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Research Article

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# Formylpeptide receptor-2 contributes to colonic epithelial homeostasis, inflammation, and tumorigenesis

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**Commensal bacteria and their products provide beneficial effects to the mammalian gut by stimulating epithelial cell turnover and enhancing wound healing, without activating overt inflammation. We hypothesized that *N*-formylpeptide receptors, which bind bacterial *N*-formylpeptides and are expressed by intestinal epithelial cells, may contribute to these processes. Here we report that formylpeptide receptor-2 (FPR2), which we show is expressed on the apical and lateral membranes of colonic crypt epithelial cells, mediates *N*-formylpeptide-dependent epithelial cell proliferation and renewal. Colonic epithelial cells in *FPR2*-deficient mice displayed defects in commensal bacterium-dependent homeostasis as shown by the absence of responses to *N*-formylpeptide stimulation, shortened colonic crypts, reduced acute inflammatory responses to dextran sulfate sodium (DSS) challenge, delayed mucosal restoration after injury, and increased azoxymethane-induced tumorigenesis. These results indicate that FPR2 is critical in mediating homeostasis, inflammation, and epithelial repair processes in the colon.**

## Introduction

Ulcerative colitis (UC) is associated with an elevated risk for colorectal cancer (1). It is believed that the chronic inflammatory process associated with UC is responsible for the neoplastic transformation of the intestinal epithelium (2). Proinflammatory cytokines and chemokines, such as TNF- $\alpha$ , IL-1, IL-6, and CXCL8, as well as matrix-degrading enzymes, growth factors, and ROS present in the tissue microenvironment of UC, enhance epithelial cell proliferation, abnormal cell turnover, leukocyte infiltration, and angiogenesis, culminating in tumorigenesis (3, 4). The association of UC with cancer involves inflammation of the submucosa of the colon, induced by direct contact with the intestinal microflora; the resulting inflammation promotes tumor growth in the overlying epithelium (5). Therefore, the capacity of the intestinal epithelial layer to cope with the intestinal flora is important not only for limiting inflammation, but for preventing tumorigenesis in the colon as well.

The microbiota in the colon is separated from the host compartment by a single layer of epithelial cells, which are able to respond to and control the threats from commensals (6, 7). Transmembrane and intracytoplasmic receptors, such as TLRs and related Nod proteins, are designated "pattern recognition receptors" (PRRs) that recognize conserved structural motifs of a wide range of microbes, termed "microbe-associated molecular patterns" (MAMPs). Formyl peptide receptors (FPRs), which are part of the 7-transmembrane G protein-coupled chemoattractant receptors (GPCRs) and were originally detected on neutrophils and macrophages (8–11), are

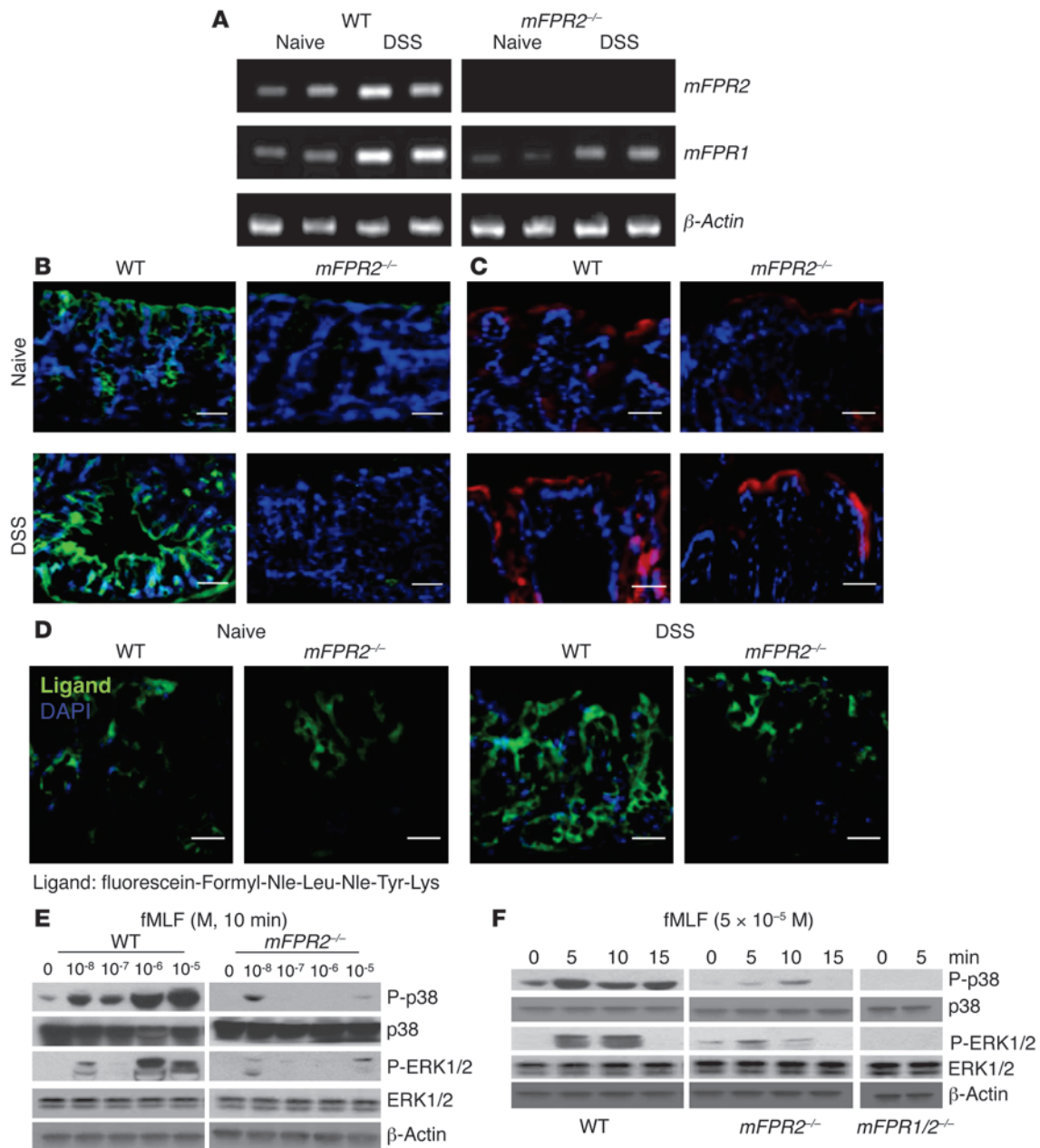
also considered a type of PRR that recognize both pathogen- and host-derived chemotactic molecular patterns (12).

In humans, there are 3 functional FPRs: FPR1, FPR2, and FPR3 (9, 11). The mouse counterparts of FPR1 and FPR2 are mFPR1 and mFPR2. The identity of mouse FPR3 is not clear. Recent studies have suggested that mFPR2 may function as both FPR2 and FPR3 (13). The best-characterized FPR ligands are *N*-formylated peptides such as *N*-formyl-methionyl-leucyl-phenylalanine (fMLF), originally identified as prokaryotic translation products. Recently, a number of host-derived formylated or nonformylated agonist peptides (FPR ligands) have been identified, including mitochondrial peptides, which play a critical role in mediating phagocyte accumulation at the sites of inflammation (9). The function of FPRs is multifaceted. Upon ligand binding, FPRs undergo a conformational change that activates pertussis toxin-sensitive (PTx-sensitive) G proteins, resulting in cell chemotaxis, transcriptional upregulation of inflammatory effectors and cytokines, activation of NADPH oxidase, and generation of ROS (12). Thus, FPRs function as PRRs that control the initial recruitment and activation of professional phagocytes to bacterial and host-derived ligands. This has been demonstrated by studies showing that *mFPR1*<sup>-/-</sup> mice are more susceptible to *Listeria* infection (14), and that *mFPR2*<sup>-/-</sup> mice have compromised innate and adaptive immune responses in allergic airway inflammation associated with defective dendritic cell recruitment into the airway and draining lymph nodes (15).

In addition to expression in phagocytes, FPR1 has been shown to localize along the lateral membranes of crypt epithelial cells in normal human colonic epithelium. Activation of FPR1 by fMLF promotes epithelial restitution (16). In mice, commensal bacterial lysates activate the ERK/MAPK pathway in an FPR-dependent

**Conflict of interest:** The authors have declared that no conflict of interest exists.

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**Figure 1**

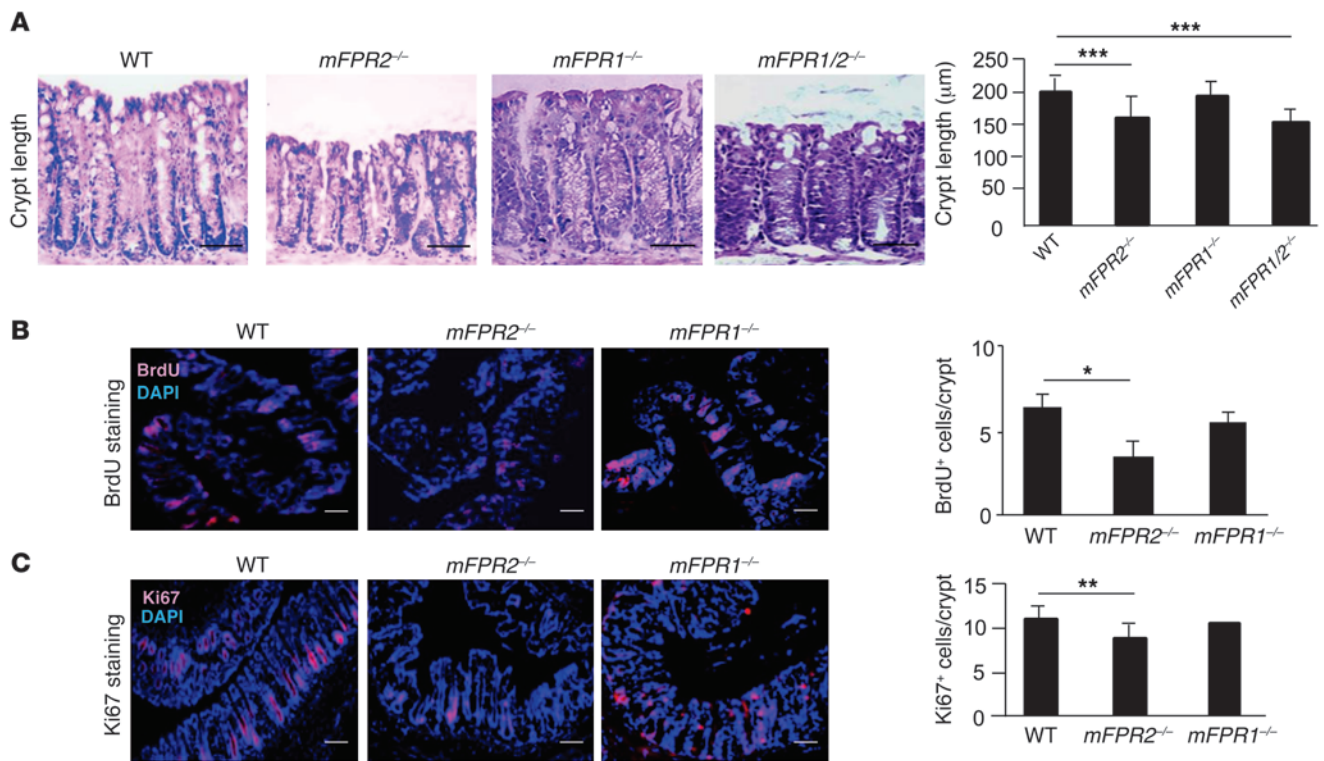
The expression of mFPR2 in epithelial cells of mouse colon. (A) *mFPR2* and *mFPR1* mRNA expression in colonic epithelial cells (composites). (B) mFPR2 protein expression in colonic epithelial cells. mFPR2 protein is in green fluorescence; nuclei are in blue (DAPI). Scale bars: 50  $\mu$ m. (C) mFPR1 protein expression in colonic epithelial cells. mFPR1 is in red fluorescence; nuclei are in blue (DAPI). Scale bars: 50  $\mu$ m. (D) Binding of fluorescein-Formyl-Nle-Leu-Nle-Tyr-Lys to colonic mucosa (green). Nuclei are in blue (DAPI). Scale bars: 100  $\mu$ m. (E and F) fMLF-induced phosphorylation of MAPKs. A 4-cm colon segment was stimulated by infusion with fMLF for indicated concentrations or time points. The colonic mucosa was scraped and lysed, and p38 and ERK1/2 phosphorylation was measured by Western blotting. (E) Effect of different doses of fMLF at 10 minutes. (F) Effect of fMLF ( $5 \times 10^{-5}$  M) at different time points. Data shown are representative of 3 independent experiments.

manner (12). However, it is not clear which of the FPRs contributes to the activation of colonic epithelial cells and their subsequent pathophysiological changes. The aim of this study was to delineate the role of FPRs in colonic epithelial homeostasis, inflammation, and colitis-associated tumor formation by using mouse strains deficient in *mFPR1*, *mFPR2*, or both receptors. Here we report that mFPR2, rather than mFPR1, plays a major role in mediating

colonic epithelial responses to bacterial ligands that are critical for inflammation, mucosal repair, and tumorigenesis.

**Results**

*Expression of functional mFPR2 on mouse colonic epithelia.* Previous studies have shown that commensal bacterial products activate the ERK/MAPK pathway in the mouse colonic epithelial layer in



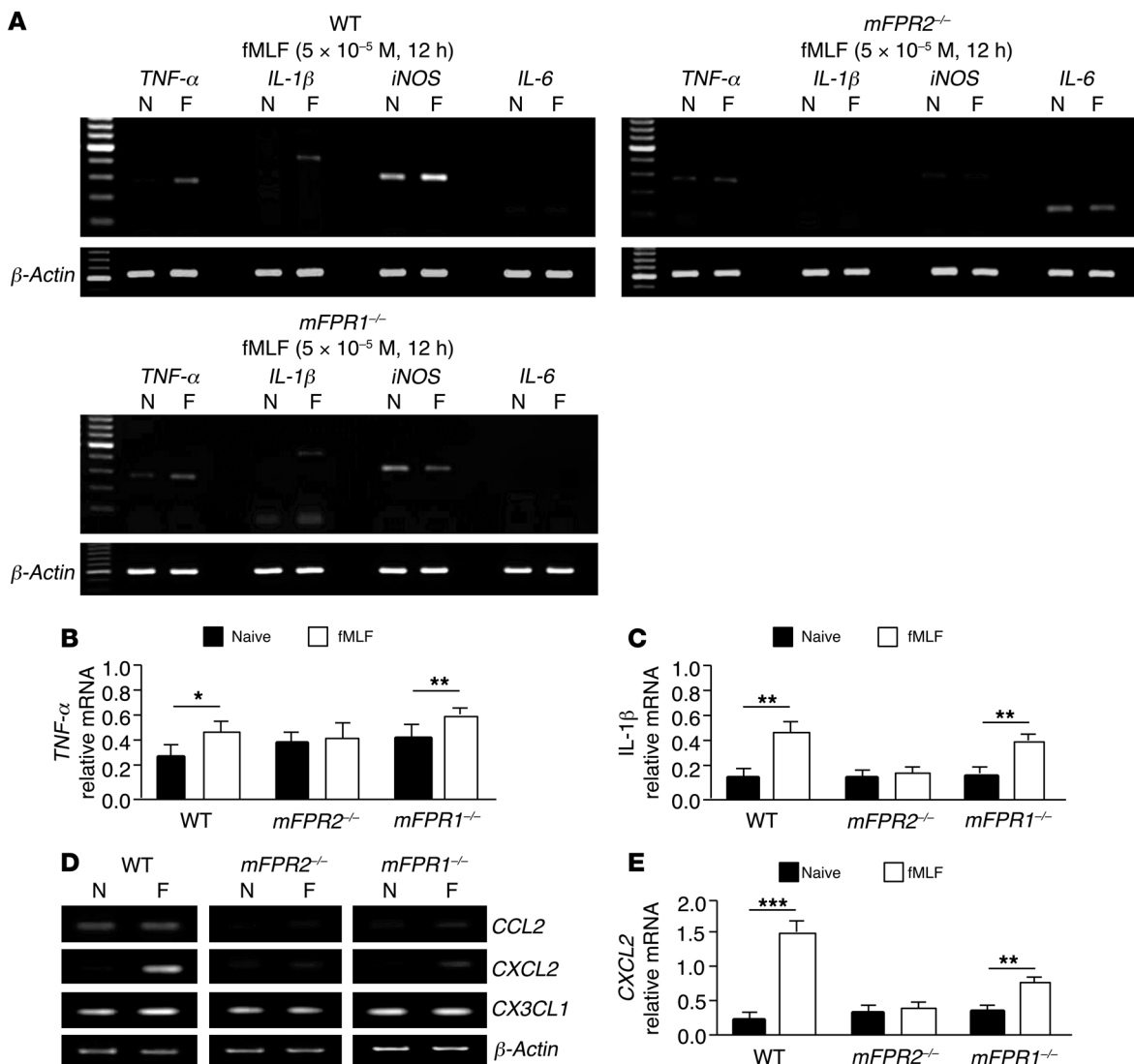
**Figure 2**

Proliferation of epithelial cells in mouse colon. (A) Reduced length of colonic crypts in *mFPR2*<sup>-/-</sup> mice. H&E-stained sections of colons from naive WT, *mFPR2*<sup>-/-</sup>, *mFPR1*<sup>-/-</sup> and *mFPR1/2*<sup>-/-</sup> mice. Scale bars: 50 µm. Right panel: Cumulative measurement of colonic crypt length. (B) DNA synthesis in colonic epithelial cells. BrdU<sup>+</sup> cells are in red; nuclei are in blue (DAPI). Scale bars: 100 µm. Right panel: Cumulative number of BrdU<sup>+</sup> cells in the mouse colonic crypts. (C) Ki67 staining of colonic epithelial cells. Ki67<sup>+</sup> cells are in red; nuclei are in blue (DAPI). Scale bars: 100 µm. Right panel: Cumulative number of Ki67<sup>+</sup> cells in the colonic crypts. Results are expressed as the mean ± SEM; *n* = 5 mice per group. \**P* < 0.05; \*\**P* < 0.01; \*\*\**P* < 0.001.

an FPR-dependent manner (12). To test the involvement of mFPR1 and mFPR2 in colonic mucosal responses, we examined the expression and function of mFPRs in mouse colon. *mFPR1* and *mFPR2* mRNAs were expressed in the colonic mucosa of WT mice, and their expression was upregulated after mice were given dextran sulfate sodium (DSS) in drinking water (Figure 1, A and B). mFPR2 was selectively expressed in normal colonic crypt epithelial cells of WT mice and was located in the apical and lateral membranes of the cells. During inflammation, the expression of mFPR2 was increased in immature as well as mature epithelial cells of the colonic crypts in WT mice (Figure 1B and Supplemental Figure 1, A and B; supplemental material available online with this article; doi:10.1172/JCI65569DS1). mFPR1 was also detected in crypt epithelial cells of WT and *mFPR2*<sup>-/-</sup> mice, and was increased after mice were given DSS in drinking water (Figure 1, A and C). In addition, the binding of fluorescein-Formyl-Nle-Leu-Nle-Tyr-Lys to colonic mucosa was enhanced in WT but not in *mFPR2*<sup>-/-</sup> mice (Figure 1D) after DSS intake, suggesting that mFPR2 plays a major role in recognizing fMLF, an agonist peptide produced by commensal bacteria in the colon. Furthermore, we detected mFPR2 expression, which was also mainly on the apical and lateral membrane surfaces of gastric (Supplemental Figure 1, C and D) and duodenal epithelial cells (Supplemental Figure 1, E and F), suggesting that mFPR2 may have an important biological function in a large part of the digestive tract (17).

To address whether DSS directly regulates the expression of mFPR2 in colonic epithelial cells, we used the murine cell line CT26, in which DSS treatment rapidly and significantly enhanced the expression of *mFPR2* mRNA (Supplemental Figure 2A). DSS treatment failed to induce the expression of TNF-α, IL-6 (Supplemental Figure 2, B and C), and IL-1β (data not shown). However, TNF-α, IL-6, and IL-1β were able to increase *mFPR2* mRNA expression by CT26 cells (Supplemental Figure 2, D-F). These results suggest that DSS has a direct effect on mFPR2 expression by colonic epithelial cells that is not dependent on induction of proinflammatory cytokines. However, at later stages of inflammatory responses, locally produced cytokines could also enhance mFPR2 expression by colonic epithelial cells. We then examined the signaling of colonic mucosa in response to fMLF stimulation. fMLF infused into the isolated mouse colon induced the phosphorylation of p38 and ERK1/2 in WT mouse gut epithelium with a more potent effect at higher fMLF concentrations. There was no effect on the colon of *mFPR2*<sup>-/-</sup> mice (Figure 1E). In contrast, phosphorylation of p38 and ERK1/2 was detected in the colon of *mFPR2*<sup>-/-</sup> mice after stimulation with a low concentration of fMLF, indicating the presence of functional mFPR1, which is a high-affinity receptor for fMLF (Figure 1E). Time-course experiments showed that a high concentration of fMLF, optimal for mFPR2, but beyond the optimal dose range for mFPR1, induced rapid phosphorylation of p38 and ERK1/2 in the colonic

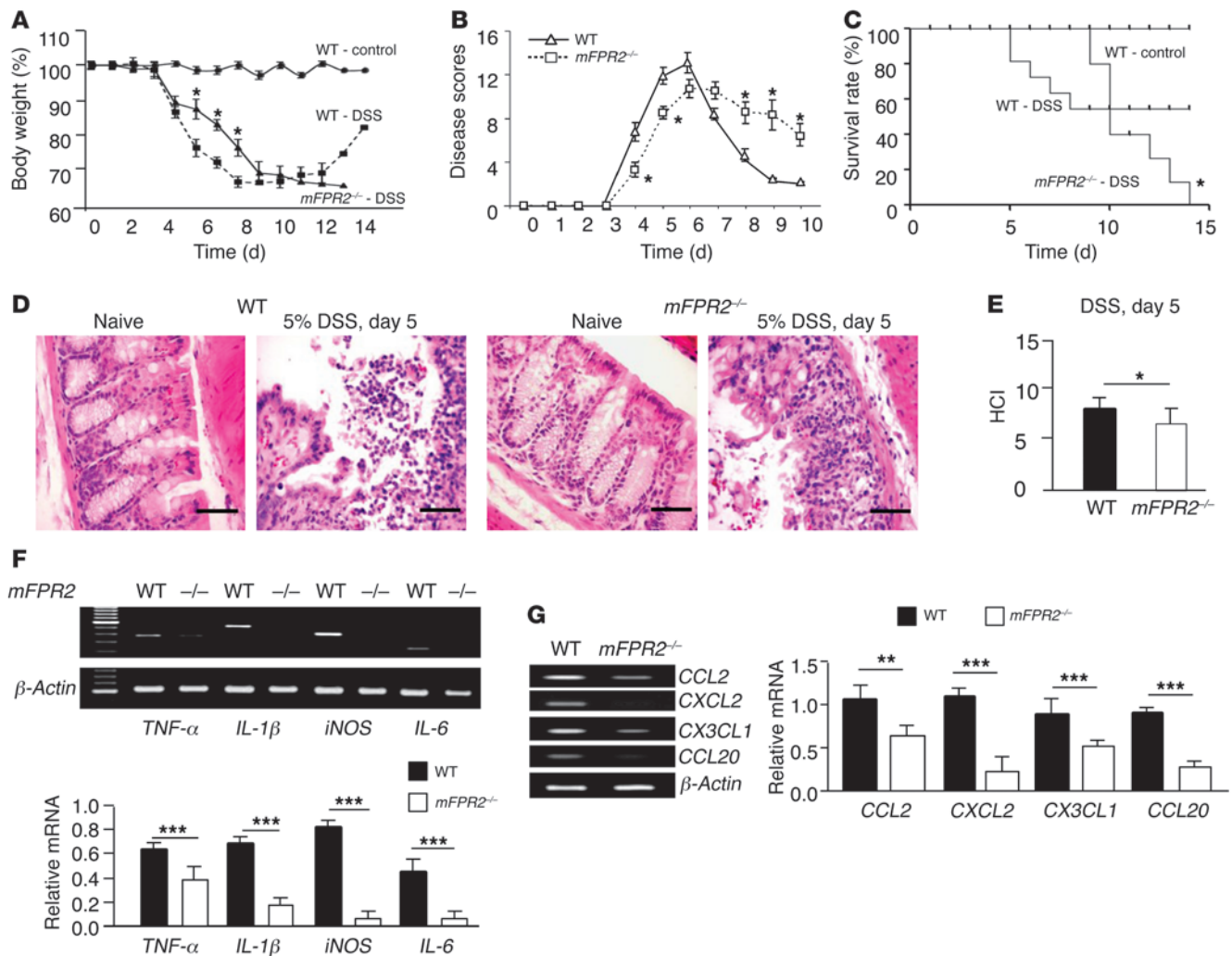


**Figure 3**

Expression of cytokine and chemokine mRNA in mouse colonic mucosa induced by fMLF. (A–E) Mice were administered fMLF ( $5 \times 10^{-5}$  M) intrarectally. After 12 hours, the expression of mRNA for cytokines, iNOS, chemokines, and  $\beta$ -actin by mouse colonic epithelial cells was examined with RT-PCR ( $\beta$ -actin blot in A is a composite). Results are expressed as the mean  $\pm$  SEM (B, C, and E);  $n = 5$  mice per group. \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ . Data shown are representative of 5 independent experiments.

mucosa of WT mice, but this response was markedly reduced in the colon of  $mFPR2^{-/-}$  mice. No phosphorylation of ERK1/2 or p38 was observed in the colon of  $mFPR1/2^{-/-}$  mice (Figure 1F). Results showing the absence of ERK1/2 phosphorylation induced by 100 nM fMLF in  $mFPR2^{-/-}$  mice could be due to the faster kinetics of the mFPR1 response combined with the lack of response of mFPR2 to the same concentration of fMLF. Also, such a dose may have caused a “desensitizing” effect on mFPR1, as observed in other biphasic biological responses of the receptor (9, 11). These results were consistent with the pattern of fMLF binding and suggest a major role for mFPR2 in mediating colonic epithelial cell response to a commensal bacterial product. The ability of colonic epithelial cells to respond to fMLF by MAPK phosphorylation was confirmed immunochemically in mouse colon (Supplemental Figure 3, A and B).

We next examined the role of mFPR2 in the homeostasis of colonic epithelium, in single or double mFPR-deficient mice. The colonic crypts of  $mFPR2^{-/-}$  mice were significantly shorter compared with those of the WT and  $mFPR1^{-/-}$  mice (Figure 2A).  $mFPR1/2^{-/-}$  mouse colons also showed a shortened crypt length similar to that of the  $mFPR2^{-/-}$  colons. The shortening of  $mFPR2^{-/-}$  mouse colonic crypts was consistent with the reduced proliferation of epithelial cells, as measured by reduced incorporation of intrarectally infused BrdU for indication of replicating cells. BrdU-positive cells were mainly located in the areas of the crypts that contain transit-amplifying cells, and the number of such cells was significantly reduced in the colons of  $mFPR2^{-/-}$  mice compared with those of the WT or  $mFPR1^{-/-}$  mice (Figure 2B).  $mFPR2^{-/-}$  mouse colonic epithelia also contained a reduced number of Ki67-positive cells (Figure 2C). These results indi-



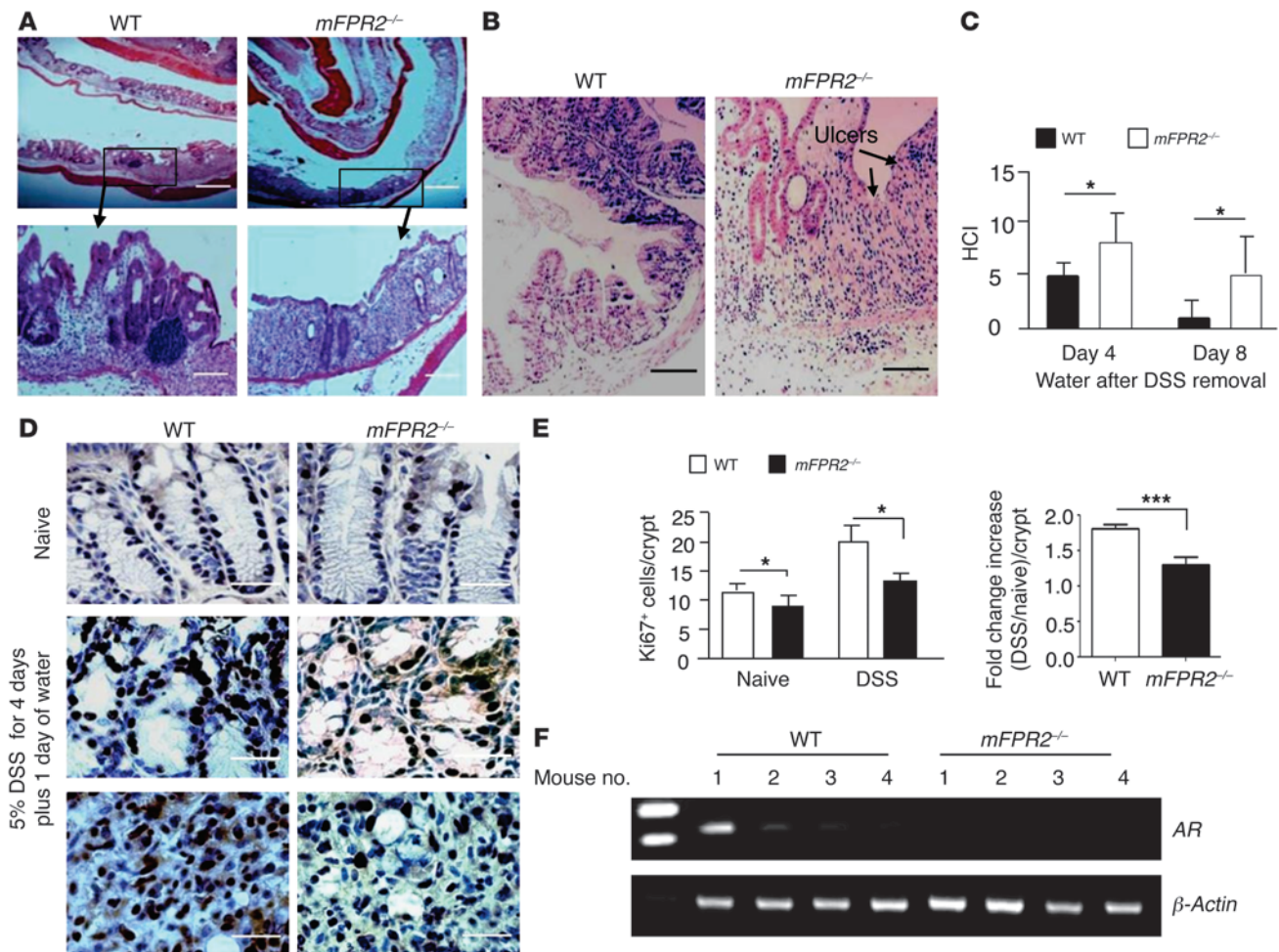
**Figure 4**  
 DSS-induced mouse acute colitis. Mice were given 5% DSS in drinking water for 5 days followed by normal water. **(A)** Body weight. \* indicates significantly reduced body weight in WT mice compared with *mFPR2*<sup>-/-</sup> mice; *n* = 12 mice per group. *P* < 0.05. **(B)** Disease scores. \* indicates a significant difference in *mFPR2*<sup>-/-</sup> mice compared with WT mice; *n* = 12 mice per group. *P* < 0.05. **(C)** Death rate of DSS-treated mice. \* indicates significantly increased death rate shown by *mFPR2*<sup>-/-</sup> mice compared with WT mice after DSS intake; *n* = 9–18 mice per group. *P* < 0.001. **(D)** H&E-stained sections of colon treated with DSS for 5 days. Scale bars: 25  $\mu$ m. **(E)** Histopathological change index (HCI). \**P* < 0.05. **(F)** Expression of cytokine mRNA in mouse colonic mucosa. Upper panel: Expression of cytokine, iNOS, and  $\beta$ -actin mRNA (blot is a composite). Lower panel: Cumulative results of relative mRNA levels of cytokines and iNOS (*n* = 5). Results are expressed as the mean  $\pm$  SEM. \*\*\**P* < 0.001. Experiments were repeated 3 times and results from representative experiments are shown. **(G)** Expression of chemokines in mouse colonic mucosa. Left panel: Chemokine mRNA expression. Right panel: Cumulative results of relative mRNA levels of chemokines. Results are expressed as the mean  $\pm$  SEM; *n* = 5 mice per group. \*\**P* < 0.01; \*\*\**P* < 0.001. Experiments were repeated 3 times and results from representative experiments are shown.

cate that the epithelial cells in the *mFPR2*<sup>-/-</sup> mouse colon are in a hypoproliferative state.

Since mFPR2 is also expressed in myeloid cells to mediate cell chemotaxis and activation, we investigated the contribution of mFPR2 expressed in myeloid cells to colonic epithelial homeostasis by using mice with myeloid cell-specific deletion of *mFPR2*. We found that the length of colonic crypts in mice with myeloid cell-specific deletion of *mFPR2* was comparable to that of the WT littermates (Supplemental Figure 3, C and D). Furthermore, we analyzed intestinal epithelial cell-specific deletion of *mFPR2* by crossing *Fpr2*-floxed mice with villin-Cre mice. The colonic mucosal crypt length of epithelia in the *mFPR2*-deletion mice was significantly shorter

than that observed in the control mice, phenocopying the systemic-deletion mice and confirming a role for mFPR2 in maintaining colonic epithelial homeostasis (Supplemental Figure 4, A and B).

In investigating the direct activity of fMLF on FPR2 (or mFPR2) expressed by colonic epithelial cells, we found that fMLF enhanced the migration and proliferation of the human colon cancer cell line T84, and the effect was blocked by the FPR antagonist Boc2 (Supplemental Figure 5, A–C). fMLF also activated p38 and ERKs and MAPKs in this cell line (Supplemental Figure 6A). fMLF-induced cell proliferation was inhibited by SB 203580, a p38 inhibitor (Supplemental Figure 6, B and C). These results confirm the function of mFPR2 expressed by colonic epithelial cells.



**Figure 5**

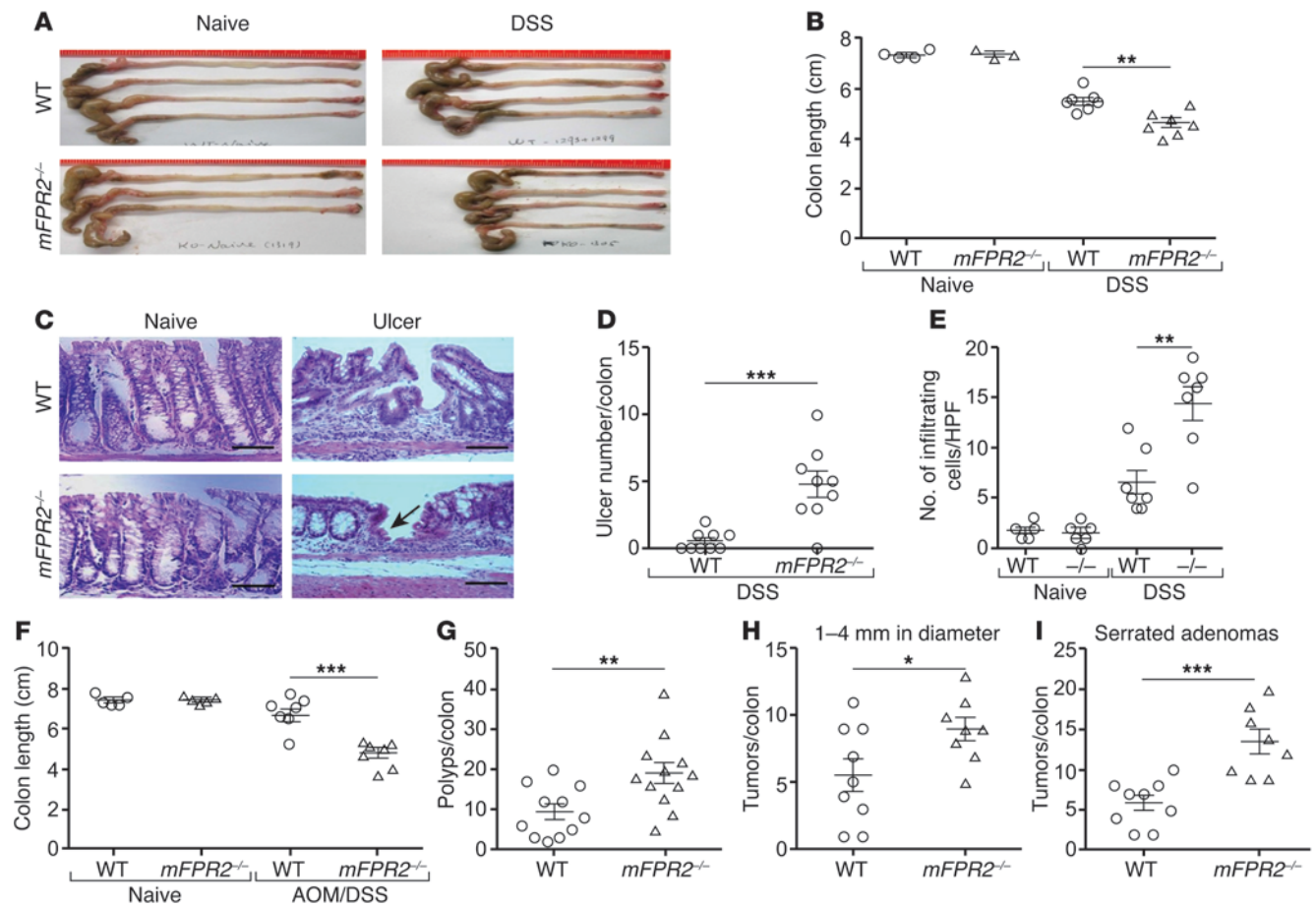
Impaired epithelial restitution in the colons of *mFPR2*<sup>-/-</sup> mice in DSS-induced acute colitis. (A) H&E-stained sections of colon from mice treated with 5% DSS for 4 days followed by normal water for 4 days. Scale bars: 200  $\mu$ m and 100  $\mu$ m, respectively. (B) H&E-stained sections of colon from mice treated with 5% DSS for 4 days followed by normal water for 8 days. Arrows indicate ulcers. Scale bars: 100  $\mu$ m. (C) HCl; *n* = 8 mice per group. \**P* < 0.05. (D) Ki67<sup>+</sup>-stained colonic crypts of mice treated with 5% DSS for 4 days followed by normal water for 1 day. Scale bars: 50  $\mu$ m. Ki67<sup>+</sup> cells are shown in brown. (E) Left panel: Cumulative number of Ki67<sup>+</sup> cells in the colonic crypts of naive and DSS-treated mice. Right panel: Fold change increase of Ki67<sup>+</sup> cells in the colonic crypts of DSS-treated mice versus naive mice. Results are expressed as the mean  $\pm$  SEM; *n* = 5 mice per group. \**P* < 0.05; \*\*\**P* < 0.001. (F) mRNA for AR in the colonic mucosa; *n* = 5 mice per group. Data shown are representative of 3 independent experiments.

*mFPR2* mediates the expression of proinflammatory cytokines by colonic mucosa. fMLF, a product of intestinal bacteria such as *E. coli* (18), is present at high (in the micromolar range) concentrations in the colon (19) and is implicated in the initiation and perpetuation of inflammatory bowel disease (IBD) (20, 21). Since TNF- $\alpha$ , IL-1 $\beta$ , IL-6, and chemokines temporally and spatially orchestrate the development and progression of IBD (22), we examined the capacity of fMLF to stimulate the expression of cytokine and chemokine mRNA (23) in mouse colonic mucosa. After intrarectal infusion of fMLF (24, 25), mRNAs for TNF- $\alpha$  and IL-1 $\beta$  were markedly increased in the colonic mucosa of WT mice, but not in that of *mFPR2*<sup>-/-</sup> mice (Figure 3, A–C). Interestingly, TNF- $\alpha$  and IL-1 $\beta$  mRNA were also upregulated by fMLF in the colonic mucosa of *mFPR1*<sup>-/-</sup> mice (Figure 3, A–C), suggesting that the effect of fMLF was mainly mFPR2 dependent. We further found that the expression of mRNA for the neutrophil-specific chemokine CXCL2 was

upregulated by fMLF in the colonic mucosa of WT mice, but not in that of *mFPR2*<sup>-/-</sup> mice. In the colonic mucosa of *mFPR1*<sup>-/-</sup> mice, fMLF was still able to increase the expression of CXCL2 mRNA, albeit at a lower level compared with its expression in WT mice (Figure 3, D and E). These results indicate that mFPR2 is the major mediator of fMLF-induced expression of mRNAs for proinflammatory cytokines and chemokines in the colonic mucosa.

*mFPR2* contributes to colonic mucosal inflammation. Having observed that mFPR2 is required to maintain the homeostasis of mouse colonic mucosa, we examined the effect of *mFPR2* deficiency on DSS-induced colitis. After intake of 5% DSS in drinking water, rectal bleeding in *mFPR2*<sup>-/-</sup> mice was markedly less severe than in the WT mice (Supplemental Figure 7, A and B). In addition, during the early phase of colitis, *mFPR2*<sup>-/-</sup> mice showed a delay in the loss of body weight (Figure 4A) and exhibited reduced colon disease scores as well as reduced mortality compared with WT





**Figure 6**

DSS-induced chronic colonic inflammation and chronic colitis-associated tumorigenesis. (A–E) Mice were given 5% DSS in drinking water for 3 days followed by normal water for 15 days for 5 cycles. (A) Length of mouse colons. (B) Cumulative measurement of colon length. Results are expressed as the mean ± SEM; *n* = 7 mice per group. (C) Number of ulcers in the colons from WT or *mFPR2*<sup>-/-</sup> mice. Scale bars: 50 μm. (D) Cumulative number of ulcers in the colon. (E) Cumulative numbers of cells infiltrating the area surrounding the ulcers. Results are expressed as the mean ± SEM; *n* = 5–7 mice per group (D and E). Mean values were calculated from each mouse in different areas in the colon. \**P* < 0.05; \*\**P* < 0.01; \*\*\**P* < 0.001 (B, D, and E). (F–I) Increased tumorigenesis in the colons of *mFPR2*<sup>-/-</sup> mice with chronic colitis. Mice treated with AOM were given 2.5% DSS for 1 week followed by 2 weeks of regular drinking water. DSS and regular water treatment were repeated for 3 additional cycles. (F) Cumulative measurement of colon length after AOM and DSS treatment. (G) Cumulative number of tumors per colon (macroscopic view), and (H) cumulative numbers (mean ± SEM) of tumors per colon (1–4 mm in diameter) in WT and *mFPR2*<sup>-/-</sup> mice. (I) Number of serrated adenomas per colon; *n* = 8–9 mice per group (H and I). \**P* < 0.05; \*\**P* < 0.01; \*\*\**P* < 0.001 (F–I).

mice (Figure 4, B and C). After DSS removal, the disease scores of WT mice were significantly improved compared with those of the *mFPR2*<sup>-/-</sup> mice up to day 10 (Figure 4B). On day 8, the surviving WT mice started to regain body weight, but it remained lower than that of the *mFPR2*<sup>-/-</sup> mice until day 10, consistent with the observation that changes in body weight lag behind the changes in disease scores. However, *mFPR2*<sup>-/-</sup> mice showed accelerated body weight loss by day 8 (Figure 4A), with deteriorated disease scores compared with WT mice (Figure 4B). All *mFPR2*<sup>-/-</sup> mice died by day 14, while more than 50% of the WT mice survived (Figure 4C).

Histological analysis showed that DSS treatment caused early recruitment of a significantly higher number of leukocytes, mainly neutrophils and macrophages, into the mucosal region of the colon of WT mice compared with *mFPR2*<sup>-/-</sup> mice (Figure 4, D and E, Supplemental Figure 8, A and B, and Supplemental Figure 9, A and B). Given the importance of proinflammatory cytokines and

chemokines in the development and exacerbation of IBD (22), we measured cytokine and chemokine mRNA expression in the mouse colonic mucosa after DSS treatment. The mRNA levels for *TNF-α*, *IL-1β*, *IL-6*, *iNOS*, *CCL2*, *CXCL2*, *CX3CL1*, and *CCL20* were markedly reduced in *mFPR2*<sup>-/-</sup> mice (Figure 4, F and G). Thus, *mFPR2* deficiency is accompanied by reduced acute inflammatory responses in the mouse colon. However, during the later phase of colitis, while WT mice showed stabilized body weight loss, improved mucosal disease scores, and a substantial level of survival, *mFPR2*<sup>-/-</sup> mice continued to lose body weight and had much slower improvement of colon disease scores in association with markedly increased mortality (Figure 4, B and C).

We also characterized the phenotype of myeloid-specific *mFPR2*-deletion mice in DSS-induced colitis. In acute colitis, while myeloid *mFPR2* deletion (*LysMCre*<sup>+</sup>*mFPR2*<sup>fllox/fllox</sup> mice) resulted in moderately reduced disease scores in mice (Supplemental Figure 10A),





with no significant differences in weight loss or mortality (Supplemental Figure 10, B and C) compared with their WT littermates (LysMCre<sup>+</sup>mFPR2<sup>fllox/fllox</sup> mice versus LysMCre<sup>-</sup>mFPR2<sup>fllox/fllox</sup>). Colon histology revealed reduced inflammation and myeloid cell recruitment in the mucosa of myeloid mFPR2-deletion mice. However, their colonic mucosa underwent a recovery similar to that of the WT mice after removal of DSS from the drinking water (Supplemental Figure 11, A and B). These results indicate that mFPR2 expressed in myeloid cells contributes to the acute inflammatory responses in the colon, but the effect on epithelial cell homeostasis and repair was limited.

**Involvement of mFPR2 in commensal bacterium-induced colonic inflammation and mucosal injury after DSS injury.** Commensal bacteria are major mediators of colitis after the disruption of mucosal integrity. We therefore investigated the capacity of mFPR2 to mediate the detrimental effect of commensal bacteria on injured colonic mucosa. Phosphorylation of MAPKs in colonic epithelia of WT mice was induced by i.p. injection of fMLF, which was inhibited by i.p. injection of the FPR antagonist Boc2. Thus, mFPR2 expressed on the lateral membranes of epithelial cells of colonic crypts also appears to be functional in response to systemically administered ligands (Supplemental Figure 12, A and B). With i.p. injection of Boc2 (26, 27), we found that leukocyte infiltration, crypt injury (Supplemental Figure 12, C–E), and CXCL2 production (Supplemental Figure 12F) in the colonic mucosa after DSS intake were reduced in WT mice, suggesting that pharmacological blockade of mFPRs resembles the effect of mFPR2 deficiency on the course of colonic inflammation.

To verify the contribution of mFPR2 in commensal bacterium-mediated inflammation in DSS-injured colon, commensal bacteria were depleted by treating WT mice with an antibiotic cocktail, which resulted in a greater than 96% reduction in colonic flora (Supplemental Figure 13A and ref. 5). This antibiotic cocktail was followed by drinking water containing 5% DSS. Mice treated with antibiotics showed reduced inflammatory responses in the colon, with fewer leukocytes infiltrating the mucosa after DSS intake, similar to the changes observed in mFPR2<sup>-/-</sup> mice in acute DSS-induced colitis (Supplemental Figure 13, B and C). Quantification of Ki-67-positive cells showed that compared with untreated WT mice, there was reduced proliferation of colonic epithelial cells in commensal-depleted WT mice in response to initial injury by DSS. This was comparable to the changes observed in the colons of mFPR2<sup>-/-</sup> mice without commensal depletion (Supplemental Figure 13D). These results suggest that DSS-induced acute colitis is likely to be elicited by commensal bacteria with the participation of mFPR2.

**Impaired epithelial proliferation in the colon of mFPR2<sup>-/-</sup> mice in DSS-induced acute colitis.** Since mFPR2 deficiency is associated with deleterious colon pathology and increased mortality in the postacute phase of colitis with impaired growth of crypts, we investigated mucosal repair in mFPR2<sup>-/-</sup> mice after DSS treatment. Four days of 5% DSS treatment followed by 4 days of water resulted in a marked reduction in colonic epithelial cell proliferation in mFPR2<sup>-/-</sup> mice compared with WT mice. In addition, while the colonic mucosa of WT mice showed progressive healing after 8 days of drinking water, the mucosal layer of mFPR2<sup>-/-</sup> mouse colon showed a much delayed restitution (Figure 5, A–C).

By using Ki-67 as a marker for proliferating epithelial cells (28), we detected increased proliferative epithelial cells in the crypts of mFPR2<sup>-/-</sup> mouse colon compared with WT mouse colon (Figure 5D). Quantification of Ki-67-positive cells demonstrated hyperprolif-

eration in the colon of WT mice in response to initial injury. In contrast, epithelial cells in the colons of mFPR2<sup>-/-</sup> mice 4 days after DSS treatment failed to proliferate and did not exceed the number found in the colons of mice receiving water alone (Figure 5E). Furthermore, the expression of mRNA for amphiregulin (AR), an EGF/TGF- $\alpha$  receptor agonist that promotes the growth of normal epithelial cells, was reduced in the colonic mucosa of mFPR2<sup>-/-</sup> mice after DSS treatment (Figure 5F). These results indicate that mFPR2 deficiency impaired the normal growth of colonic crypts as well as their restoration after acute injury. This conclusion is supported by results obtained in mice treated with DSS for 3 days followed by normal water for 15 days over 5 cycles, in which the colons of mFPR2<sup>-/-</sup> mice were significantly shorter than those of their WT littermates (Figure 6, A and B). The number of ulcers in the colonic mucosa was increased in mFPR2<sup>-/-</sup> mice compared with WT mice (Figure 6, C and D), and there was more inflammatory cell infiltration under ulcers in the colonic mucosa of mFPR2<sup>-/-</sup> mice than in WT mice (Figure 6E). The thickness of the muscularis propria was in general increased in the colons of mice with chronic inflammation. However, the increased thickness of the muscularis propria in mFPR2<sup>-/-</sup> mice was greater than in WT mice (Supplemental Figure 14, A and B). These results confirm that mFPR2 deficiency impairs restoration of the colonic epithelial layer after injury.

**Increased tumorigenesis in mFPR2<sup>-/-</sup> mouse colon.** Since the linkage between chronic inflammation and cancer has been well established (1, 2), we examined the impact of mFPR2 deficiency on colitis-associated tumorigenesis. Mice were injected with AOM followed by administration of DSS in drinking water for 3 cycles (29). Consistent with the results observed in chronic colitis, the colon length in mFPR2<sup>-/-</sup> mice was significantly reduced compared with the colon length in WT mice (Figure 6F and Supplemental Figure 15A). This was associated with a markedly increased number of adenomas in the mFPR2<sup>-/-</sup> mouse colon (Figure 6G and Supplemental Figure 15, B and C). mFPR2 was detected on adenoma cells from WT mouse colon (Supplemental Figure 15D). Histological examination revealed 2 major types of adenomas in the colons of AOM/DSS-treated mice, as well as hyperplastic polyps and serrated adenomas (Supplemental Figure 15, E and F), the latter having a higher rate of malignant transformation (30). In mFPR2<sup>-/-</sup> mice colons, the number of large tumors (1–4 mm in diameter) and serrated adenomas was significantly higher than in the WT littermate colons (Figure 6, H and I). Thus, mFPR2 deficiency increased the susceptibility to chronic inflammation-associated tumors in the mouse colon.

## Discussion

The innate immune system provides first-line defenses against invading microbial organisms and endogenous danger signals by activating pathways that result in inflammation, microbial clearance, and tissue repair. This is especially important in the intestinal mucosa in which PRRs protect against microbial intrusion and serve to maintain epithelial barriers in the presence of commensal microorganisms. Bacterial sensing by PRRs is increasingly recognized to be critical for intestinal homeostasis and injury-induced tissue repair (31, 32). Experimental animal models of IBD have demonstrated that resident luminal bacteria are necessary for the induction and perpetuation of intestinal inflammation, since genetically susceptible mice are protected from colitis when raised in germ-free conditions (33). Depletion of commensal bac-



teria (5, 34) and TLR4 antagonist Abs (35), or depletion of TLR4 (36, 37) ameliorates colonic inflammation. However, depletion of commensal bacteria or TLR adaptor protein MyD88 also results in profound defects in the ability to repair DSS-induced mucosal damage (32, 38). Similar to TLRs, NOD2 has recently been shown to protect mice from DSS-induced acute injury (39). Stimulation of TLRs or NOD2 activates MAPKs and NF- $\kappa$ B (39, 40), which are associated with colonic inflammation and epithelial regeneration. These studies suggest that these innate immune receptors that interact with commensal bacteria are essential for colonic tissue homeostasis, inflammatory responses, and repair.

In addition to TLRs and NOD2, FPRs have also been defined as PRRs based on their capacity to recognize a plethora of bacteria and host-derived agonists that are not recognized by TLRs or NODs (12). Our study shows that mFPR2 was expressed on the apical and lateral membrane of mouse colonic epithelial cells and was activated by fMLF infused into the colon lumen or by peritoneal injection of fMLF, suggesting that both apical and lateral FPR2 are functional. This property of mFPR2 may have important biological significance, as it enables the epithelial cells to respond to both locally and systemically available ligands under various pathophysiological conditions. We also found that in WT mice, mFPR2 is selectively expressed in mature epithelial cells of the colonic crypts. DSS intake increased the expression of mFPR2 in immature epithelial cells as well as in mature epithelial cells of colonic crypts. Although both mFPR1 and mFPR2 are expressed on the mouse colonic epithelial cells, our observations demonstrate that mFPR2 plays a more prominent role in maintaining the normal growth of colonic epithelial cells than mFPR1, since *mFPR2*<sup>-/-</sup> mouse colon contained significantly shortened crypts and a diminished number of proliferating epithelial cells. In contrast, although mFPR1 also has the capacity to mediate fMLF-triggered MAPK signaling in colonic epithelial cells, *mFPR1*<sup>-/-</sup> mouse colons showed crypt lengths equivalent to those found in WT mouse colons. Moreover, mice deficient in both mFPR1 and mFPR2 displayed a colonic phenotype similar to that seen in mice with an *mFPR2* single deficiency. This distinction between the roles of mFPR1 and mFPR2 in the colon may be attributable to the fact that colonic epithelial cells express higher levels of mFPR2 than mFPR1.

IBD, in particular, UC, is characterized by recurrent or chronic inflammation of the intestinal tract in the absence of a specific inciting pathogen. The histological hallmarks of UC are the invasion of crypt epithelium and lamina propria by polymorphonuclear neutrophils (PMNs), disruption of epithelial lining, and ulceration in the colon. PMNs are believed to play a pivotal role in mediating tissue damage in UC (41–43). In humans, PMN accumulation is also observed in rectal biopsies of active UC patients (44, 45). The infiltration of PMNs in inflamed colon is attributed to increased expression of the chemokine CXCL8 (46), which is detected in the colonic mucosa of UC patients (47, 48). In mice, CXCL2, a functional homolog of CXCL8 (49), is not expressed in normal colonic epithelia, but is expressed after stimulation with LPS or proinflammatory cytokines (50, 51). In addition to CXCL2, cytokines such as TNF- $\alpha$ , IL-1 $\beta$ , and IL-6 are pivotal to the development, recurrence, and exacerbation of the inflammatory process in colitis (22). Our study shows that colonic mucosal cells from naive *mFPR2*<sup>-/-</sup> mice failed to respond to stimulation by the bacterial-derived fMLF. Also, production of inflammatory cytokines and chemokines was diminished in DSS-induced colitis

in *mFPR2*<sup>-/-</sup> mice. In addition, *mFPR2*<sup>-/-</sup> mice showed diminished PMN infiltration into the colonic mucosa in acute colitis. Furthermore, depletion of commensal bacteria and administration of the FPR antagonist BOC2 reduced inflammatory responses, crypt damage, and CXCL2 production in the WT mouse colonic mucosa treated with DSS. These results suggest that a critical role exists for mFPR2 in mediating commensal bacteria-induced acute colitis following DSS stimulation and that the role of mFPR2 antecedes that of CXCL2, other chemokines, and cytokines.

It has been reported that fMLF stimulation enhanced intestinal epithelial cell restitution in vitro (16). Our present study also shows that mFPR2 is involved in colonic mucosal repair and that the absence of mFPR2 prolongs chronic inflammation and increased tumorigenesis in the colon. The importance of mFPR2 in epithelial cells was confirmed by results obtained in mice with epithelial-specific deletion of mFPR2 in which the colonic crypt length was shorter than that of their WT littermates. In contrast, although myeloid-specific mFPR2 deletion resulted in a moderate reduction of DSS-induced acute colitis, the effect on epithelial cell homeostasis and regeneration was limited. DSS-induced mouse colitis also enhances the expression of AnxA1, a putative host-derived FPR2 agonist in the intestinal epithelial cells. In *AnxA1*<sup>-/-</sup> mice, the recovery of colonic mucosa following withdrawal of DSS was also delayed (52). However, the amino terminal peptides of AnxA1 have been reported to activate mFPR1 as well as mFPR2 (9). In our study, mFPR2 plays a more important role in maintaining colonic homeostasis and inflammatory responses than mFPR1, as shown by decreased epithelial cell proliferation and shortened crypts in the naive state. In contrast, *mFPR1*<sup>-/-</sup> mice showed normal colonic crypts, while double deficiency in mFPR1 and mFPR2 resulted in a phenotype resembling mFPR2 single deficiency. Thus, mFPR2 is nonredundant in colonic mucosal homeostasis and inflammation. It also requires a proliferative burst for healing and is one of the major receptors for AnxA1 in the colon.

A critical role for mFPR2 in colonic mucosal repair is further supported by the observation that *mFPR2* deficiency markedly increases the tumor formation associated with chronic inflammation. It has been well established that chronic inflammation is a major causative factor for cancer in the intestine (1, 2). Our study shows that *mFPR2*<sup>-/-</sup> mice were more susceptible to tumorigenesis induced by the carcinogen azoxymethane (AOM), which was followed by DSS injury. We also observed activation of the  $\beta$ -catenin pathway in mouse tumors and an increased number of larger-sized adenomas with a propensity to develop into invasive tumors in *mFPR2*<sup>-/-</sup> mice. Thus, mFPR2 is a leukocyte chemoattractant receptor that not only plays an essential role in host immune responses (15), but is also involved in controlling colonic mucosal homeostasis, inflammation, and the repair of injured mucosa to prevent tumor formation.

Our study raises important new questions for future research, most importantly, whether FPR2 is a factor in human IBD and colonic neoplasia. We have found that FPR2 is functionally expressed by a human colon cancer cell line; however, whether primary human colonic epithelia and cancer cells express one or more functional FPR2s remains to be determined. Together with our previous studies in the lung (15), the present work supports a general model in which FPR2 may function not only in immune cells, but also in epithelial cells to regulate inflammation and restitution of injured mucosal surfaces. It may do this in response to distinct, locally produced ligands and in part by orchestrating cell proliferation and production of chemokines and cytokines.



## Methods

**Animals.** *mFPR1*<sup>-/-</sup> and *mFPR2*<sup>-/-</sup> mice were generated as described (14, 15). To construct a targeting vector for the generation of *mFPR1* and *mFPR2* double knockout mice (*mFPR1/2*<sup>-/-</sup>), an approximately 18-kb DNA fragment covering the *mFPR1* and *mFPR2* gene locus was first retrieved from the mouse BAC clone RP23-77H6 in the pLMJ235 vector. A 7-kb fragment containing exon 1 of the *mFPR1* gene, the promoter region located between the *mFPR1* and *mFPR2* genes, and exon 1 of the *mFPR2* gene was then replaced with a neo gene cassette. The procedures were performed using a recombinogenic cloning method. The targeting vector was subsequently electroporated into mouse ES cells for subsequent generation of *mFPR1/2*<sup>-/-</sup> mice. The neo gene was deleted by crossing to a  $\beta$ -actin Cre transgenic mouse. Myeloid-specific *mFPR2*-deletion mice were generated by crossing lysozyme-Cre (*LysMCre*) mice with *mFPR2*-floxed mice (15). Epithelial-specific *mFPR2*-deletion mice were generated by crossing villin-Cre mice with *mFPR2*-floxed mice. All mice were housed in the animal facility at Frederick National Laboratory for Cancer Research (Frederick, Maryland, USA) and were used at 8 to 12 weeks of age.

**Reagents.** An anti-*mFPR2* Ab was obtained from Novus Biologicals and goat anti-rabbit Ig-PE was obtained from Santa Cruz Biotechnology. Bromodeoxyuridine (BrdU), biotin goat anti-rabbit Ig Ab, biotin polyclonal anti-rat Ig Ab, streptavidin-PE, and streptavidin-FITC were all obtained from BD. Anti-Ki67 rabbit monoclonal Ab was from Epitomics. PE-anti-BrdU Ab (clone: Bu 20a) and biotin anti-BrdU Ab were from BioLegend. Abs against phosphorylated (P)-p38 MAPK (Thr<sup>180</sup>/Tyr<sup>182</sup>), p38, P-ERK1/2 (Thr<sup>202</sup>/Tyr<sup>204</sup>), ERK1/2, and  $\beta$ -actin were obtained from Cell Signaling Technology. Mouse CXCL2/MIP2 and CCL2/JE/MCP-1 ELISA kits were from eBioscience; fMLF was obtained from Sigma-Aldrich; and fluorescein-Formyl-Nle-Leu-Nle-Tyr-Lys was from Invitrogen.

**Induction of colitis.** To induce acute colitis, mice were administered 5% DSS (4,000 kDa, USB, Affymetrix) in drinking water. The disease activity indexes (DAIs) were defined as reported with minor modifications (ref. 53 and Supplemental Table 1). For observation of colonic mucosal recovery, animals were treated with DSS for 4 to 6 days and then were allowed to recover on normal drinking water for an additional 4 to 12 days. For the induction of chronic colitis, animals were given DSS in drinking water at a concentration of 5% (w/v) for 3 days, and then were given normal drinking water for an additional 15 days with 5 cycles of treatment.

**Tumorigenesis.** Colitis-associated tumors were induced by intraperitoneal injection of mice on day 0 with 12.5 mg/kg azoxymethane (AOM; Sigma-Aldrich). On day 7, mice were given 2.5% DSS in drinking water for 1 week, followed by 2 weeks of normal drinking water. The DSS treatment was repeated for 3 cycles. At week 17, mice were sacrificed and their colons were removed, flushed with cold PBS and cut longitudinally. Pictures were taken with a PowerShot G3 Digital Camera (Canon) and tumor size was measured with a caliper.

**Histopathology.** Colonic tissues were embedded in paraffin and stained with H&E. Histological severity of acute colitis in DSS-treated mice was determined using combined scores as reported (54), with minor modifications (Supplemental Table 2).

**BrdU labeling.** BrdU (BD) was infused intrarectally (5 mg in 100  $\mu$ l PBS) into mice. Mice were kept in an inverted position for 1 minute after each

administration to prevent leakage of BrdU from the anus. After 45 minutes, the mice were sacrificed. Colonic tissues were fixed in Tissue-Tek OCT compound and frozen sections were stained with anti-BrdU-PE or Biotin anti-BrdU Ab, followed by streptavidin-PE or streptavidin-FITC and DAPI (Invitrogen).

**RT-PCR.** An RNeasy mini kit (QIAGEN) was used to extract total mRNA from mucosal tissues that were isolated by scraping. mRNA expression of *mFPR2*, *mFPR1*, *TNF- $\alpha$* , *IL-1 $\beta$* , *IL-6*, and *iNOS* was examined by RT-PCR (Supplemental Table 3). The expression of mRNA for *CCL2*, *CXCL2*, *CCL20*, *CX3CL1*, and AR was examined with previously reported primers (35, 55). All PCR products were resolved on 1.5% agarose gel by electrophoresis and visualized after ethidium bromide staining. For quantitation, gels were scanned, and the pixel intensity for each band was determined using ImageJ software (NIH Image) and normalized to the amount of  $\beta$ -actin.

**Analysis of colonic mucosal cell responses to fMLF.** Mice were intrarectally infused with fMLF ( $5 \times 10^{-5}$  M in PBS, 100  $\mu$ l per mouse). Control mice received PBS only. All mice were euthanized 12 hours after infusion. Total RNA was extracted from mucosal tissues that were isolated via scraping for analysis of cytokine and chemokine mRNA expression. For immunoblotting, immunofluorescence, and immunohistochemistry, 4-cm colon segments were ligated at two ends and infused with fMLF in 100  $\mu$ l PBS. Colonic mucosa were then scraped and lysed. P-p38 and P-ERK1/2 were detected by immunoblotting. Colonic tissues were also frozen in OCT compound (Tissue-Tek) for staining of phosphorylated p38 and ERK1/2.

**Statistics.** Unless otherwise specified, all experiments were performed at least 3 times. Data are presented as the mean  $\pm$  SEM. A two-tailed Student's *t* test or ANOVA was used for evaluating statistical significance between testing and control groups. Kaplan-Meier analysis was used to measure mouse survival rates. A *P* value less than 0.05 was considered significant.

**Study approval.** All animals received proper care in agreement with experimental protocols approved by the Institutional Animal Care and Use Committee of Frederick National Laboratory for Cancer Research.

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