Fos Proteins Suppress Dextran Sulfate Sodium-Induced Colitis through Inhibition of NF-κB

Yasunari Takada, Neelanjan Ray, Eiji Ikeda, Tomohiro Kawaguchi, Masayoshi Kuwahara, Erwin F. Wagner and Koichi Matsuo

*J Immunol* 2010; 184:1014-1021; Prepublished online 16 December 2009;
doi: 10.4049/jimmunol.0901196
http://www.jimmunol.org/content/184/2/1014

Supplementary Material  
http://www.jimmunol.org/content/suppl/2009/12/15/jimmunol.0901196.DC1

Why *The JI*?  
- **Rapid Reviews!** 30 days* from submission to initial decision  
- **No Triage!** Every submission reviewed by practicing scientists  
- **Speedy Publication!** 4 weeks from acceptance to publication

*average

References  
This article cites 46 articles, 14 of which you can access for free at:  
http://www.jimmunol.org/content/184/2/1014.full#ref-list-1

Subscription  
Information about subscribing to *The Journal of Immunology* is online at:  
http://jimmunol.org/subscription

Permissions  
Submit copyright permission requests at:  
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts  
Receive free email-alerts when new articles cite this article. Sign up at:  
http://jimmunol.org/alerts
Fos Proteins Suppress Dextran Sulfate Sodium-Induced Colitis through Inhibition of NF-κB

Yasunari Takada,*† Neelanj Ray,* Eiji Ikeda,‡† Tomohiro Kawaguchi,§ Masayoshi Kuwahara,* Erwin F. Wagner,§ and Koichi Matsuo*†

The Fos family proteins, c-Fos and Fra-1, are components of the dimeric transcription factor AP-1, which is typically composed of Fos and Jun family proteins. We have previously shown that mice lacking c-Fos (Fos−/− mice) respond more strongly to LPS injection than do wild-type (wt) controls. We then examined the sensitivity of Fos−/− mice to acute inflammatory stress in a dextran sulfate sodium (DSS)-induced colitis model. We found that Fos−/− mice exhibited more severe weight loss, bleeding, diarrhea, and colon shortening than did wt mice, in association with higher TNF-α production and NF-κB activity in colon segments of DSS-treated Fos−/− mice. Furthermore, NF-κB inhibition suppressed severe DSS-induced colitis in Fos−/− mice. In contrast, Fra-1 transgenic (Tg) mice responded poorly to LPS injection, and Fra-1—overexpressing macrophages and fibroblasts showed reduced production of proinflammatory cytokines, NO, and NF-κB activity. Remarkably, in the DSS-induced colitis model, Fra-1 Tg mice showed less severe clinical scores of colitis than did wt mice. Consistently, proinflammatory cytokine production and NF-κB activity in colon segments of DSS-treated Fra-1 Tg mice were lower than in wt controls. These findings reveal that the absence of c-Fos and overexpression of Fra-1 respectively enhance and suppress the activation of NF-κB in DSS-induced inflammatory stress. In this paper, we propose that AP-1 transcription factors containing c-Fos or Fra-1 are negative regulators of NF-κB-mediated stress responses. The Journal of Immunology, 2010, 184: 1014–1021.

Inflammatory bowel disease (IBD), a chronic and relapsing inflammation of the gastrointestinal tract, affects millions of people worldwide. Although the causes of IBD are still unknown, genetic and environmental factors, infectious agents, impairment of local tolerance, and mucosal imbalance leading to activation of the mucosal immune system have been suggested to play roles (1). The resulting imbalance of the mucosal immune system causes the overproduction of inflammatory cytokines, reactive oxygen metabolites, and infiltration of neutrophils into the intestine, thus leading to uncontrolled intestinal inflammation and tissue damage (1). Dextran sulfate sodium (DSS) is commonly used in rodent models to chemically induce acute intestinal inflammation, and the DSS-induced colitis is characterized by weight loss, bloody diarrhea, epithelial cell damage, and immune cell infiltration, as well as an increased production of inflammatory mediators including TNF-α, IL-6, IL-12, and interferons. Two stress-responsive transcription factors—AP-1 and NF-κB—were activated to induce such inflammatory mediators (2, 3). Acute DSS-induced colitis does not require T cells or B cells because it occurs in SCID mice that lack these cells (4). Colitis may result from DSS toxicity to colonic epithelial cells.

AP-1 is a collection of dimeric transcription factors typically composed of one of four Fos family proteins (c-Fos, FosB, Fra-1, and Fra-2) and one of three Jun family proteins (c-Jun, JunB, and JunD). Unlike Fos proteins, Jun proteins can also form dimers within the family. AP-1 is one of the major regulators of bone and immune cells, among other cell types. A wide range of cellular stresses, including serum, UV, and pathogen-associated molecular patterns, such as the cell-wall component LPS of Gram-negative bacteria, sequentially induce transcription of the Fos family genes (2, 5). The activity of both newly synthesized and pre-existing AP-1 components is modulated through their posttranslational modification. Key enzymes are various MAPKs, including JNK, ERK, and p38, which phosphorylate AP-1 components and thereby regulate transcriptional activity.

c-Fos is implicated in the regulation of innate immune responses. Genes encoding proinflammatory mediators, such as TNF-α, IL-6, and IL-12, are transcriptionally activated in response to LPS or TNF-α, mainly through NF-κB activation (6). We previously reported that NF-κB activity is elevated in Fos−/− mice, resulting in augmented inflammatory responses (7). Injection of LPS into mice lacking c-Fos results in more prominent hypothermia and bradycardia than occur in wild-type (wt) controls, owing to the elevated production of proinflammatory cytokines, especially TNF-α (7). Therefore, in contrast to NF-κB, c-Fos appears to be a negative regulator of proinflammatory responses. Fra-1 is a Fos family protein that is structurally related to, but smaller than, c-Fos and that lacks so-called transactivation domains within the molecule (8). Fra-1 and ΔFosB dramatically enhance osteoblast differentiation.

Abbreviations used in this paper: DSS, dextran sulfate sodium; IBD, inflammatory bowel disease; IKK, IkB kinase; MDM, CSF-dependent macrophage; MEF, mouse embryonic fibroblast; ODN, oligodeoxynucleotide; qRT-PCR, quantitative RT-PCR; ROS, reactive oxygen species; Tg, transgenic; wt, wild-type.

Copyright © 2010 by The American Association of Immunologists, Inc. 0022-1767/10516.00

Accepted for publication November 4, 2009. Received for publication April 15, 2009.

The Journal of Immunology
when overexpressed in mice (9, 10). However, roles of Fra-1 in inflammatory responses in vivo remain to be determined.

In this study, we hypothesized that Fos proteins may protect cells from excessive stress responses by suppressing NF-κB activity. Our results suggest that these Fos proteins are negative regulators of NF-κB–mediated stress responses.

Materials and Methods

Mice

Fos<sup>−/−</sup> mice (11) were bred on a mixed background of 129 and C57BL/6J. A powder diet was provided to Fos<sup>−/−</sup> mice. Fra-1 transgenic (Tg) mice (9) were backcrossed onto C57BL/6J mice (Crea, Tokyo, Japan) >10 generations, and both mice groups were maintained under specific pathogen-free conditions. Wt littermates were used as controls. Fra-1–conditional generations, and both mice groups were maintained under specific pathogen-free conditions. Wt littermates were used as controls. Fra-1–conditional knockout mice on a C57BL/6J × 129 mixed background were produced by crossing Fos<sup>−/−</sup> mice with MORE-Cre mice (12). All experiments were conducted in accordance with the institutional review board-approved protocols of Keio University.

DSS administration

Mice were given distilled drinking water containing 2% (wt/vol) DSS (m.w. = 36,000–50,000; MP Biomedicals, Irvine, CA) ad libitum. DSS was administered to Fos<sup>−/−</sup> mice for 7 d and to Fra-1 Tg mice for 13 d. Bay 11-7085 solution (Biomol International, Plymouth Meeting, PA; final 4% DMSO in PBS) or solution control (mock) was injected 1 h before and each day during DSS administration by i.p. injection at a concentration of 20 mg/kg mouse body weight. The clinical scores of colitis, such as weight change, diarrhea, and colorectal bleeding, and survival were observed. The whole colon was extracted to measure its length and to examine its histological status.

Histological analysis

The extracted colon was fixed in 4% paraformaldehyde, embedded in paraffin, and cut into 4-μm sections on slide glasses. The slides were stained with H&E (Wako, Tokyo, Japan) and analyzed under a microscope (Axiostar 135; Carl Zeiss, Jena, Germany). Frozen 3-μm sections were also prepared. The histological examination of colonic tissue was performed as described (13). The tissues were scored in a blinded fashion on a scale from 0 to 4, according to intensity of lesions: 0, normal; 1, mild; 2, moderate; 3, severe; and 4, very severe. Intensity measures included the number of infiltrating cells, depth of inflammation, crypt damage, and percent involvement. The average disease activity and the total histological inflammation score are shown.

Immunohistochemistry

Frozen sections were stained with rabbit anti-c-Fos (Abcam, Cambridge, UK), mouse anti-Fra-1 (Santa Cruz Biotechnology, Santa Cruz, CA), or Armenian hamster anti-CD11c (Abcam) Abs, followed by incubation with biotin-labeled Armenian hamster anti-CD11c (Abcam) Abs, respectively. The slide was developed by diaminobenzidine solution (EnVision Kit, Dako, Carpinteria, CA), counterstained by hematoxylin (Wako), and mounted with Softmount (Wako). For control purpose, DSS-treated colon slides were stained with the specific isotype IgG as a replacement for the first Ab, and followed general staining procedures.

Quantitative RT-PCR

Mice were sacrificed, and total RNAs were extracted from colon fragments, using ISOGEN (Nippon Gene, Toyama, Japan). cDNA was synthesized from 1–2 μg total RNA, using random hexamers (Qiagen, Valencia, CA) with PrimeScript (Takara Bio, Tokyo, Japan). Quantitative RT-PCR (qRT-PCR) was performed on an ABI Prism 7000 machine (Applied Biosystems, Foster City, CA). mRNA levels were quantified using a standard curve generated with serially diluted plasmids containing the PCR amplicon and normalized to Gapdh expression. Experiments were repeated three times with three independent cDNA syntheses.

ELISA analysis

Mouse serum and culture supernatants were collected, and protein levels of TNF-α, IL-6, IL-10, and IL-12 p40 were measured using ELISA sets (BD Biosciences, San Diego, CA). The production levels of TGF-β and IL-4 also were measured using ELISA sets (eBioscience, San Diego, CA).

EMSA

NF-κB or AP-1 DNA-binding activity was assessed with EMSA, as described previously (14). Briefly, 3 μg nuclear extracts prepared from M-CSF–dependent macrophages (MDMs) and colon fragments was incubated for 30 min at 37°C with [32P]–end-labeled 27-mer double-stranded oligonucleotide containing the kB3 binding site of mouse TNF-α promoter (5′-AGCTGAGGGAGGGAGACCCCCCTTTGG-3′) (15) or 28-mer double-stranded oligonucleotide containing the consensus AP-1 binding site of the human collagenase promoter (16). The DNA-protein complex formed was then separated from free oligonucleotide on 6.6% native polyacrylamide gels. Dried gels were visualized using a BAS5000 imaging analyzer (Fuji Film, Tokyo, Japan), and bands were quantified using MultiGauge software (Fuji Film).

Locomotor activity

A telemetric radio transmitter (TA10ETA-F20; Data Sciences, Saint Paul, MN) was used to measure locomotor activity. A transmitter was implanted subcutaneously on the back of each mouse, as previously described (7). The locomotor activity was monitored continuously by receivers (CTR-86; Data Sciences) below the cages and recorded every 5 min by a Data Quest analysis system (Data Sciences). A stock solution of 10 mg/ml LPS from Salmonella enterica serovar Typhimurium (S. Minnesota Re595; Sigma-Aldrich, St. Louis, MO) was prepared in water at 70°C. Before the experiment, LPS was further diluted. In consideration of circadian rhythm, LPS was administered i.p. at 13:00 h for each experiment.

Cell culture

Bone marrow from tibiae and femora of wt and Fra-1 Tg mice (basically at 6 wk old) was harvested by flushing with α-minimal essential medium solution containing 10% FBS and penicillin-streptomycin. After passage through a 70-μm–cell strainer, cells were cultured overnight in 15-cm tissue culture dishes (Greiner, Frickenhausen, Germany). Floating cells were then collected and expanded in the presence of 10 μg/ml M-CSF (R&D Systems, Minneapolis, MN) for 3 d. These cells were used as MDMs, and were seeded at a concentration of 1 × 10<sup>6</sup> cells in a 24-well plate and treated with 0.1 μg/ml LPS for 24 h. The supernatants were subjected to ELISA to analyze the expression of indicated cytokines and the production of NO. Mouse embryonic fibroblasts (MEFs) were prepared from wt and Fra-1 Tg mice, seeded at a concentration of 1 × 10<sup>5</sup> cells in a 24-well plate, and treated with 0.1 μg/ml LPS for 24 h. The supernatants were then subjected to ELISA.

NO production assay

The concentration of stable nitrite was determined by Griess reaction, as previously described (14). The absorbance at 530 nm was determined on a plate reader (680XR; BioRad Laboratories, Hercules, CA). A sodium nitrite standard curve was generated in each experiment.

Western blot analysis

To determine protein expression levels, we prepared whole-cell extracts and fractionated them on 4–12% SDS-polyacrylamide gels (Novex; Invitrogen, Carlsbad, CA) and transferred them onto Hybond nitrocellulose membranes (Amersham, Little Chalfont, UK). The following primary Abs were used: polyclonal anti-TLR4 goat Ab (Santa Cruz Biotechnology), polyclonal anti-CD14 rabbit Ab (Santa Cruz Biotechnology), monoclonal phospho-IκB mouse Ab (Cell Signaling Technology), monoclonal anti-phospho-p65 mouse Ab (Cell Signaling Technology), monoclonal anti-β-actin goat Ab (Santa Cruz Biotechnology), monoclonal anti-phospho-JNK mouse Ab (Cell Signaling Technology), monoclonal anti-β-actin mouse Ab (Santa Cruz Biotechnology), monoclonal anti-phospho-ERK1/2 rabbit Ab (Cell Signaling Technology), monoclonal anti-phospho-p38 mouse Ab (Cell Signaling Technology), and monoclonal anti-phospho-ERK1/2 rabbit Ab (Cell Signaling Technology). Where indicated, blots were stripped and reprobed with a polyclonal anti-Actin goat Ab (Santa Cruz Biotechnology) to monitor protein loading.

Statistical analysis

All data were expressed as the mean ± SEM. The statistical significance of differences between values was evaluated by the Student t test.

Results

DSS treatment induces c-Fos and Fra-1 expression

We first examined the expression of stress-responsive transcription factor AP-1 in DSS-induced colitis. We administered 2% DSS in drinking water to wt mice for 7 d to induce acute intestinal


inflammation. DSS induced infiltration of inflammatory cells and crypt damage in the colon, and c-Fos and Fra-1 proteins were detected by immunohistochemistry in various cell types, including infiltrating macrophages (Fig. 1A). We also found that DSS induced expression of CD11c-positive APCs or dendritic cells (Fig. 1A). Furthermore, we analyzed mRNA levels of AP-1 genes by qRT-PCR and noted that expression of all seven AP-1 family members was elevated (Fig. 1B). These data suggest that c-Fos and Fra-1 are expressed mainly in infiltrating cells, including APCs, during the course of DSS injury.

Deletion of c-Fos enhances DSS-induced colitis

We investigated the susceptibility of Fos^{-/-} mice to DSS-induced colitis. We administered 2% DSS in drinking water to Fos^{-/-} mice and their wt littermates, and monitored clinical parameters, cytokine profiles, and NF-κB activity. Some of the Fos^{-/-} mice died within 6 d after DSS administration (Fig. 2A), and surviving Fos^{-/-} mice showed more severe clinical colitis profiles than did wt controls (Fig. 2B–D). Seven days after DSS administration, the histological status of each colon was analyzed. In the Fos^{-/-} mice, the percentage of colon shortening, which reflects inflammation at the site, was four times higher (Fig. 2F), and invasiveness of inflammatory lesions and crypt damage were significantly greater than in wt controls (Fig. 2F). DSS-induced cytokine production in serum was also examined. We found that production of proinflammatory cytokines such as TNF-α, IL-6, and IL-12 was significantly enhanced in DSS-treated Fos^{-/-} mice. Although TGF-β expression was increased, anti-inflammatory cytokines IL-4 and IL-10 were not elevated in DSS-treated Fos^{-/-} mice compared with wt controls (Fig. 2G). We also noted that TNF-α and IL-6 production (Supplemental Fig. 1), NF-κB-binding activity (Fig. 2H), and MAPK activity (Fig. 2I) were higher in colon fragments of DSS-treated Fos^{-/-} mice than in those of DSS-treated wt mice. The enhanced production of TNF-α and IL-6 in the absence of c-Fos was also observed in colon fragments treated with LPS (Supplemental Fig. 2). These results demonstrate that the loss of c-Fos protein enhances susceptibility to DSS-induced colitis.

Inhibition of NF-κB suppresses DSS-induced colitis in Fos^{-/-} mice

To clarify the role of NF-κB activation in DSS-induced colitis in Fos^{-/-} mice, we administered the NF-κB inhibitor Bay 11-7085 along with DSS treatment, using wt and Fos^{-/-} mice. The Fos^{-/-} mice that were administered Bay 11-7085 demonstrated lower clinical colitis profiles than did mock-treated Fos^{-/-} mice (Fig. 3A–C). The histological status of the colon showed that treatment of Bay 11-7085 suppressed colon shortening in Fos^{-/-} mice (Fig. 3D), and invasiveness of inflammatory lesions and crypt damage were significantly lower than in mock-treated Fos^{-/-} mice (Fig. 3E). The production levels of inflammatory cytokines TNF-α and IL-6 were significantly suppressed in Fos^{-/-} mice administered Bay 11-7085 (Fig. 3F). Therefore, the loss of c-Fos enhances DSS-induced colitis mainly through NF-κB. Together with enhanced LPS-induced cytokine production in the absence of c-Fos (7), these results strongly suggest that c-Fos is an anti-inflammatory transcription factor in response to administration of both DSS and LPS.

Reduced cytokine production of Fra-1 Tg mice

We next asked whether the loss of Fra-1, another Fos family protein, also enhances inflammatory responses. Because Fra-1 knockout mice are embryonic lethal owing to a placental defect, we examined the LPS response of Fra-1–conditional knockout (Fosl1^{−/−}) mice, in which Fra-1 was deleted in the entire body but not in the placenta (12). We found that serum levels of TNF-α and IL-6 in response to LPS injection were comparable between Fosl1^{−/−} and control mice, suggesting that, as long as c-Fos is present, the loss of Fra-1 does not result in increased cytokine production (data not shown).

We then wondered whether overexpression of Fos proteins might suppress the inflammatory response. Whereas c-Fos Tg mice develop osteosarcoma (17), Fra-1 Tg mice, which widely overexpress Fra-1 under the control of the H-2\(^{k}\) promoter, show enhanced bone formation (9). We next determined the effects of forced Fra-1 expression on pro- and anti-inflammatory cytokine production, using Fra-1 Tg mice. To prove the principle of acute inflammatory responses, we injected 4 mg/kg of LPS i.p. into wt and Fra-1 Tg mice, and measured cytokine levels in serum. Levels of proinflammatory cytokines, TNF-α, IL-6, and IL-12, were significantly lower in Fra-1 Tg mice than in wt control mice (Fig. 4A). The production levels of anti-inflammatory cytokines TGF-β and IL-4 were also lower in LPS-injected Fra-1 Tg mice, whereas that of IL-10 was not altered (Fig. 4B). LPS injection is known to reduce the locomotor activity of mice through the induction of inflammatory mediators. To determine whether reduced proinflammatory cytokine production in Fra-1 Tg mice could block the LPS-induced decrease in locomotor activity, we implanted a telemetric radio transmitter into wt and Fra-1 Tg mice. After injection of a low dose (1 mg/kg) of LPS, wt control mice often stayed in a crouched position, resulting in reduced locomotor activity, but Fra-1 Tg mice tolerated LPS injection and remained active (Fig. 4C). Therefore, Fra-1 overexpression reduces the production of proinflammatory cytokines such as TNF-α in vivo.

Reduced cytokine production and NF-κB activity in Fra-1–overexpressing cells

We then determined how Fra-1–overexpressing cells respond to LPS. We prepared MDMs and MEFs from wt and Fra-1 Tg mice. Fra-1–overexpressing MDMs and MEFs produced lower levels of
Cytokine production in sera. Sera were prepared from mice. A score was evaluated by histological grading of colitis. (wt, n = 11) and Fos−/−mice (C, n = 10) were administered 2% DSS in drinking water. B, Percentage weight changes. Initial weight of each mouse was set as 100%. Data are means ± SEM. p < 0.05. C, Diarrhea scores. p < 0.05. D, Bleeding scores. p < 0.05. E, Percentage of colon shortening of mice treated with 2% DSS for 7 d (wt, n = 11, Fos−/−, n = 7) relative to colon length of untreated mice (wt, n = 5, Fos−/−, n = 5). Data are means ± SEM, p = 0.12. F, Histological section of colon. H&E staining. Lower panels show a higher magnification of the box in the upper panels. Scale bars, 1 mm (upper panel), 200 μm (lower panel). Inflammation score was evaluated by histological grading of colitis. G, Cytokine production in sera. Sera were prepared from mice untreated (wt, n = 4, Fos−/−, n = 4) or treated with 2% DSS for 7 d (wt, n = 4, Fos−/−, n = 3). Cytokine production in sera was analyzed by ELISA. Data are means ± SEM. p < 0.05 versus DSS-treated wt mice. H, NF-κB activation in colon fragments without (wt, n = 2, Fos−/−, n = 2) or with DSS treatment (wt, n = 2, Fos−/−, n = 3). Nuclear proteins were prepared and subjected to EMSA for NF-κB activation. Data are means ± SEM. p < 0.05 versus DSS-treated wt mice. I, MAPK activation in colon fragments without (wt, n = 2, Fos−/−, n = 2) or with DSS treatment (wt, n = 2, Fos−/−, n = 2). Whole-cell extract was prepared and subjected to Western blot analysis.

IL-6 than did control wt cells in response to increasing LPS concentration (Fig. 5A). NO production was also reduced (Fig. 5B), suggesting that various inflammatory responses are diminished in Fra-1 Tg mice. To examine the molecular mechanisms underlying reduced inflammatory responses, we compared AP-1 and NF-κB binding activities between wt and Fra-1 Tg MEFs treated with LPS, using EMSA. In Fra-1 Tg MEFs, AP-1–binding activity was higher than in the control mice, as expected. In contrast, LPS-induced NF-κB–binding activity was lower in Fra-1 Tg than in wt MEFs (Fig. 5C). These findings suggest that diminished inflammatory responses to LPS in Fra-1–overexpressing cells result from NF-κB suppression.

Overexpression of Fra-1 suppresses the LPS signaling pathway

To gain insight into the molecular mechanisms underlying the suppression of NF-κB by Fra-1, we used MEFs to analyze protein levels of LPS receptors, NF-κB, and MAPKs. As shown in Fig. 6A, expression levels of two LPS receptors, TLR4 and CD14, were comparable between wt and Fra-1–overexpressing cells. Activation of NF-κB requires the sequential activation of IκB kinase (IKK), degradation of IκBα, and phosphorylation and nuclear translocation of p65 (18). We therefore treated wt and Fra-1–overexpressing MEFs with 0.1 μg/ml of LPS for different time periods, prepared the whole-cell extracts, and analyzed the activation status of these molecules. In wt MEFs, we observed the LPS-induced phosphorylation and degradation of IκBα, as well as the phosphorylation of p65. Interestingly, the phosphorylation of IκBα was decreased in Fra-1–overexpressing MEFs, and degradation of IκBα and phosphorylation of p65 were also reduced (Fig. 6B). To explore the role of Fra-1 in LPS-induced MAPK activation, we analyzed LPS-induced phosphorylation of JNK, ERK, and p38. In wt MEFs, phosphorylated forms of JNK, ERK, and p38 were observed in a time-dependent manner, with maximum activation at 15 to 30 min. In contrast, in Fra-1–overexpressing MEFs, the phosphorylation of each MAPK was reduced and delayed (Fig. 6C). Thus, our results suggest that Fra-1 overexpression suppresses the activation of NF-κB and MAPKs by LPS.

Overexpression of Fra-1 suppresses DSS-induced colitis

Because mice overexpressing Fra-1 showed reduced LPS-induced inflammatory responses, we studied whether Fra-1 overexpression could protect mice from DSS-induced colitis. We treated Fra-1 Tg mice and their wt littersmates with 2% DSS in drinking water and induced acute intestinal inflammation. The Fra-1 Tg mice and wt
Fra-1 reduces susceptibility to DSS-induced colitis. 2). Taken together, these results demonstrate that overexpression of Fra-1 Tg mice showed decreased production of proinflammatory cytokines and NO, compared with wt control cells. The magnitude of DSS-induced colitis, proinflammatory cytokine production, and NF-κB activation were diminished in Fra-1 Tg mice. Therefore, the Fra-1–containing AP-1 transcription factor acts as a negative regulator of NF-κB and inflammatory responses.

Because bone formation is enhanced in Fra-1 Tg mice (9, 12, 19), we separately analyzed the healing process after tibial transverse fracture and consistently found that production of inflammatory cytokines, including TNF-α and IL-6, around the bone fracture site is impaired in these mice (20). Furthermore, using the murine macrophage cell line RAW264.7 overexpressing Fra-1 by retrovirus, Morishita et al. (21) reported that Fra-1 negatively regulates LPS-induced production of TNF-α, IL-1, IL-6, and NO. In contrast, RAW264.7 cells transfected with Fra-1 small interfering RNA showed increased LPS-induced expression of proinflammatory cytokines (21). These observations indicate that Fra-1 is a negative regulator of proinflammatory cytokine production.

Because c-Fos is also a negative regulator in LPS-induced expression of proinflammatory cytokines (7, 22–28), we examined the effect of c-Fos disruption in DSS-induced colitis. Fra-1−/− mice exhibited higher susceptibility to DSS-induced colitis associated with higher levels of proinflammatory cytokines and NF-κB activity than did wt mice. Furthermore, administration of NF-κB inhibitor Bay 11-7085 suppressed the enhancement of DSS-induced colitis in Fra-1−/− mice. These findings establish that both c-Fos and Fra-1 are negative regulators in LPS-induced proinflammatory cytokine production and NF-κB activation.

In this study, we observed that the enhanced response to LPS is associated with an increased susceptibility to DSS in mice lacking derived from Fra-1 Tg mice showed reduced activation of NF-κB and MAPKs, as well as reduced production of proinflammatory cytokines and NO, compared with wt control cells. The magnitude of DSS-induced colitis, proinflammatory cytokine production, and NF-κB activation were diminished in Fra-1 Tg mice. Therefore, the Fra-1–containing AP-1 transcription factor acts as a negative regulator of NF-κB and inflammatory responses.

Discussion
In this study, we determined the functions of Fos proteins, c-Fos and Fra-1, in inflammatory stress responses using LPS injection and a DSS-induced colitis model. Upon LPS injection, Fra-1 Tg mice showed decreased production of proinflammatory cytokines and attenuated endotoxin shock as illustrated by continuous locomotor activity. Moreover, when treated with LPS, the MDMs and MEFs

![FIGURE 3. NF-κB inhibitor Bay 11-7085 suppresses DSS-induced colitis in Fox−/− mice.](Image)

![FIGURE 4. Reduced LPS responses in Fra-1 Tg mice. A. Serum levels of proinflammatory cytokines. Wt (wt) and Fra-1 Tg mice (n = 4 each) were injected i.p. with 4 mg/kg LPS. After 3 h, TNF-α, IL-6, and IL-12 levels in sera were measured by ELISA. Data are means ± SEM. *p < 0.05. B. Serum levels of anti-inflammatory cytokines. TGF-β, IL-4, and IL-10 levels in serum were measured by ELISA. Data are means ± SEM. *p < 0.05. C. Telemetry analysis of locomotor activity. Light-dark cycles are indicated using the 24-h system. Horizontal movement was monitored in wt and Fra-1 Tg mice (n = 3 each) after i.p. injection of 1 mg/kg LPS (arrowhead). Representative data are shown.](Image)
c-Fos. In contrast, mice lacking TLR4, TLR2, or MyD88 also show
increased susceptibility to mucosal inflammation (29–31). How
can these two observations be reconciled? First, the enhanced
susceptibility of *Fos*2/2 mice to DSS is apparently mediated not
only by TLR-MyD88 signaling but also by other mechanisms such
as direct damage to colon epithelial cells after DSS treatment.
Second, the mixed genetic background of mice lacking c-Fos (129
and C57BL/6J), which was unavoidable owing to the reduced
fertility of *Fos*2/2 mice on a C57BL/6J background, might affect
DSS susceptibility. It will be informative to study the roles of Fos
proteins in various chemically induced colitis models such as
those using acetic acid and trinitrobenzene sulfuric acid (32).

How do Fra-1 and c-Fos negatively regulate NF-κB–mediated
proinflammatory cytokine production? In the DSS-induced colitis
model in *Fos*−/− mice and increased susceptibility to mucosal inflammation (29–31). How can these two observations be reconciled? First, the enhanced susceptibility of *Fos*2/2 mice to DSS is apparently mediated not only by TLR-MyD88 signaling but also by other mechanisms such as direct damage to colon epithelial cells after DSS treatment. Second, the mixed genetic background of mice lacking c-Fos (129 and C57BL/6J), which was unavoidable owing to the reduced fertility of *Fos*2/2 mice on a C57BL/6J background, might affect DSS susceptibility. It will be informative to study the roles of Fos proteins in various chemically induced colitis models such as those using acetic acid and trinitrobenzene sulfuric acid (32).

How do Fra-1 and c-Fos negatively regulate NF-κB–mediated
proinflammatory cytokine production? In the DSS-induced colitis
model in *Fos*−/− mice and increased susceptibility to mucosal inflammation (29–31). How can these two observations be reconciled? First, the enhanced susceptibility of *Fos*2/2 mice to DSS is apparently mediated not only by TLR-MyD88 signaling but also by other mechanisms such as direct damage to colon epithelial cells after DSS treatment. Second, the mixed genetic background of mice lacking c-Fos (129 and C57BL/6J), which was unavoidable owing to the reduced fertility of *Fos*2/2 mice on a C57BL/6J background, might affect DSS susceptibility. It will be informative to study the roles of Fos proteins in various chemically induced colitis models such as those using acetic acid and trinitrobenzene sulfuric acid (32).

How do Fra-1 and c-Fos negatively regulate NF-κB–mediated
proinflammatory cytokine production? In the DSS-induced colitis
model in *Fos*−/− mice and increased susceptibility to mucosal inflammation (29–31). How can these two observations be reconciled? First, the enhanced susceptibility of *Fos*2/2 mice to DSS is apparently mediated not only by TLR-MyD88 signaling but also by other mechanisms such as direct damage to colon epithelial cells after DSS treatment. Second, the mixed genetic background of mice lacking c-Fos (129 and C57BL/6J), which was unavoidable owing to the reduced fertility of *Fos*2/2 mice on a C57BL/6J background, might affect DSS susceptibility. It will be informative to study the roles of Fos proteins in various chemically induced colitis models such as those using acetic acid and trinitrobenzene sulfuric acid (32).

How do Fra-1 and c-Fos negatively regulate NF-κB–mediated
proinflammatory cytokine production? In the DSS-induced colitis
model in *Fos*−/− mice and increased susceptibility to mucosal inflammation (29–31). How can these two observations be reconciled? First, the enhanced susceptibility of *Fos*2/2 mice to DSS is apparently mediated not only by TLR-MyD88 signaling but also by other mechanisms such as direct damage to colon epithelial cells after DSS treatment. Second, the mixed genetic background of mice lacking c-Fos (129 and C57BL/6J), which was unavoidable owing to the reduced fertility of *Fos*2/2 mice on a C57BL/6J background, might affect DSS susceptibility. It will be informative to study the roles of Fos proteins in various chemically induced colitis models such as those using acetic acid and trinitrobenzene sulfuric acid (32).

How do Fra-1 and c-Fos negatively regulate NF-κB–mediated
proinflammatory cytokine production? In the DSS-induced colitis
model in *Fos*−/− mice and increased susceptibility to mucosal inflammation (29–31). How can these two observations be reconciled? First, the enhanced susceptibility of *Fos*2/2 mice to DSS is apparently mediated not only by TLR-MyD88 signaling but also by other mechanisms such as direct damage to colon epithelial cells after DSS treatment. Second, the mixed genetic background of mice lacking c-Fos (129 and C57BL/6J), which was unavoidable owing to the reduced fertility of *Fos*2/2 mice on a C57BL/6J background, might affect DSS susceptibility. It will be informative to study the roles of Fos proteins in various chemically induced colitis models such as those using acetic acid and trinitrobenzene sulfuric acid (32).
to external stimuli, including LPS and TNF-α. ROS, such as H2O2, induces NF-κB activation by tyrosine phosphorylation of Syk, followed by IKK activation (40), and downregulates the activity of AP-1 (41). The antioxidants pyrrolidine dithiocarbamate and N-acetyl-cysteine, which inhibit NF-κB activation, enhance DNA binding and transactivation of AP-1 in response to phorbol ester (42). Hirata et al. (43) reported that pyrrolidine dithiocarbamate suppressed DSS-induced TNF-α production and NF-κB activation. These studies raised the possibility that NF-κB and AP-1 activities are regulated by mutual negative feedback mechanisms in stress responses, including those during DSS-induced colitis. Curiously, mutual negative regulation between AP-1 and NF-κB has been studied; the NF-κB and AP-1 have been shown for discussion, and Mayako Asakawa for technical support.

Disclosures
The authors have no financial conflicts of interest.

References

FIGURE 7. DSS-induced colitis in Fra-1 Tg mice. Wt (n = 3) and Fra-1 Tg mice (n = 4) were administered 2% DSS in drinking water. A, Survival curve after DSS administration. Wt (n = 3) and Fra-1 Tg mice (n = 3) were administered 2% DSS in drinking water. B, Percentage weight changes. Initial weight of each mouse is set as 100%. Data are means ± SEM. p < 0.05. C, Diarrhea score. p < 0.05. D, Bleeding score. p < 0.05. E, Percentage of colon shortening in mice treated with 2% DSS for 13 d (wt, n = 3, Fra-1 Tg, n = 4) relative to colon length of untreated mice (wt, n = 3, Fra-1 Tg, n = 3). Data are means ± SEM. p < 0.05 versus DSS-treated wt mice. F, Histological section of colon. H&E staining. Scale bar, 200 μm. The inflammation score was evaluated by histological grading of colitis. G, Cytokine production in serum analyzed by ELISA. Sera were prepared from mice untreated (wt, n = 3, Fra-1 Tg, n = 3) or treated with 2% DSS for 13 d (wt, n = 3, Fra-1 Tg, n = 4). Data are means ± SEM. p < 0.05 versus DSS-treated wt mice. H, NF-κB activation in colon fragments without DSS treatment (wt, n = 2, Fra-1 Tg, n = 3) or with DSS treatment (wt, n = 2, Fra-1 Tg, n = 4). Nuclear proteins were then prepared and subjected to EMSA for NF-κB activation. p < 0.05 versus DSS-treated wt mice. I, MAPK activation in colon fragments without or with DSS treatment (wt, n = 2, Fra-1 Tg, n = 2 for each group). Whole-cell extract was prepared and subjected to Western blot analysis.

Acknowledgments
We thank Toru Yamaguchi for producing Fra-1 Tg mice, Kenta Maruyama for discussion, and Mayako Asakawa for technical support.

Downloaded from http://www.jimmunol.org/ by guest on October 23, 2017


Supplemental Figure 1
Cytokine production by colon ex vivo. Colon fragments were prepared in triplicate from mice untreated (wt, n=4, Fos-; n=6) or treated with 2% DSS for 7 days (wt, n=4, Fos-; n=6). The fragments were washed and incubated in culture medium supplied with antibiotics for 16 h. Supernatants were subjected to ELISA for TNF-α and IL-6. Values were normalized by total protein. Data are means ± SEM.
*p<0.05 vs. DSS-treated wt mice.
Supplemental Figure 2
Cytokine production by colon ex vivo. Colon fragments were prepared in triplicate from wild-type mice (n=3). The fragments were washed and stimulated by 1 μg/ml LPS in culture medium supplied with antibiotics for 16 h. Supernatants were subjected to ELISA for TNF-α and IL-6. Values were normalized by total protein. Data are means ± SEM. *p<0.05 vs. DSS-treated wt mice.