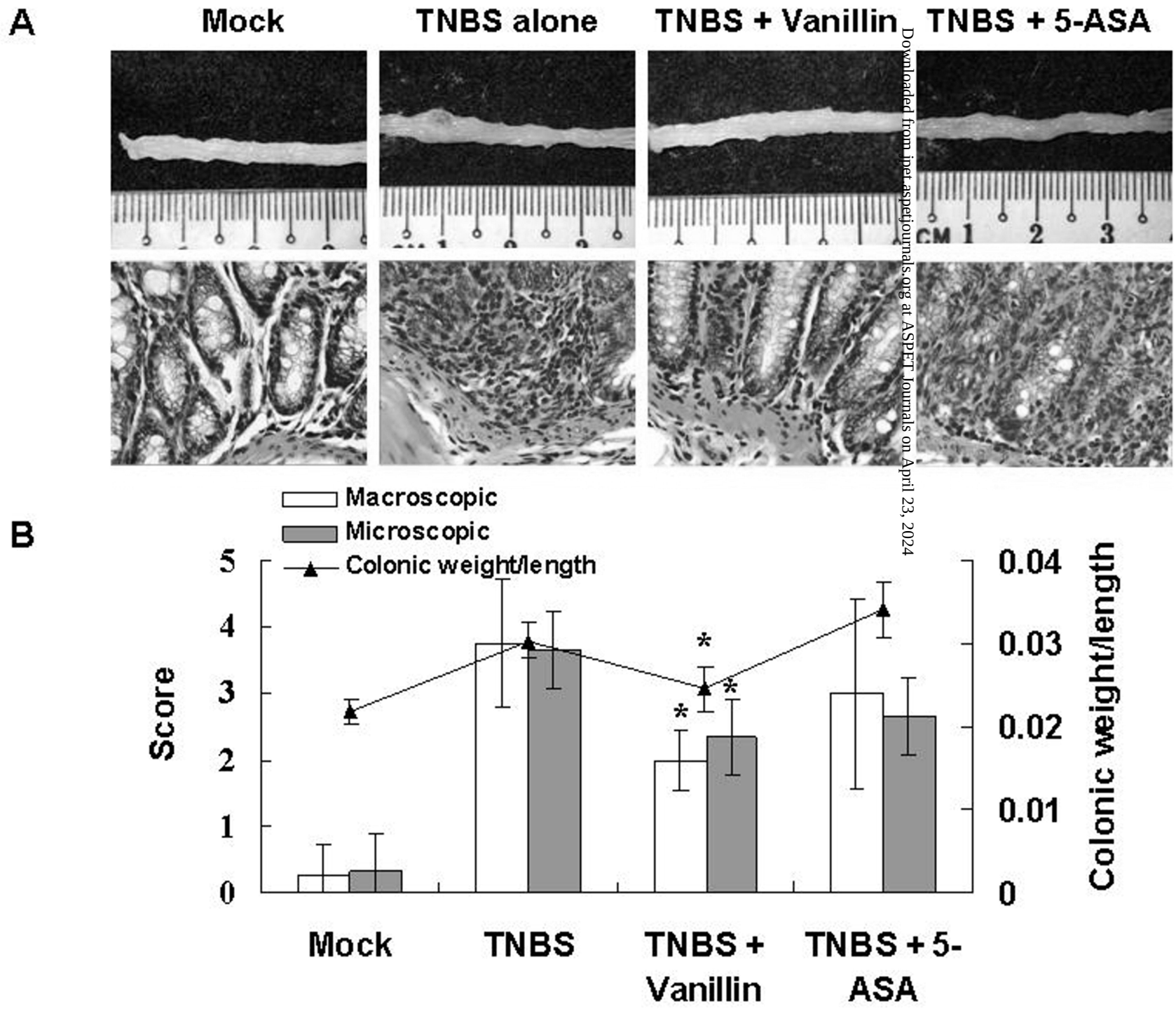

Figure 4

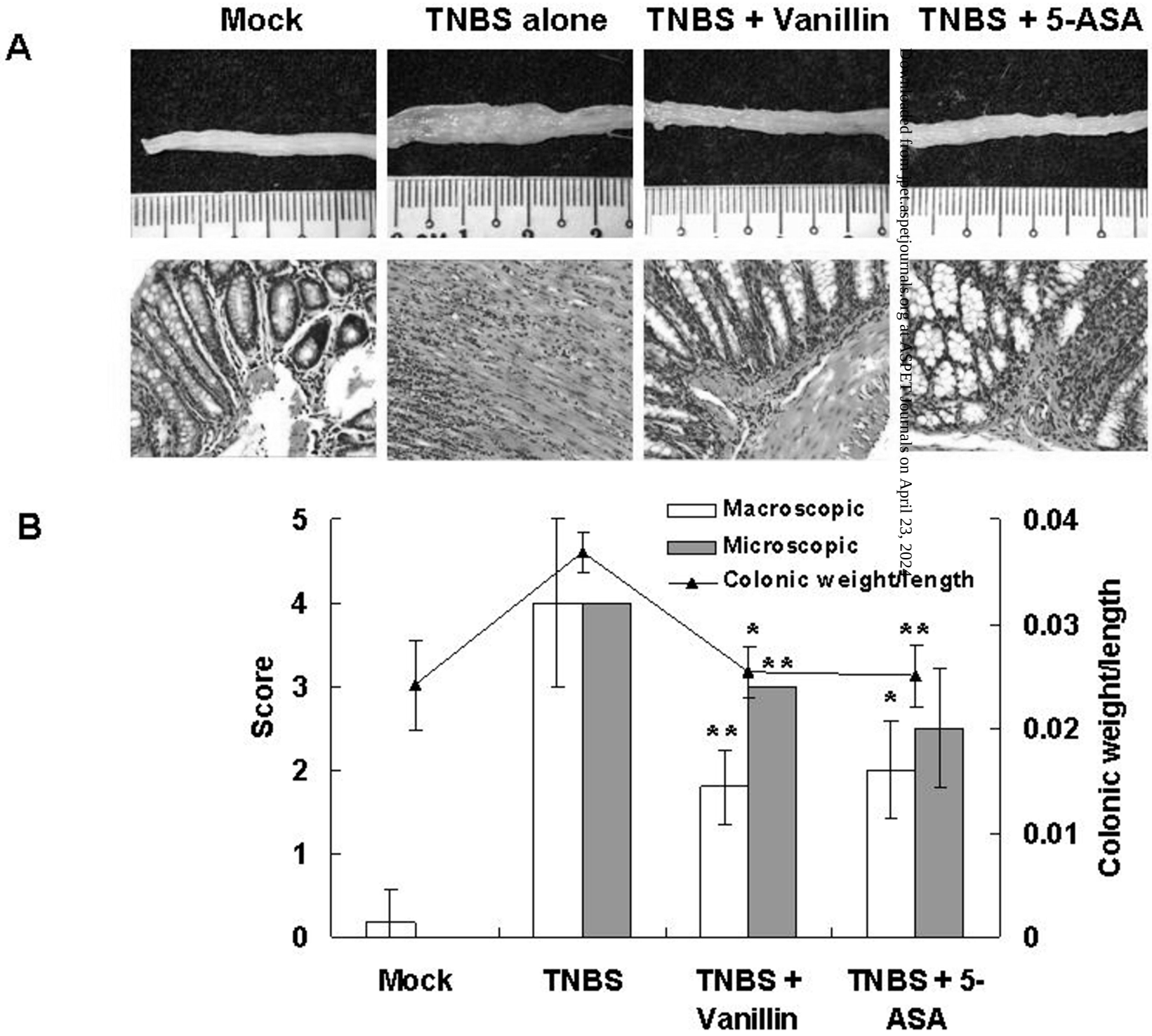

Figure 5

JPET Fast Forward. Published on May 7, 2009 as DOI: 10.1124/jpet.109.152835

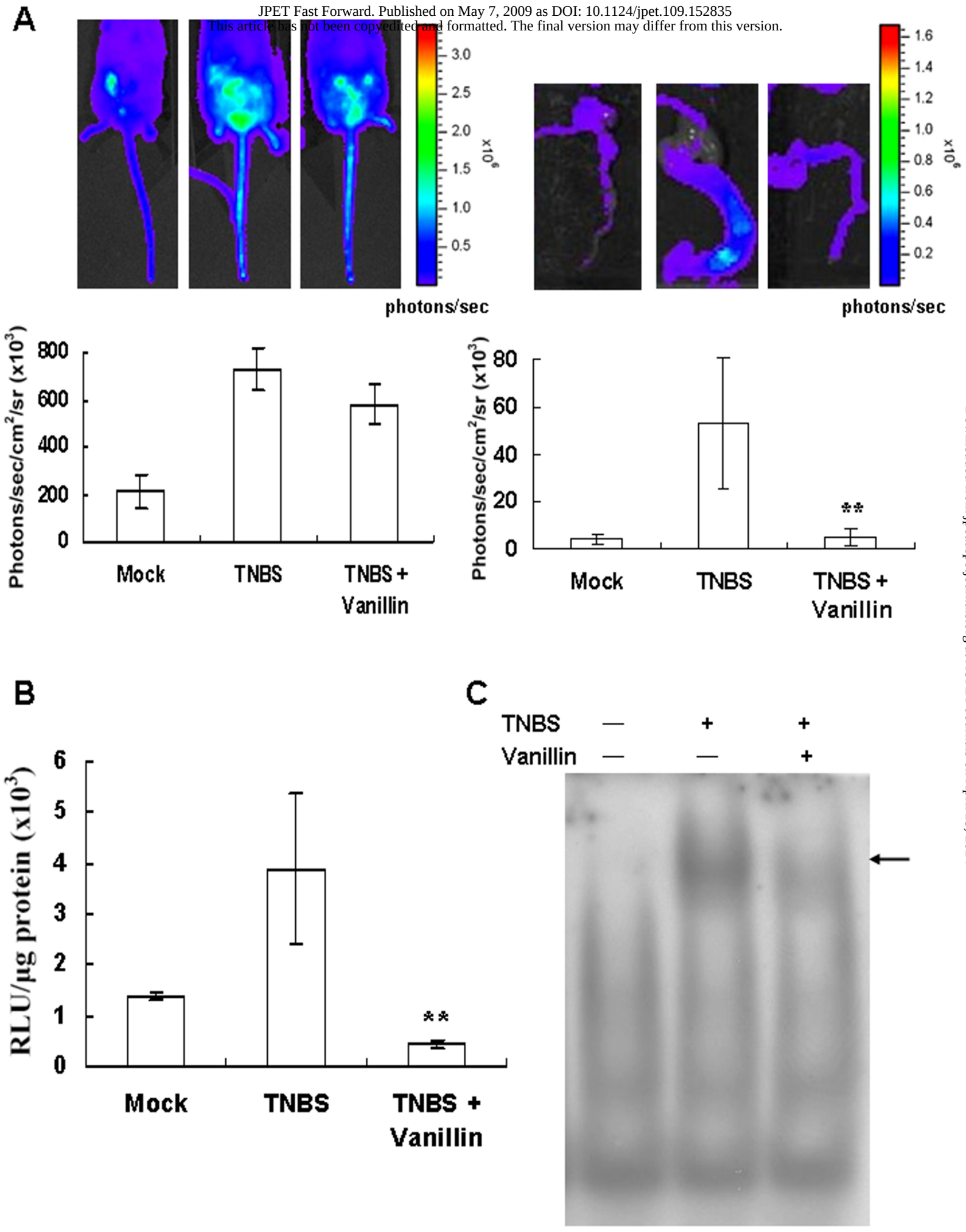

Figure 6

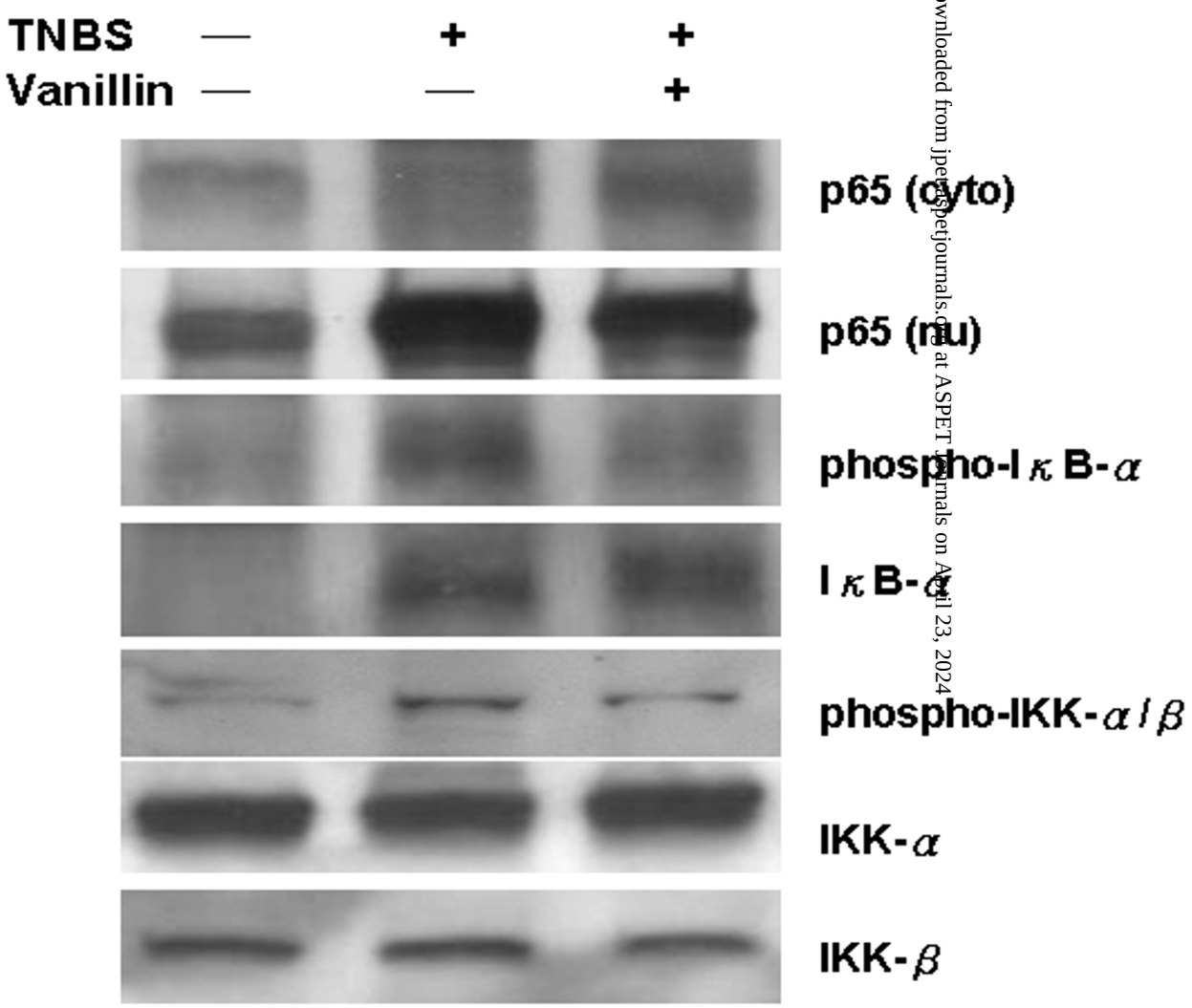

Figure 7