

# Formyl peptide receptor 2 regulates monocyte recruitment to promote intestinal mucosal wound repair

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**ABSTRACT:** Mucosal wound repair is coordinated by dynamic crosstalk between endogenous and exogenous mediators and specific receptors on epithelial cells and infiltrating immune cells. One class of such receptor-ligand pairs involves formyl peptide receptors (FPRs) that have been shown to influence inflammatory response and repair. Here we explored the role of murine *Fpr2/3*, an ortholog of human FPR2/receptor for lipoxin A4 (ALX), in orchestrating intestinal mucosal repair. Compared with wild-type (WT) mice, *Fpr2/3*<sup>-/-</sup> mice exhibited delayed recovery from acute experimental colitis and perturbed repair after biopsy-induced colonic mucosal injury. Decreased numbers of infiltrating monocytes were observed in healing wounds from *Fpr2/3*<sup>-/-</sup> mice compared with WT animals. Bone marrow transplant experiments revealed that *Fpr2/3*<sup>-/-</sup> monocytes showed a competitive disadvantage when infiltrating colonic wounds. Moreover, *Fpr2/3*<sup>-/-</sup> monocytes were defective in chemotactic responses to the chemokine CC chemokine ligand (CCL)20, which is up-regulated during early phases of inflammation. Analysis of *Fpr2/3*<sup>-/-</sup> monocytes revealed altered expression of the CCL20 receptor CC chemokine receptor (CCR)6, suggesting that *Fpr2/3* regulates CCL20-CCR6-mediated monocyte chemotaxis to sites of mucosal injury in the gut. These findings demonstrate an important contribution of *Fpr2/3* in facilitating monocyte recruitment to sites of mucosal injury to influence wound repair.—Birkl, D., O'Leary, M. N., Quiros, M., Azcutia, V., Schaller, M., Reed, M., Nishio, H., Keeney, J., Neish, A. S., Lukacs, N. W., Parkos, C. A., Nusrat, A. Formyl peptide receptor 2 regulates monocyte recruitment to promote intestinal mucosal wound repair. *FASEB J.* 33, 000–000 (2019). [www.fasebj.org](http://www.fasebj.org)

**KEY WORDS:** epithelium • GPCRs • FPR2 • inflammation • inflammatory bowel disease

Intestinal epithelial cells are at the interface between the gut lumen and underlying tissue compartments, creating a dynamic, selectively permeable barrier that not only plays an important role in host defense but in mediating crosstalk between the mucosal immune system and luminal antigens and microbes. Pathologic states that occur during chronic inflammation, ischemia, and mechanical injury are associated with disruption of the epithelial barrier and

mucosal ulceration. Efficient repair of the epithelium is critical in restoring the barrier and in regaining mucosal homeostasis (1–4).

Mucosal repair is a dynamic process orchestrated by a spatiotemporal network of mediators that include secreted factors that crosstalk with epithelial and immune cells (2–7). Chemoattractants, such as chemokines and cytokines, are secreted by immune cells and injured epithelial cells and signal through receptors to recruit other immune cells, thus orchestrating the inflammatory response. After an initial recruitment of neutrophils, there is an influx of monocytes to sites of mucosal injury in response to locally produced chemoattractants, such as CC chemokine ligand (CCL)2 and CCL20. In later phases of the acute inflammatory response, cells in healing wounds secrete factors that mediate active resolution of inflammation and repair (8, 9). For example, in response to bacterial LPS stimulation, IL-1 $\beta$  or TNF- $\alpha$  signaling *via* NF $\kappa$ B activation induces CCL20 expression in intestinal epithelial cells (10–13). CCL20-mediated signaling through the receptor

**ABBREVIATIONS:** ALX, receptor for lipoxin A4; APC, allophycocyanin; BM, bone marrow; CBC, complete blood cell; CCL, CC chemokine ligand; CCR, CC chemokine receptor; CXCL, chemokine (C-X-C motif) ligand; DAI, Disease Activity Index; DSS, dextran sodium sulfate; FBS, fetal bovine serum; FPR, formyl peptide receptor; PE, phycoerythrin; qPCR, quantitative PCR; Siglec, sialic acid-binding immunoglobulin-like lectin; TBP, TATA-box-binding protein; UC, ulcerative colitis; WT, wild type

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CC chemokine receptor (CCR)6 (14) induces chemotaxis of CCR6-expressing monocytes, dendritic cells, and macrophages to sites of infection that influence resolution and repair (15, 16).

In addition to expressing CCR6, monocytes express a wide variety of GPCRs, including formyl peptide receptors (FPRs) and CCRs (17, 18). FPRs serve as important receptors during wound repair because they regulate recruitment of immune cells to sites of inflammation and promote migration of epithelial cells (19, 20). Although 3 human FPRs (FPR1–3) have been identified, mice express at least 8 *Fpr* genes. Human FPR2, also referred to as the receptor for lipoxin A4 (*ALX*) gene, has been proposed to function as 2 mouse genes referred to as *Fpr2* and *Fpr3*, which share their first 2 exons (21). FPRs are expressed on epithelial (22–27) and immune cells (neutrophils, monocytes, macrophages, and dendritic cells) (20, 28, 29). *Fpr2*-deficient mice have shortened colonic crypts, reduced epithelial proliferation, and delayed recovery from acute colitis compared with wild-type (WT) mice (25). Previous work has suggested that *Fpr2/3* plays a role in monocyte recruitment during inflammation induced by polymicrobial sepsis (30) and chronic airway inflammation in a manner dependent on CCR2 (6, 31).

CCR signaling has been shown to cooperate with activation of FPRs to play an important role in coordinating immune cell recruitment to sites of injury and inflammation to initiate resolution. Specifically, the chemokine CCL3 and the FPR1/2 ligand fMLF have been shown to synergistically activate chemotaxis of human monocytes (32). The mechanisms by which these receptors cooperate to recruit immune cells and facilitate intestinal mucosal repair are not well understood. Here, we used *Fpr2/3*<sup>-/-</sup> mice to identify the role of *Fpr2/3* in facilitating recruitment of monocytes to sites of mucosal injury to promote wound repair. We found that *Fpr2/3* expression in epithelial and immune cells was required for colonic mucosal wound repair. Deletion of *Fpr2/3* resulted in reduced monocyte recruitment to sites of mucosal injury due to an alternate CCR6-CCL20 signaling axis, demonstrating that FPR2/3 regulates monocyte migration to influence colonic mucosal repair.

## MATERIALS AND METHODS

### Mice

Experiments were performed on mice 8–12 wk of age. Male C57BL/6J (*Fpr2/3*<sup>+/+</sup>, WT) mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). *Fpr2/3*<sup>-/-</sup> mice on a C57BL/6 background were generated as previously described by Dufton *et al.* (19). *Ccr6*<sup>-/-</sup> mice (*Ccr6*<sup>tm(EGFP)lrv</sup>; 013061, The Jackson Laboratory) were generated as previously described by Kucharzik *et al.* (33). Animals were housed under a standard day-night cycle with free access to food and water. Animal protocols were approved by the Institutional Animal Care and Use Committee of the University of Michigan and were done in accord with accepted national guidelines.

### Dextran sodium sulfate–induced colitis

Mice were provided with 2.5% (w/v) dextran sodium sulfate (DSS) dissolved in drinking water for 3 d followed by 5 d of recovery on water. To assess response to chronic DSS-induced colitis, the treatment was repeated twice. Daily clinical assessment of DSS-treated animals was monitored by a Clinical Disease Activity Index (DAI) ranging from 0 to 4 (34), which was calculated using stool consistency, presence or absence of fecal blood (Hemocult; Thermo Fisher Scientific, Waltham, MA, USA), and weight loss. Mice were euthanized after 2 cycles of treatment, and colons were isolated for histology and analysis of pathobiology.

### In vivo wounding of colonic mucosa

Mice were anesthetized by intraperitoneal injection of a ketamine (100 mg/kg)/xylazine (10 mg/kg) solution. A high-resolution, miniaturized colonoscope system equipped with biopsy forceps (Coloview Veterinary Endoscope; Karl Storz, Tuttlingen, Germany) was used to injure the colonic mucosa at 5–10 sites along the dorsal artery, and healing was quantified on d 1 and 3 post-injury. Endoscopic procedures were viewed with high-resolution (1024 × 768 pixels) images on a flat-panel color monitor. Wound size averaged ~1 mm<sup>2</sup>, which is equivalent to removal of ~250–300 crypts. For each analysis, 20–25 lesions from 5 mice/group were examined. Mucosal wounds and intact mucosa were harvested for quantitative PCR (qPCR) analysis of mRNA expression or flow cytometry. Wound area was quantified using ImageJ (National Institutes of Health, Bethesda, MD, USA).

### Bone marrow transplantation

For total bone marrow (BM) transplant experiments, donor BM cells were harvested from WT (C57BL/6 CD45.1; 002014, The Jackson Laboratory) and *Fpr2/3*<sup>-/-</sup> (CD45.2) mice. Recipient mice were sublethally irradiated using 2 times 5 Gy 4 h apart. 1 × 10<sup>6</sup> donor BM cells were transplanted by tail vein injection into recipient mice. For competitive BM transplant experiments, a 1:1 ratio of CD45.1 (WT) and CD45.2 (*Fpr2/3*<sup>-/-</sup>) at 5 × 10<sup>5</sup> was used for each genotype for a total of 1 × 10<sup>6</sup> BM donor cells/recipient. Blood samples were collected from the recipients 5 wk after BM transplantation to confirm engraftment. Experiments using the recipients were conducted 8–10 wk after BM transplantation and cardiac puncture was used to collect blood for engraftment and complete blood cell (CBC) analysis.

### Lamina propria isolation

Punch biopsies (2 mm) of wounded or intact colon tissue from each experimental condition were placed in 10 ml of Rosewell Park Memorial Institute (RPMI) 1640 medium containing 150 μl Liberase stock (2.5 mg/ml) and 150 μl DNase I stock (2 × 10<sup>4</sup> Kuntz U/ml) (both from MilliporeSigma, Burlington, MA, USA). Biopsied tissue samples were digested at 37°C for 30 min, passed several times through an 18-gauge needle plus a 3-cc syringe, and then filtered through a 70-μm cell strainer into a clean 50-ml tube on ice. Samples were centrifuged to pellet the immune cells then resuspended in PBS containing 2% fetal bovine serum (FBS)/1 mM EDTA.

### Flow cytometry and cell sorting

Isolated lamina propria cells from colonic wounds were resuspended in flow buffer (PBS containing 2% FBS/1 mM EDTA), filtered through 70-μm nylon mesh, and then incubated for 30

min at 4°C with a Live/Dead dye (eBioscience Fixable Viability Dye eFluor 780; Thermo Fisher Scientific). After being washed thoroughly, cells were stained with a labeled primary Ab cocktail in the presence of Fc block for 30 min at 4°C. Cells were then washed and fixed in 4% paraformaldehyde for 10 min at room temperature in the dark. Flow cytometric analysis was performed on a NovoCyte Flow Cytometer (Acea Biosciences, San Diego, CA, USA). The results were plotted and analyzed using FlowJo software (Becton Dickinson, Franklin Lakes, NJ, USA). For blood, 200  $\mu$ l was collected into 1 ml PBS/2 mM EDTA and kept on ice. Blood was centrifuged, and RBC were lysed in 400  $\mu$ l of ACK lysis buffer for 2–3 min on ice. After being washed in flow buffer, cells were stained and analyzed as previously described.

## Monocyte isolation

Monocytes were isolated from spleen by antibody-based negative selection. Briefly, spleens were collected in PBS supplemented with 1% FBS and 1 mM EDTA, passed through a 70- $\mu$ m Falcon strainer (Corning, Corning, NY, USA), and collected in a 50-ml conical tube. Cell suspension was centrifuged at 400 *g* for 5 min and red blood cells were lysed with 1 ml of distilled water for 30 s. Lysing reaction was stopped by adding 1 ml of 1.8% NaCl. Subsequently, monocytes were isolated from the cell suspension according to EasySep Mouse Monocyte Isolation protocol (Stemcell Technologies, Vancouver, BC, Canada).

## Monocyte migration assay

Transcollagen migration experiments were performed using collagen-coated (10  $\mu$ g/ml rat tail collagen type I), permeable, 0.33- $\text{cm}^2$  polycarbonate Transwells (5  $\mu$ m pore size; Costar, Cambridge, MA, USA), in the presence of a chemotactic gradient of 100 ng/ml CCL9 or 100 ng/ml CCL20 (PeproTech, Rocky Hill, NJ, USA).  $2 \times 10^5$  of splenic monocytes were added to the upper chambers of Transwell, and migration to the lower chamber was assayed for 2 h at 37°C. Migrated monocytes were fixed and stained with crystal violet. Representative images from 5 fields were acquired and migrated cells were quantified. The rate of migration is represented as the percentage of the total monocytes added to the upper chamber of the Transwell that have migrated into the bottom.

## CBC analysis

CBC analysis was performed by the University of Michigan Animal Diagnostic Laboratory. Whole blood samples (50  $\mu$ l) were collected by cardiac puncture into EDTA microtainers (Becton Dickinson) and stored at 4°C until analysis.

## Reagents

The following antibodies were used for flow cytometry analysis: eFluor 450-conjugated Ly-6C (48-5932-82), phycoerythrin (PE)-

Cyanine7-conjugated CD19 (25-0193-82), allophycocyanin (APC)-conjugated CD45.1 (17-0453-82), FITC-conjugated CD45.2 (11-0454-85), APC-eFluor 780-conjugated CD4 (47-0041-82), and PE-conjugated F4/80 (MF48004) antibodies from Thermo Fisher Scientific; BV510-conjugated CD11b (562950), PE-conjugated CD3e (553063), PerCP-conjugated CD8a (553036), APC-Cy7-conjugated sialic acid-binding immunoglobulin-like lectin (Siglec)-F (565527), and BD Fc Block CD16/CD32 (553142) antibodies from BD Biosciences (San Jose, CA, USA); and BV608-conjugated Ly-6G (127639), PE/Cy7-conjugated CD64 (139314), APC-conjugated CCR1 (152504), and BV785-conjugated CCR6 (129823) antibodies from BioLegend (San Diego, CA, USA). Antibodies were used at 1:200 for flow cytometry.

## qPCR

Total RNA was isolated from colonic wounds or isolated monocytes using the RNeasy Kit (Qiagen, Germantown, MD, USA) with DNase I treatment following the manufacturer's protocol. Equal amounts of total RNA (500 ng–1  $\mu$ g) were transcribed into cDNA using the iScript Reverse Transcription Supermix (Bio-Rad, Hercules, CA, USA). Individual gene expression was determined by qPCR using SsoAdvanced Universal SYBR Green (Bio-Rad) with a Bio-Rad CTX Cyclor measuring SYBR green incorporation for product detection. Reactions were performed in triplicate with at least 3 biologic replicates. The relative expression of the gene of interest was calculated by  $2^{-\Delta\Delta C_t}$  and normalized to the housekeeping gene TATA-box-binding protein (TBP). The primer sequences are shown in Table 1.

## Luminex assay

Intestinal tissue CCL-20 protein concentration was determined using Bio-Plex Pro Mouse Chemokine Assay (12009159) purchased from Bio-Rad following the manufacturer's instructions.

## Statistical analysis

Statistical comparisons were performed by 1- or 2-way ANOVA with Bonferroni's multiple comparison, or unpaired 2-tailed Student's *t* test, as appropriate. A value of *P* < 0.05 was considered significant.

## RESULTS

### *Fpr2/3*<sup>-/-</sup> mice display delayed colonic mucosal wound repair

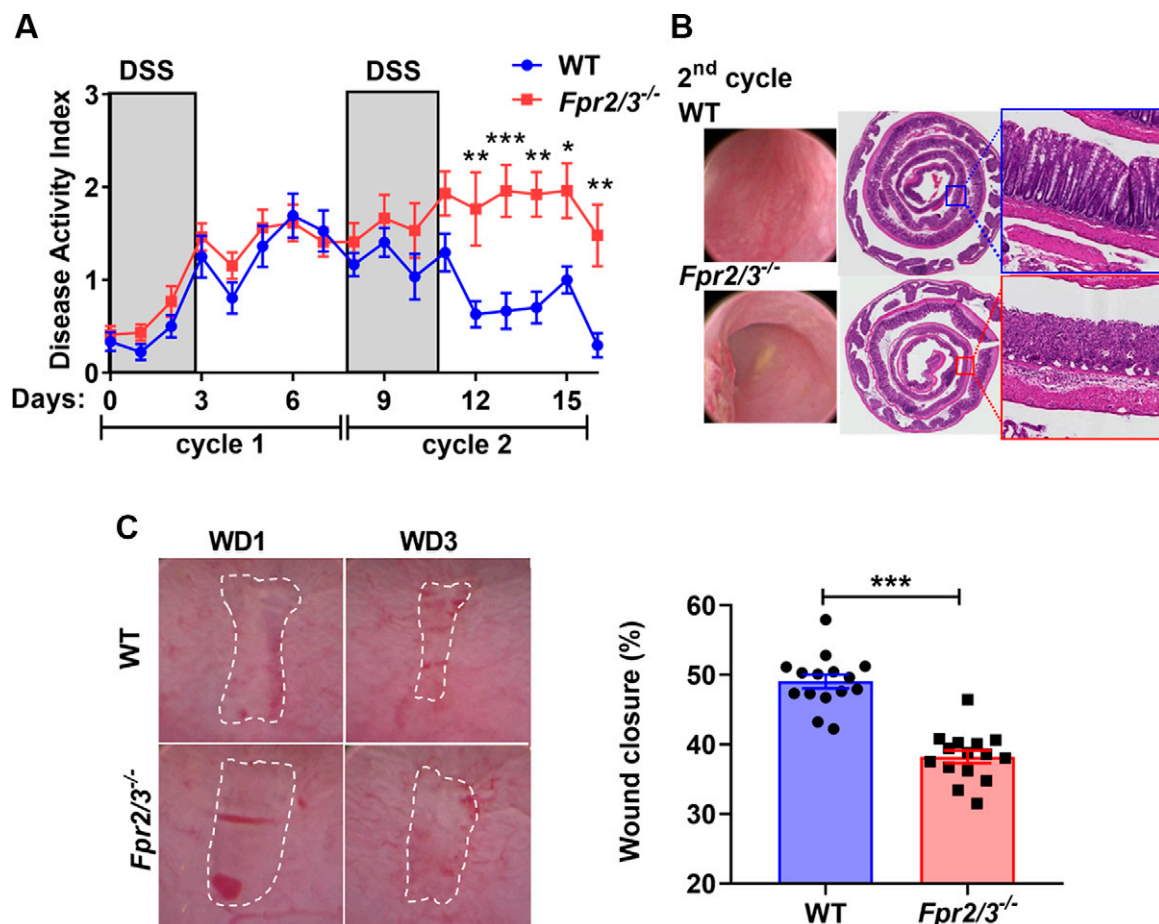
Intestinal epithelial expression of FPR2 has been reported to regulate epithelial homeostasis and response to mucosal

TABLE 1. Primers

Gene	Primer name	Primer sequence, 5'–3'	
		Forward	Reverse
<i>Ccr1</i>	Mm <i>Ccr1</i>	AGGAACTGGTCAGGAATAATAGC	CAAAGGCCCCAGAAACAAAGTC
<i>Ccr2</i>	Mm <i>Ccr2</i>	ACTGAGGTAACATATATTGTCTTCCA	GAGCCATACCTGTAAATGCCA
<i>Ccr6</i>	Mm <i>Ccr6</i>	GTCACGTGCATGCTTACTTGAATG	CTTAGGACTGGAAGCCTGGATA
<i>FPR2</i>	Mm <i>Fpr2</i>	AAGGAGACCTCAGCTGGTTGTG	TCCACAGAACTCTGGAGATGGTAG
<i>TBP</i>	Mm <i>Tbp</i>	GGAATTGTACCGCAGCTTCAAA	GATGACTGCAGCAAATCGCTT

inflammation (25). Inflammatory bowel disease is commonly associated with repeated bouts of active inflammation followed by reparative events. To model the process of repetitive bouts of mucosal inflammation and repair, we subjected *Fpr2/3*<sup>-/-</sup> and WT mice to cycles of DSS administration in drinking water followed by recovery on water alone. Mice were administered 2 cycles of 2.5% (w/v) DSS for 3 d in the drinking water with a recovery period of 5 d/cycle. The concentration of DSS and duration of administration were based on close monitoring of disease activity. We determined that administration of 2.5% DSS resulted in optimal conditions that allowed for evaluation of resolution of inflammation and repair in FPR2/3 mice. Higher DSS concentration or longer exposure resulted in severe disease activity that necessitated euthanasia during the repair phase in FPR2/3 mice. The DAI was monitored by evaluating body weight, stool consistency, and fecal blood. Mice lacking *Fpr2/3* (*Fpr2/3*<sup>-/-</sup>) displayed similar DAI to that observed with WT mice

during the first cycle of DSS treatment. However, increased DAI was noted in *Fpr2/3*<sup>-/-</sup> mice during the second DSS cycle, as evidenced by a marked delay in recovery from colitis (d 11–16) (Fig. 1A). Endoscopic images of the colonic mucosa showed increased hyperemia and ulceration in *Fpr2/3*<sup>-/-</sup> mice compared with WT controls (C57BL/6J) at the termination of the experiments (Fig. 1B). Histologic evaluation showed increased mucosal ulceration and delayed mucosal healing in *Fpr2/3*<sup>-/-</sup> mice compared with WT mice (Fig. 1B). Colonic mucosa from *Fpr2/3*<sup>-/-</sup> mice displayed loss of epithelial architecture, increased immune cell infiltration, and decreased epithelial repair (Fig. 1B). These results are consistent with delayed healing responses in *Fpr2/3*<sup>-/-</sup> mice. To further evaluate the contribution of *Fpr2/3* in controlling colonic mucosal wound repair, we analyzed mucosal wound healing using a colon biopsy-induced injury model. As shown in Fig. 1C, *Fpr2/3*<sup>-/-</sup> mice demonstrated delayed mucosal wound healing compared with control mice



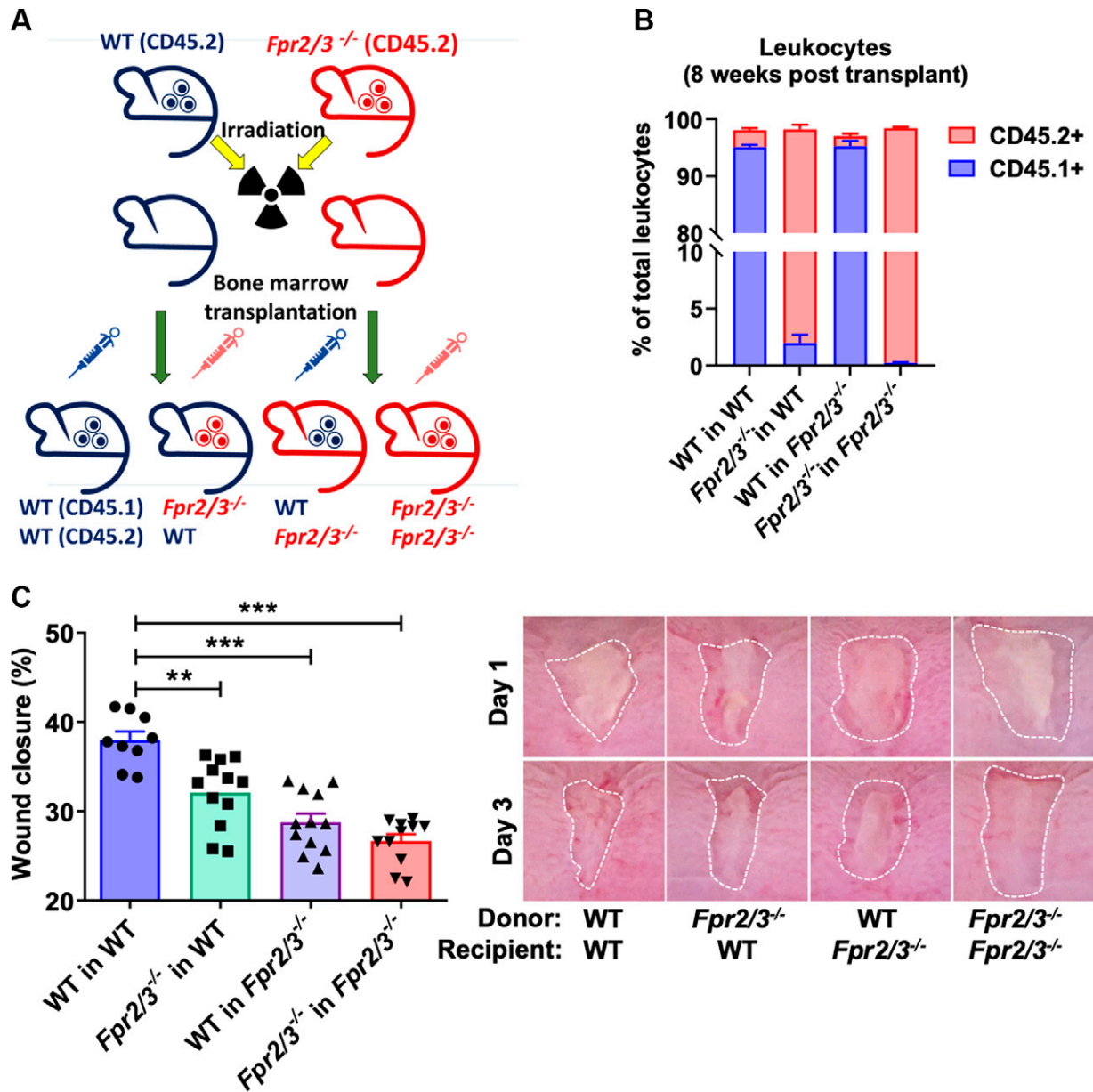
**Figure 1.** *Fpr2*<sup>-/-</sup> mice show decreased acute and chronic wound healing. **A**) Disease activity index comparing C57BL/6J (WT) with *Fpr2/3*<sup>-/-</sup> mice after 2 cycles of DSS treatment consisting of 3 d of 2.5% DSS followed by 5 d of recovery with water consumption. **B**) Representative images of endoscopic videos and hematoxylin and eosin–stained histologic sections of WT or *Fpr2/3*<sup>-/-</sup> colonic mucosa after the second cycle of DSS treatment. Boxed areas are magnified in insets equidistant from the rectum. The total magnification of the photomicrographs is original magnification,  $\times 2$ , and the inset is original magnification,  $\times 40$ . **C**) Endoscopic images of colonic mucosal wounds 1 and 3 d after biopsy-induced injury in *Fpr2/3*<sup>-/-</sup> ( $n = 15$ ) or WT control ( $n = 15$ ) mice. Wound area was quantified using ImageJ. WD, wound day. Graph shows quantification of wound closure. Statistical comparisons were performed using an unpaired 2-tailed Student's *t* test with Welch's correction. \*\*\* $P < 0.001$  (means  $\pm$  SEM).

( $38.2 \pm 3.5$ ,  $Fpr2/3^{-/-}$ ;  $49.0 \pm 3.8$ , WT;  $P < 0.001$ ). Taken together, these results support an important role of  $Fpr2/3^{-/-}$  in colonic mucosal wound repair.

### Fpr2/3 expressed in epithelial and immune cells regulates colonic mucosal wound repair

Mucosal repair is orchestrated by the epithelium as well as infiltrating immune cells at sites of injury. Because epithelial cells and leukocytes express FPR2 (28, 35, 36), we evaluated the relative contributions of epithelial and immune cell expression of  $Fpr2/3$  in regulating mucosal

repair. Mice were subjected to BM transplantation followed by biopsy-induced mucosal wound healing experiments. Irradiated WT or  $Fpr2/3^{-/-}$  recipient mice were reconstituted with BM from either WT or  $Fpr2/3^{-/-}$  donor mice (Fig. 2A). Engraftment was assessed by flow cytometry to evaluate allelic markers 8 wk posttransfer (Fig. 2B), and reconstitution of hematopoietic cells was determined by CBC analyses at the end of the experiment (Supplemental Fig. S1). Analysis of healing wounds at d 3 post-injury revealed delayed wound repair in  $Fpr2/3^{-/-}$  mice reconstituted with WT BM (WT in  $Fpr2/3^{-/-}$ ) (Fig. 2C), supporting a role of epithelial  $Fpr2/3$  in regulation of



**Figure 2.** Expression of  $Fpr2/3$  on epithelial and immune cells is necessary for colonic mucosal wound repair. **A)** Illustration of BM transplant experiment. **B)** Engraftment verification after BM transplantation by flow cytometry analysis of leukocytes in irradiated WT or  $Fpr2/3^{-/-}$  host mice that were reconstituted with BM from either WT (CD45.1<sup>+</sup>) or  $Fpr2/3^{-/-}$  (CD45.2<sup>+</sup>) donor mice (WT→WT;  $Fpr2/3^{-/-}$ →WT; WT→ $Fpr2/3^{-/-}$ ;  $Fpr2/3^{-/-}$ → $Fpr2/3^{-/-}$ ;  $n = 6$ ; means  $\pm$  SEM). **C)** Quantification and endoscopic images of colonic mucosal wound repair after biopsy-induced injury, comparing irradiated WT or  $Fpr2/3^{-/-}$  host mice that were reconstituted with BM from either WT or  $Fpr2/3^{-/-}$  donor mice (WT→WT,  $n = 9$ ;  $Fpr2/3^{-/-}$ →WT,  $n = 12$ ; WT→ $Fpr2/3^{-/-}$ ,  $n = 12$ ;  $Fpr2/3^{-/-}$ → $Fpr2/3^{-/-}$ ,  $n = 11$ ).

wound healing. Additionally, WT mice that received a BM transplant from *Fpr2/3*<sup>-/-</sup> mice (*Fpr2/3*<sup>-/-</sup> in WT) had delayed wound repair (Fig. 2C), suggesting that immune cell-expressed *Fpr2/3* also plays a role in regulating mucosal repair.

### **Fpr2/3 influences monocyte recruitment into healing mucosal wounds**

Given that previous reports supported a role of epithelial-expressed FPR2 in regulating mucosal wound repair (23–25), we investigated the contribution of FPR2 in regulating immune cell recruitment to sites of injury and mucosal repair *in vivo*. We first examined wound-associated immune cells in the colonic mucosa 1–3 d after biopsy-induced injury of WT and *Fpr2/3*<sup>-/-</sup> mice using flow cytometric approaches. Immune cells were flow sorted using the following gating strategy (Supplemental Fig. S3) to analyze immune cells' infiltration into the wounds: neutrophils (CD45<sup>+</sup> Ly6G<sup>+</sup> Siglec-F<sup>-</sup>), eosinophils (CD45<sup>+</sup> Ly6G<sup>-</sup> Siglec-F<sup>+</sup>), and monocytes/macrophages (CD45<sup>+</sup> CD11b<sup>+</sup> Ly6G<sup>-</sup> Siglec-F<sup>-</sup>). Monocyte and macrophage populations were further sorted into 3 populations: macrophages (CD45<sup>+</sup> CD11b<sup>+</sup> Ly6G<sup>-</sup> Siglec-F<sup>-</sup> F4/80<sup>+</sup>), Ly6C<sup>lo</sup> monocytes (CD45<sup>+</sup> CD11b<sup>+</sup> Ly6G<sup>-</sup> Siglec-F<sup>-</sup> F4/80<sup>lo</sup> Ly6C<sup>lo</sup>), and Ly6C<sup>hi</sup> monocytes (CD45<sup>+</sup> CD11b<sup>+</sup> Ly6G<sup>-</sup> Siglec-F<sup>-</sup> F4/80<sup>lo</sup> Ly6C<sup>hi</sup>). CBC analyses revealed no differences between immune cells in blood from *Fpr2/3*<sup>-/-</sup> mice compared with WT (Supplemental Fig. S2). As shown in Fig. 3A, on d 1 and 2 after wounding, mice lacking *Fpr2/3* had reduced numbers of inflammatory monocytes (defined as viable, CD45<sup>+</sup> CD11b<sup>+</sup> Ly6G<sup>-</sup> Siglec-F<sup>-</sup> F4/80<sup>lo</sup> Ly6C<sup>hi</sup>). Although changes in other immune cell populations were also noted (Supplemental Fig. S3), monocytes have been strongly linked to playing important roles in regulating wound repair in a number of organ systems (37). We therefore focused on defining the role of FPR2/3 in regulating monocyte migration and infiltration to sites of intestinal mucosal injury.

Based on the above results, we hypothesized reduced recruitment of Ly6C<sup>hi</sup> monocytes into healing colonic mucosal wounds of *Fpr2/3*<sup>-/-</sup> mice. To explore the role of *Fpr2/3* in monocyte recruitment, competitive BM chimera experiments were performed. Irradiated WT or *Fpr2/3*<sup>-/-</sup> host mice were reconstituted with a 1:1 mixture of BM from WT (CD45.1) and *Fpr2/3*<sup>-/-</sup> (CD45.2) donor mice (Fig. 3). Engraftment was assessed by allelic markers at 10-wk posttransfer, and reconstitution of hematopoietic cells was determined by CBC analyses at the end of experiments (Supplemental Fig. S4). Colonic mucosal wounds were induced by biopsy, and infiltrating immune cell populations were evaluated after injury. The ratio of WT (CD45.1) to *Fpr2/3*<sup>-/-</sup> (CD45.2) Ly6C<sup>hi</sup> monocytes in normal colonic mucosa and after injury was compared with the ratio of CD45.1:CD45.2 cells in blood. Results of such experiments demonstrated that WT CD45.1 cells have a competitive advantage in recruitment from blood to wounded mucosa compared with cells from *Fpr2/3*-deficient CD45.2 mice that is independent of the genetic background (Fig. 3D). There was no difference in

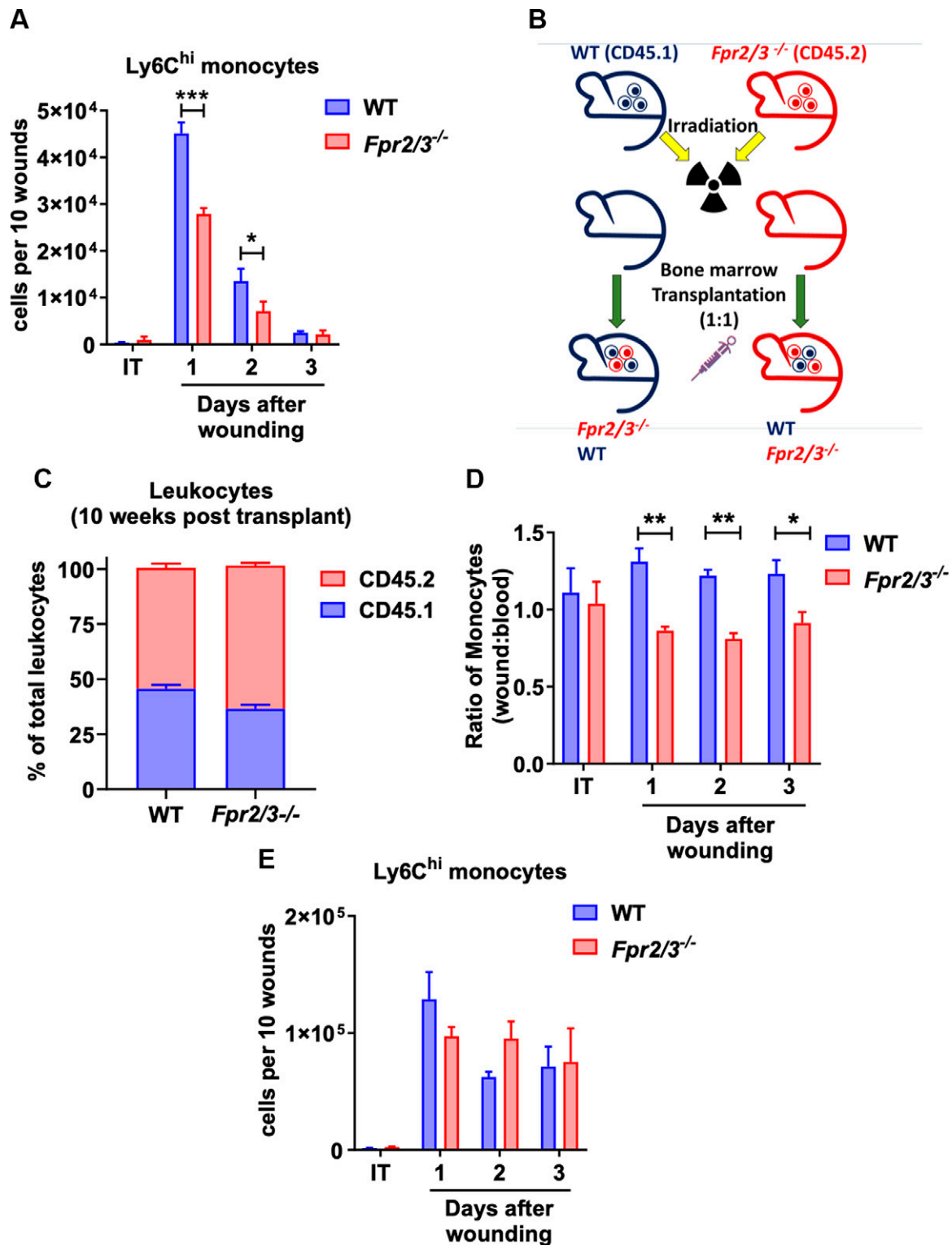
recruitment of Ly6C<sup>hi</sup> monocytes into wound beds of *Fpr2/3*<sup>-/-</sup> mice reconstituted with 1:1 WT:*Fpr2/3*<sup>-/-</sup> BM compared with WT recipients (Fig. 3E), suggesting that the presence of WT cells may compensate for reduced monocyte recruitment in *Fpr2/3*<sup>-/-</sup> mice. Together, these data suggest that *Fpr2/3* expression influences inflammatory monocyte recruitment into healing mucosal wounds.

### **Fpr2/3 impacts intestinal wound cytokine and chemokine expression profile**

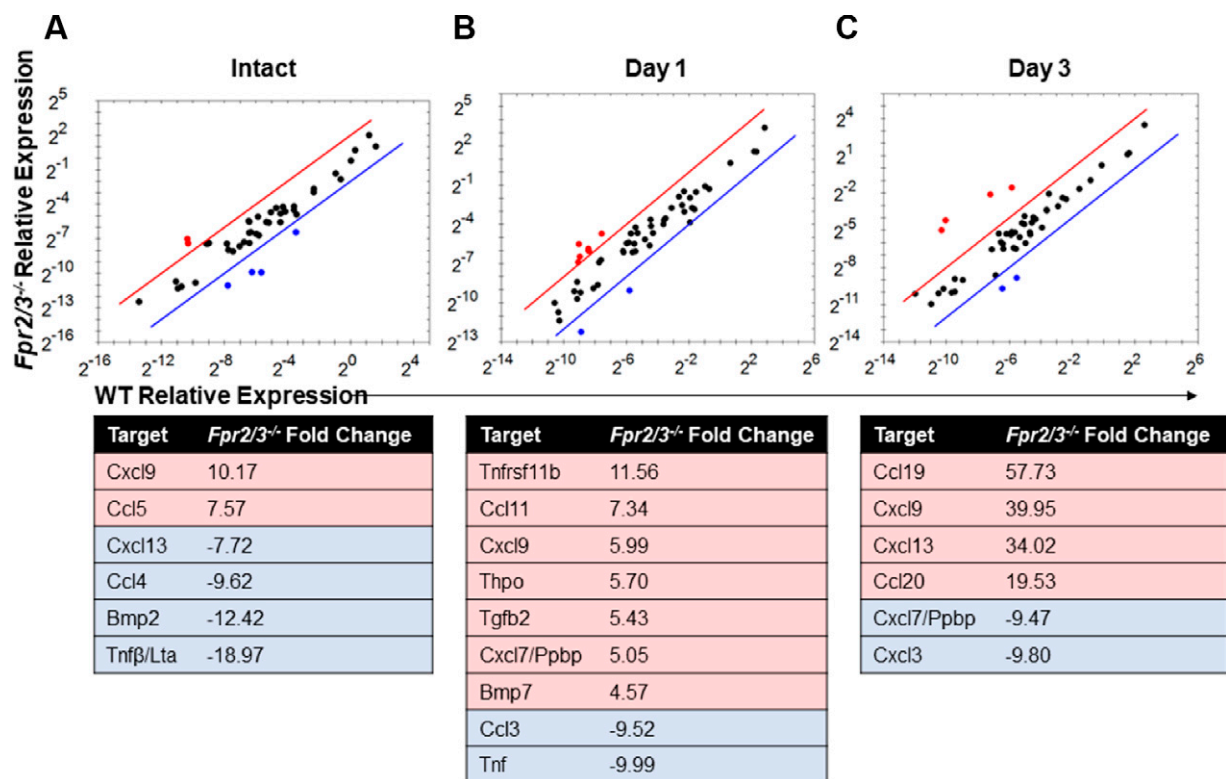
To determine the basis of delayed *Fpr2/3*<sup>-/-</sup> monocyte recruitment into healing wounds, we analyzed expression of cytokines and chemokines using a PCR array. RNA was isolated from intact colonic mucosa and healing biopsy-induced wounds 1 and 3 d after biopsy-induced injury. Pooled samples from multiple mice were reverse transcribed and analyzed. In intact mucosa, expression of the leukocyte chemoattractants *Ccl5* and chemokine (C-X-C motif) ligand (*Cxcl9*)*Cxcl9* was up-regulated, whereas expression of *Bmp2*, *Ccl4*, *Cxcl13*, and *Tnf-β* was down-regulated relative to WT (Fig. 4A). Analysis of colonic mucosa 1 d after injury revealed up-regulation of targets linked to regulation of immune cell recruitment (*Ccl11*, *Cxcl7*, and *Cxcl9*) (40, 41) (Fig. 4B). Additionally, in *Fpr2/3*<sup>-/-</sup> wounded mucosa isolated on d 1 after injury, *Tnf-α* expression was down-regulated relative to WT along with *Ccl3*, a ligand for CCR1, CCR4, and CCR5 (Fig. 4B). In wounded mucosa isolated on d 3 after injury, up-regulation of T-cell and B-cell chemoattractants (*Ccl19*, *Cxcl9*, and *Cxcl13*) and down-regulation of chemoattractants involved in neutrophil recruitment (*Cxcl7* and *Cxcl3*) were found in *Fpr2/3*<sup>-/-</sup> wounded mucosa relative to WT (Fig. 4C). The chemokine *Ccl20* was found to be highly up-regulated in wounds 3 d after injury in *Fpr2/3*<sup>-/-</sup> mice when compared to WT (Fig. 4C). CCL20 has been described to have an important role in migration of Ly6C<sup>hi</sup> monocytes in inflamed tissues through its interaction with the CCR6 receptor (15, 38, 39). Furthermore, CCL20 protein in healing wounds was increased 3 d postinjury in *Fpr2/3*<sup>-/-</sup> mice compared with WT mice (Supplemental Fig. S5). Overall, these data suggest there is a different chemokine and cytokine milieu in *Fpr2/3*<sup>-/-</sup> mice compared with WT animals. Because monocytes play an important role in repair, our study was focused on identifying contribution of *Fpr2/3*<sup>-/-</sup> in monocyte recruitment to healing wounds.

### **Fpr2/3 promotes monocyte tissue migration cooperatively with CCR6 signaling**

Given the delayed recruitment of inflammatory monocytes in healing wounds of *Fpr2/3*<sup>-/-</sup> mice, we evaluated whether elevated *Ccl20* mRNA levels might be secondary to an altered CCL20-CCR6 signaling axis, offering a possible explanation for the defect in monocyte recruitment observed. Expression of monocyte chemokine receptors previously shown to influence Ly6C<sup>+</sup> monocyte migration from blood to inflamed tissues was evaluated (42, 43). To



**Figure 3.** FPR2 expression is required for competitive advantage of monocytes to migrate to sites of mucosal injury. *A*) Analysis of monocytes from intact (IT) or wounded lamina propria tissue on different postinjury days isolated from WT or *Fpr2/3*<sup>-/-</sup> ( $n = 6$ ). *B*) Illustration of competitive BM transplant experiment. *C*) Engraftment verification after competitive BM transplantation by flow cytometry analysis of leukocytes in irradiated WT or *Fpr2/3*<sup>-/-</sup> host mice that were reconstituted with a 1:1 mixture of BM from WT (CD45.1) and *Fpr2/3*<sup>-/-</sup> (CD45.2) donor mice ( $n = 19-20$ , means  $\pm$  SEM). WT, C57BL/6J. *D*, *E*) Analysis of monocytes isolated from IT or wounded lamina propria tissue on different postinjury days isolated from irradiated WT or *Fpr2/3*<sup>-/-</sup> host mice were reconstituted with a 1:1 mixture of BM from WT (CD45.1) and *Fpr2/3*<sup>-/-</sup> (CD45.2) donor mice. *D*) Graphs represent the ratio of WT (CD45.1) or *Fpr2/3*<sup>-/-</sup> (CD45.2) Ly6C<sup>hi</sup> cells in the wound *vs.* in the blood ( $n = 10$ ) of combined WT and *Fpr2/3*<sup>-/-</sup> mice submitted to a competitive BM transplant. *E*) Numbers of Ly6C<sup>hi</sup> monocytes on different postinjury days and in IT lamina propria tissue ( $n = 5$ ). IT, intact tissue; WT, C57BL/6J. Statistical comparisons performed using 2-way ANOVA with Bonferroni's multiple comparison. \* $P < 0.5$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$  (means  $\pm$  SEM).



**Figure 4.** Cytokine and chemokine expression analysis in intact and injured tissue. Scatter plots comparing gene expression changes determined by PCR array performed on RNA isolated from 3-mm punch biopsies of WT or *Fpr2/3*<sup>-/-</sup> intact tissue (A) or resealing colonic wounds on d 1 (B) and d 3 (C) postinjury isolated from mice. Tables detail the fold change in expression observed in *Fpr2/3*<sup>-/-</sup> samples relative to WT (C57BL/6j).

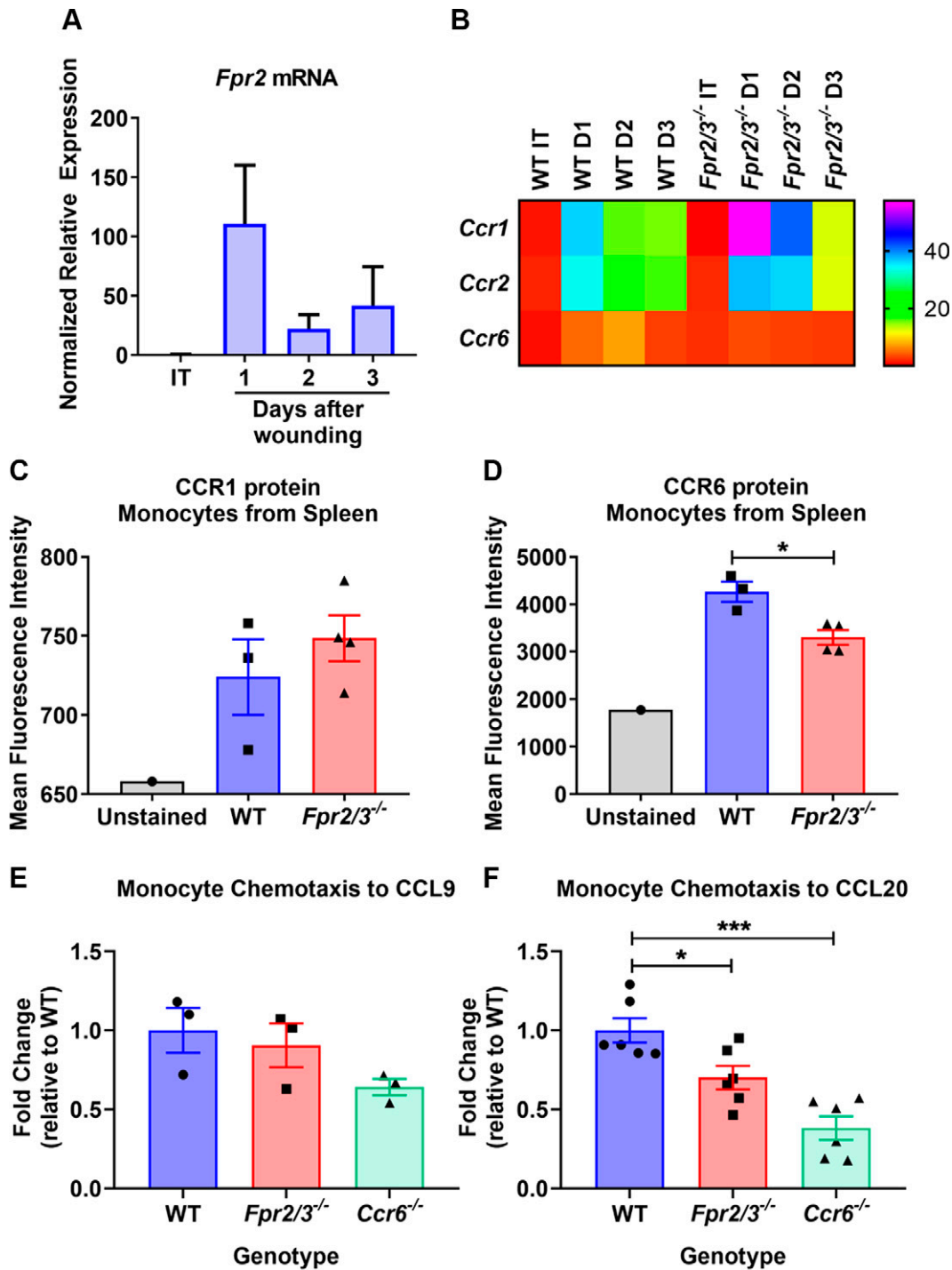
specifically evaluate the expression of chemokine receptors on wound-associated monocytes, intact mucosa or wound beds 1–3 d after biopsy-induced injury were obtained from WT or *Fpr2/3*<sup>-/-</sup> mice. Monocytes were isolated from the lamina and qPCR was performed. Notably, increased *Fpr2* mRNA expression was detected in WT monocytes isolated from colonic wounds on d 1 after biopsy-induced injury relative to monocytes in intact tissue (Fig. 5A). Interestingly, *Ccr6* mRNA was up-regulated on d 1 postinjury in WT controls but not *Fpr2/3*<sup>-/-</sup> monocytes (Fig. 5B). These data suggest that in the absence of *Fpr2/3*<sup>-/-</sup>, monocyte recruitment to healing wounds may be impaired secondary to altered expression of *Ccr6*.

To determine if monocytes lacking *Fpr2/3* have reduced chemokine receptor expression, we harvested splenic monocytes from *Fpr2/3*<sup>-/-</sup> or WT mice and determined expression of CCR1 and CCR6 receptors by flow cytometry. Although there was no change in CCR1, CCR6 expression was indeed reduced on *Fpr2/3*<sup>-/-</sup> splenic monocytes (Fig. 5C, D and Supplemental Fig. S6). Because CCR6 receptor expression was reduced in *Fpr2/3*<sup>-/-</sup> monocytes, we compared WT or *Fpr2/3*<sup>-/-</sup> monocyte chemotaxis toward the CCR6 ligand CCL20 using Transwell migration assays. Although we did not detect a significant change in chemotaxis of *Fpr2/3*<sup>-/-</sup> or *Ccr6*<sup>-/-</sup> monocytes toward the CCR1 ligand CCL9 (Fig. 5E), there was decreased *Fpr2/3*<sup>-/-</sup> chemotaxis toward CCL20 compared with WT monocytes (Fig. 5F). As an additional control, *Ccr6*<sup>-/-</sup> monocytes displayed significantly

reduced chemotaxis toward CCL20 compared with WT monocytes (Fig. 5F). These data are consistent with *Fpr2/3* indirectly regulating monocyte chemotaxis through the CCL20-CCR6 signaling axis to influence mucosal repair.

## DISCUSSION

Gastrointestinal mucosal injury associated with wounds is observed in a number of pathologic states, including inflammatory bowel disease. The murine ortholog of FPR2/ALX, *Fpr2/3*, has been implicated in resolution of inflammation in models of polymicrobial sepsis, mesenteric ischemia–reperfusion injury, and carrageenan-induced paw edema (19, 30). In this study, we report that *Fpr2/3*<sup>-/-</sup> mice have 2 distinct functions that extend our knowledge of mucosal repair: 1) FPR2/3 has a central role in monocyte recruitment into colonic mucosal wounds to promote healing responses, and 2) FPR2/3 further promotes recruitment and positioning of prorepair myeloid cells through regulation of other chemokine receptors (*i.e.*, CCR6). These observations are consistent with other studies using *Fpr2/3*<sup>-/-</sup> mice that demonstrate impaired recruitment of monocytes to apoptotic murine neutrophils (44) and reduced peritoneal recruitment of monocytes in a murine sepsis model (30). In the skin, immune cell *Fpr1/2* depletion has been shown to impair repair (45), whereas stimulation with the FPR2-specific peptide agonist WKYMVM increases macrophage infiltration into



**Figure 5.** Monocytes lacking FPR2 expression have a deficit in their chemotactic response. *A*) *Fpr2* mRNA levels were determined by qPCR of RNA isolated from monocytes collected from the lamina propria of WT intact tissue or resealing colonic wounds isolated from mice ( $n = 3-4$ ). *B*) Heat map representation of expression levels of *Ccr1*, *Ccr2*, and *Ccr6* mRNA in monocytes was determined by qPCR analysis of RNA ( $n = 3-4$ ). *C*, *D*) Flow cytometric analysis of CCR1 (*C*) and CCR6 (*D*) expression on monocytes isolated from spleens of WT or *Fpr2*<sup>-/-</sup> mice. *E*, *F*) *In vitro* migration of spleen monocytes isolated from WT or *Fpr2*<sup>-/-</sup> mice toward CCL9 ( $n = 3$ ) (*E*) or CCL20 ( $n = 6$ ) (*F*). The rate of migration is represented as the fold change relative to WT based on the ratio of total monocytes added to the upper chamber of the Transwell to the number of cells migrated into the bottom. MFI, mean fluorescence intensity. Statistical comparisons performed using 1-way ANOVA with Bonferroni's multiple comparison or paired Student's *t* test. \* $P < 0.5$ , \*\*\* $P < 0.001$  (means  $\pm$  SEM).

wounds and promotes repair (46). Liu *et al.* (45) reported decreased wound repair associated with defects in neutrophil migration to sites of dermal injury that was attributed to lack of FPR1 and FPR2 receptors. In our study,

we also observed decreased neutrophil recruitment in healing intestinal mucosal wounds of mice lacking FPR2/3 receptors (Supplemental Fig. S3). Given the current appreciation of mononuclear cells in regulating tissue repair

and our previous findings showing the importance of inflammatory monocytes in orchestrating colonic mucosal wound repair (47), we performed studies to investigate the contribution FPR2/3 in monocyte-mediated intestinal mucosal wound repair.

Infiltrating monocytes/macrophages accumulate in close proximity to wound-associated healing epithelial cells (2, 48). In the current study, we observed delayed recovery from DSS-induced colitis and biopsy-induced mucosal wound repair in *Fpr2/3*<sup>-/-</sup> compared with WT mice associated with decreased recruitment of Ly6C<sup>hi</sup> inflammatory monocytes into healing mucosal wounds normally observed within a day following biopsy-induced injury. BM transplant experiments revealed contribution of epithelial and immune cell expression of *Fpr2/3* in orchestrating mucosal repair. A previous study reported shortened colonic crypts with reduced epithelial proliferation, decreased acute inflammatory response, and delayed recovery from acute DSS colitis in *Fpr2*-deficient mice compared with WT mice (25). Additionally, mice with myeloid-specific *Fpr2* deletion (*Fpr2*<sup>ff</sup>/*LysMcre*) displayed a moderate reduction in disease associated with decreased myeloid cell recruitment into the colonic mucosa without a change in mucosal recovery (25). The differences in our findings compared with those reported (25) might be related to the reparative experimental models, mouse strains (19), and institutional animal facilities.

The importance of FPRs on immune cells in the context of host defense during inflammatory diseases has been previously described (49–52). Monocyte recruitment to specific tissues is mediated through activation of specific surface receptors by various ligands, including chemokines. Chemokine receptor signaling is critical for chemotactic movement and cell activation, and FPR signaling has been implicated in desensitization and internalization of chemotactic receptors, such as CXCR1, PAFR, C5aR, CCR1, CCR5, and CXCR4 (53–58).

We observed selective up-regulation of *Ccr6* in monocytes isolated from wounds on d 1 of WT mice, which was not detected in wounds from *Fpr2/3*<sup>-/-</sup> mice, suggesting a functional relationship between these 2 receptors. Furthermore, we observed reduced CCR6 surface receptor expression on *Fpr2/3*<sup>-/-</sup> splenic monocytes compared with WT cells, suggesting that the expression of CCR6 is linked to *Fpr2/3*. Although CCL20 expression is tightly regulated under normal physiologic processes, aberrant expression of CCR6 and CCL20 has also been linked to pathologic inflammation, as reviewed in ref. 59. Epithelial expression of CCL20 has been shown to induce chemotaxis of inflammatory monocytes and monocyte-derived dendritic cells in response to inflammation (15, 38, 39). Inflammatory mediators, such as TNF- $\alpha$ , have been shown to up-regulate *Ccl20* mRNA and protein expression in colonic epithelial cells (13) as well as play an important role in intestinal wound repair in humans and mice (60, 61). Indeed, we found elevated *Ccl20* mRNA and protein levels in harvested mucosal wounds. In humans, CCL20 levels are elevated in the injured/inflamed colonic mucosa of individuals with active ulcerative colitis (UC) (62).

In summary, we found that epithelial and immune cell expression of FPR2/3 is required for colonic mucosal

repair by regulating monocyte chemotaxis through the CCL20-CCR6 signaling axis. The reduced monocyte influx into the wound bed might cause a lack of neutrophil efferocytosis, altered production of SPMs, and reduced macrophage differentiation, thus interrupting the process of resolution and resulting in chronic inflammation and impaired wound healing. Our results show that FPR2 promotes resolution of inflammation through the recruitment of inflammatory monocytes into the sites of injury, therefore transducing proinflammatory responses. Under physiologic conditions, this acute inflammatory response sets the stage for a complex process leading to resolution. In chronic diseases, such as UC, or conditions associated with vascular compromise, including ischemia or atherosclerosis, the shift from inflammation to resolution is impaired, leading to a nonresolving state (chronic inflammation) and persistent tissue damage (2–5). Under such conditions, there is persistent high expression of FPR2, as observed in biopsies of patients with UC as well as in atherosclerotic lesions (63). Due to altered resolution signals, down-regulation of FPR2 expression does not occur and serves to perpetuate a chronic inflammatory response. An example is highlighted by the increased macrophage infiltration observed in *Fpr2*<sup>+/+</sup> compared with *Fpr2*<sup>-/-</sup> mice suffering from atherosclerosis (63). It is possible that during long-term disease processes, proinflammatory and anti-inflammatory responses resolve, leading to UC remission or plaque stabilization even with persistent elevation of FPR2 receptors. Our results highlight the importance of *Fpr2* in mediating resolution of inflammation by influencing monocyte cell trafficking and could have clinical implications that include the use of FPR2 agonists to promote monocyte migration and neutrophil clearance and enhance intestinal mucosal wound repair in chronic intestinal inflammatory disorders, such as inflammatory bowel disease. FJ

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## AUTHOR CONTRIBUTIONS

D. Birkl, M. N. O'Leary, M. Quiros, N. W. Lukacs, C. A. Parkos, and A. Nusrat designed research and analyzed the data; D. Birkl, M. N. O'Leary, M. Quiros, V. Azcutia, M. Schaller, M. Reed, H. Nishio, and J. Keeney performed experiments; D. Birkl, M. N. O'Leary, M. Quiros, A. S. Neish, N. W. Lukacs, C. A. Parkos, and A. Nusrat wrote, reviewed, and edited the manuscript; and A. S. Neish and A. Nusrat acquired funding.

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