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Resident and inflammatory mononuclear phagocytes (MPhs) with functional plasticity in the intestine are critically involved in the pathology of inflammatory bowel diseases (IBDs), the mechanism of which remains incompletely understood. In the present study, we found that increased expression of the E3 ligase F-box and WD repeat domain–containing 7 (FBXW) in the inflamed intestine was significantly correlated with IBD severity in both human diseases and in mouse models. Myeloid Fbxw7 deficiency protected mice from colitis induced by dextran sodium sulfate (DSS) or 2,6,4-trinitrobenzene sulfonic acid (TNBS). Fbxw7 deficiency resulted in decreased production of the chemokines CCL2 and CCL7 by colonic CX3CR1$^{hi}$ resident macrophages and reduced the accumulation of CX3CR1$^{int}$ proinflammatory MPhs in colitis-affected colon tissue. Mice that received adeno-associated virus–shFbxw7 (AAV-shFbxw7) showed significantly improved survival rates and alleviation of colitis. Mechanism screening demonstrated that FBXW7 suppressed H3K27me3 modification and promoted Ccl2 and Ccl7 expression via degradation of the histone-lysine N-methyltransferase enhancer of zeste homolog 2 (EZH2) in macrophages. Taken together, our results indicate that FBXW7 degrades EZH2 and increases Ccl2 and Ccl7 in CX3CR1$^{hi}$ macrophages, thereby promoting the recruitment of CX3CR1$^{int}$ proinflammatory MPhs into local colon tissues with colitis. Targeting FBXW7 might represent a potential therapeutic approach for the treatment of intestinal inflammation.

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Resident and inflammatory mononuclear phagocytes (MPhs) with functional plasticity in the intestine are critically involved in the pathology of inflammatory bowel diseases (IBDs), the mechanism of which remains incompletely understood. In the present study, we found that increased expression of the E3 ligase F-box and WD repeat domain–containing 7 (FBXW7) in the inflamed intestine was significantly correlated with IBD severity in both human diseases and in mouse models. Myeloid Fbxw7 deficiency protected mice from colitis induced by dextran sodium sulfate (DSS) or 2,6,4-trinitrobenzene sulfonic acid (TNBS). Fbxw7 deficiency resulted in decreased production of the chemokines CCL2 and CCL7 by colonic CX3CR1hi resident macrophages and reduced the accumulation of CX3CR1int proinflammatory MPhs in colitis–affected colon tissue. Mice that received adeno-associated virus–shFbxw7 (AAV-shFbxw7) showed significantly improved survival rates and alleviation of colitis. Mechanism screening demonstrated that FBXW7 suppressed H3K27me3 modification and promoted Ccl2 and Ccl7 expression via degradation of the histone-lysine N-methyltransferase enhancer of zeste homolog 2 (EZH2) in macrophages. Taken together, our results indicate that FBXW7 degrades EZH2 and increases Ccl2 and Ccl7 in CX3CR1hi macrophages, thereby promoting the recruitment of CX3CR1int proinflammatory MPhs into local colon tissues with colitis. Targeting FBXW7 might represent a potential therapeutic approach for the treatment of intestinal inflammation.

Introduction

Chronic and progressive inflammation is the key pathogenesis of inflammatory bowel disease (IBD) including Crohn’s disease (CD) and ulcerative colitis (UC), which involve dysregulation of the genetic, environmental, or microbial factors and immune responses. A macrophage-induced innate immune response within the intestinal mucosa is the first line of defense against the invading pathogens (1–3). Inefficient or overactivation of gastrointestinal macrophage subsets participate in IBD development by regulating the initiation, amplification, and resolution of local inflammation (4–6).

Monocyte–derived mononuclear phagocytes (MPhs) in colonic lamina propria (CLP) express certain levels of the chemokine CX3C receptor CX3CR1 and CD11b (7, 8). In a healthy colon, Ly6Cint (Ly6C high-positive) monocytes mainly give rise to CX3CR1hi (CX3CR1 high-positive) resident macrophages that contribute to the maintenance of gut homeostasis and protect the host from certain pathogens (9, 10). However, the phenotype and fate of mucosal monocyte–derived MPhs change dramatically in inflammatory environments. Under these circumstances, CX3CR1int resident macrophages that dominate in the healthy colon are replaced by inflammation-elicited plastic CX3CR1int (CX3CR1 intermediate-positive) MPhs that are the progeny of rapidly infiltrating Ly6Cint circulating peripheral blood monocytes (11), which express higher levels of proinflammatory mediators and TLRs, NO, reactive oxygen intermediates, cathepsins, and metalloproteases than do their resident counterparts, forming an intense infiltration into CLP. As a result, CX3CR1int proinflammatory MPhs aggravate the intestinal inflammatory response and play crucial roles in the pathogenesis of CD and UC (7). Although the cells in this MPh pool are on a differentiation waterfall that may include cell populations that have predominantly more macrophage-like functions, there are also a small number of cells with DC-like functions (10). Therefore, herein, we refer to CLP CX3CR1int macrophages as CX3CR1int MPhs.

The infiltration of CX3CR1int proinflammatory MPhs can be triggered by dysregulated expression of chemokines in inflamed colon tissues through recruitment of Ly6Cint monocytes, which differentiate into CX3CR1int proinflammatory MPhs and produce proinflammatory cytokines including IL-6 and TNF-α (12, 13). Chemokines are produced by a wide variety of cells, including the inflammatory cells present in IBD lesions, fibroblasts, and endothelial and epithelial cells in the gastrointestinal system. The potent capacity of macrophages to secrete chemokines to mediate immune cell recruitment is critical for the inflammation cascade and has been demonstrated in previous studies (14–16). It has been reported that the increase of inflammatory macrophages in patients with IBD who do not respond to anti–TNF-α therapy is associated with an upregulation of the TREM-1/CCL7/CCR2 axis (17). The induction of chemokines has been reported to

Authorship note: JH, YS, and GL contributed equally to this work.
Conflict of Interest: The authors have declared that no conflict of interest exists.
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Submitted: July 10, 2018; Accepted: June 18, 2019; Published: August 19, 2019.
Reference information: J Clin Invest. https://doi.org/10.1172/JCI123374.
occur via activation of the NF-κB signaling cascade (18–20), however, mechanisms by which the complex cytokine and chemokine network in the colonic microenvironment influences inflammatory Mph infiltration are not well defined and represent a fundamental gap in the understanding of homeostatic immune function and IBD development.

F-box and WD repeat domain–containing 7 (FBXW7) is a component of the SKP1, CUL1, and F-box protein type ubiquitin ligase (SCF) complex. Fbxw7 mutations have been identified in various types of cancers (21). FBXW7 is also reported to regulate lipid metabolism (22), target osteogenic and chondrogenic transcriptional factors (23), interact with parkin, and play important roles in Parkinson’s disease (24). Our previous study showed that FBXW7 is critical for promoting innate antiviral immunity by mediating the ubiquitination of SHP2 (25). However, the function and mechanisms of FBXW7 in inflammation responses have not been clarified. In this study, we found that FBXW7 expression was markedly increased in inflamed intestinal tissues from patients with UC or CD. Fbxw7 deficiency in myeloid cells reduced inflammation and disease severity in the colitis mouse model. FBXW7 promoted enhancer of zeste homolog 2 (EZH2) ubiquitination and methylation and disease severity in the colitis mouse model (26). However, EZH2 expression in myeloid cells was not associated with the severity of inflammation (27). These findings indicate that myeloid FBXW7 deficiency attenuates experimental colitis. To investigate the role of Fbxw7 in macrophages in colitis, LysM-Cre+ Fbxw7fl/fl (LysM+ Fbxw7fl/fl) mice and their control littermates (Fbxw7fl/fl) were subjected to acute colitis induction using 3% DSS. Colitis-induced macroscopic changes (body weight loss, diarrhea, and rectal bleeding) were significantly alleviated in the LysM+ Fbxw7fl/fl mice compared with Fbxw7fl/fl littermates (Figure 3A). LysM+ Fbxw7fl/fl mice sacrificed on day 9 displayed significantly longer colons (Figure 3B), milder epithelial damage, and decreased areas of mucosal ulceration (Figure 3C) compared with Fbxw7fl/fl littermates. Moreover, expression levels of the tight junction genes Cldn1, Cldn2, Ocln, and Tjp1 (Supplemental Figure 2B) and of TJP1 protein (Supplemental Figure 2C) were significantly higher in the epithelia of LysM+ Fbxw7fl/fl mice compared with Fbxw7fl/fl littermates after DSS treatment, which indicated that the epithelial barrier integrity was less disrupted in mice with myeloid-specific Fbxw7 deficiency. At the same time, LysM+ Fbxw7fl/fl mice showed significantly improved survival rates compared with Fbxw7fl/fl littermates after 4% DSS treatment (Figure 3D), indicating that Fbxw7 deficiency protects mice from DSS-induced colitis.

During the recovery period of intestinal inflammation, the rate of body weight gain was more rapid in LysM+ Fbxw7fl/fl mice than in Fbxw7fl/fl littermates (Figure 3E). Moreover, LysM+ Fbxw7fl/fl mice had longer colon lengths than did their Fbxw7fl/fl littermates (Figure 3F) on day 15. Similarly, TNBS-induced colon shortening, the disease activity index (DAI), body weight loss, and epithelial damage were also alleviated in LysM+ Fbxw7fl/fl mice compared with Fbxw7fl/fl littermates (Supplemental Figure 3, A–D). These findings indicate that myeloid Fbxw7 deficiency attenuates experimental colitis.

Fbxw7 deficiency decreases proinflammatory Mph accumulation. Microbiota-induced inflammation is critical for the regulation of intestinal homeostasis. To determine whether the decreased DSS colitis susceptibility in LysM+ Fbxw7fl/fl mice was mediated by shifts in gut microbiota, we analyzed the fecal microbiota composition in LysM+ Fbxw7fl/fl mice and Fbxw7fl/fl littermates. The relative abundance of bacteria at the phylum level and the species composition cluster according to operational taxonomic units (OTUs) among LysM+ Fbxw7fl/fl mice and Fbxw7fl/fl littermates are shown in Supplemental Figure 4A and Figure 4A, respectively. Moreover, microbial community diversity (Figure 4B) and microbial community composition (Supplemental Figure 4B) were not significantly different between LysM+ Fbxw7fl/fl mice and Fbxw7fl/fl littermates. Furthermore, fecal microbiota were transferred from LysM+ Fbxw7fl/fl mice into Fbxw7fl/fl mice by cohousing. At the end of the colitis induction period, cohoused LysM+ Fbxw7fl/fl mice still showed clear alleviation of DSS-induced colitis (Supplemental Figure 4, C–G) compared with cohoused Fbxw7fl/fl or single-housed Fbxw7fl/fl mice. These results demonstrated that decreased DSS-induced colitis susceptibility in LysM+ Fbxw7fl/fl mice was not due to the microbiome change.
We next investigated the changes in myeloid cell subpopulations in CLP during colitis development by analyzing CD11b+ CX3CR1hi resident macrophages and CD11b+CX3CR1int proinflammatory MPhs in the colon. We found that the accumulation of CX3CR1int cell population compared with Fbxw7fl/fl littermates during the colitis period (Figure 4, C and D). In addition, the percentage and number of Ly6C+CX3CR1int monocytes (Figure 4E) and the percentage of CD11b+Ly6G+ neutrophils in CLP (Supplemental Figure 5A) were lower in LysM+ Fbxw7fl/fl mice than in Fbxw7fl/fl mice at the later, but not the early, stage of colitis, whereas the accumulation of CD103+ or CD103+ DCs was similar in CLP of LysM+ Fbxw7fl/fl mice and their Fbxw7fl/fl littermates (Supplemental Figure 5B).

Given that neutrophils express high levels of Lyz2 and can be recruited to inflamed tissue to exert antimicrobial effects, the expression of Fbxw7 was also increased in peripheral blood and CLP neutrophils from mice with colitis compared with healthy mice. We then examined whether the attenuated colitis in LysM+ Fbxw7fl/fl mice was related to the neutrophils. We found that depletion of neutrophils by anti-Ly6G Ab could not abrogate the reduced inflammation phenotype of LysM+ Fbxw7fl/fl mice (Supplemental Figure 6, C–G). These findings indicated that the function of Fbxw7 in colitis was not dependent on neutrophils.

To further confirm that the function of Fbxw7 in colitis was dependent on macrophages, we depleted macrophages using clodronate-containing liposomes (CLs) (26, 27). Flow cytometric analysis confirmed a marked reduction in the percentages of local macrophages in mice treated with CLs (Supplemental Figure 7, A and B). The protective effect of myeloid...
as the production of inflammatory cytokines during colonic inflammation (Supplemental Figure 7D). We also confirmed the increased expression of \( \text{Fbxw7} \) in sorted CX3CR1 \text{hi} resident macrophages and CX3CR1 \text{int} MPhs from C57BL/6 mice with \( \text{Fbxw7} \) deficiency in colitis disappeared in the absence of macrophages compared with that seen in the control cohort (Figure 4, F–I), which was further confirmed by analysis of tight junction protein expression (Supplemental Figure 7C) as well as the production of inflammatory cytokines during colonic inflammation (Supplemental Figure 7D). We also confirmed the increased expression of \( \text{Fbxw7} \) in sorted CX3CR1 \text{hi} resident macrophages and CX3CR1 \text{int} MPhs from C57BL/6 mice with
therefore examined whether Fbxw7 deficiency decreases the expression of cytokines. An ELISA revealed comparable inflammatory cytokine production levels (TNF-α, IL-6, IL-10) in LysM + Fbxw7fl/fl and Fbxw7fl/fl colon tissue 5 days after DSS challenge (Figure 5A and Supplemental Figure 9A), and analysis of mRNA expression of cytokine genes in BMDMs from LysM + Fbxw7fl/fl and Fbxw7fl/fl mice revealed no significant differences (Supplemental Figure 9B). Local inflammation and tissue damage in UC and CD are affected by local expression of specific chemokines within IBD tissues (29), and DSS-induced colitis correlates directly with the CCR2-mediated accumulation of MPhs (30). To address the mechanisms involved in the downregulation of intestinal inflammation by myeloid deficiency of Fbxw7, we sorted CX3CR1 hi resident macrophages, CX3CR1int proinflammatory MPhs, and Ly6Chi monocyte populations. We found that CX3CR1 hi resident macrophages expressed markedly higher amounts of chemokines than did those in CX3CR1int proinflammatory MPhs and Ly6Chi monocyte populations. We found that CX3CR1hi resident macrophages expressed markedly higher amounts of chemokines than did those in CX3CR1int proinflammatory MPhs and Ly6Chi monocyte populations. We found that CX3CR1hi resident macrophages expressed markedly higher amounts of chemokines than did those in CX3CR1int proinflammatory MPhs and Ly6Chi monocyte populations. We found that CX3CR1hi resident macrophages expressed markedly higher amounts of chemokines than did those in CX3CR1int proinflammatory MPhs and Ly6Chi monocyte populations. We found that CX3CR1hi resident macrophages expressed markedly higher amounts of chemokines than did those in CX3CR1int proinflammatory MPhs and Ly6Chi monocyte populations. We found that CX3CR1hi resident macrophages expressed markedly higher amounts of chemokines than did those in CX3CR1int proinflammatory MPhs and Ly6C2hi monocyte populations. We found that CX3CR1hi resident macrophages expressed markedly higher amounts of chemokines than did those in CX3CR1int proinflammatory MPhs and Ly6C2hi monocyte populations. We found that CX3CR1hi resident macrophages expressed markedly higher amounts of chemokines than did those in CX3CR1int proinflammatory MPhs and Ly6C2hi monocyte populations. We found that CX3CR1hi resident macrophages expressed markedly higher amounts of chemokines than did those in CX3CR1int proinflammatory MPhs and Ly6C2hi monocyte populations. We found that CX3CR1hi resident macrophages expressed markedly higher amounts of chemokines than did those in CX3CR1int proinflammatory MPhs and Ly6C2hi monocyte populations. We found that CX3CR1hi resident macrophages expressed markedly higher amounts of chemokines than did those in CX3CR1int proinflammatory MPhs and Ly6C2hi monocyte populations. We found that CX3CR1hi resident macrophages expressed markedly higher amounts of chemokines than did those in CX3CR1int proinflammatory MPhs and Ly6C2hi monocyte populations. We found that CX3CR1hi resident macrophages expressed markedly higher amounts of chemokines than did those in CX3CR1int proinflammatory MPhs and Ly6C2hi monocyte populations. We found that CX3CR1hi resident macrophages expressed markedly higher amounts of chemokines than did those in CX3CR1int proinflammatory MPhs and Ly6C2hi monocyte populations. We found that CX3CR1hi resident macrophages expressed markedly higher amounts of chemokines than did those in CX3CR1int proinflammatory MPhs and Ly6C2hi monocyte populations.
**Figure 4.** Fbxw7 deficiency decreases the accumulation of CX3CR1\(^{hi}\) proinflammatory MPhs. (A) Venn diagram of Fbxw7\(^{fl/fl}\) (WT) and LysM\(^{+}\) Fbxw7\(^{fl/fl}\) (KO) groups showing unique and shared OTUs for bacterial sequences based on normalized sequences and 97% sequence similarity (n = 3). (B) Analysis of α diversity (observed species) in WT and KO groups (n = 3). The P value was calculated from analysis of an independent samples Student’s t test. (C) Representative flow cytometric plots gated on CD45\(^{+}\) living cells isolated from CLP from WT and KO mice on days 0, 3, 6, and 9 after DSS challenge. CD11b\(^{+}\)CX3CR1\(^{hi}\) resident macrophages (blue); CD11b\(^{+}\)CX3CR1\(^{int}\) MPhs (red). (D) Dynamics of CD11b\(^{+}\)CX3CR1\(^{hi}\) macrophages and CD11b\(^{+}\)CX3CR1\(^{int}\) MPh infiltration into CLP after DSS treatment (n = 5 for any time point). (E) Percentage and number of Ly6C\(^{hi}\)CX3CR1\(^{int}\) monocytes in inflamed colon after DSS treatment (n = 5 for any time point). Then, macrophages were depleted by CLs in WT and KO mice that were subjected to 3% DSS treatment for 7 days, and the mice were sacrificed on day 9. Diarrhea score (F) and body weight changes (G) were assessed daily (n = 5). (H) Gross morphology of colons from mice on day 9 after DSS treatment and colon length measurements (n = 5). (I) H&E-stained images of colon sections from mice with colitis. Scale bars: 200 μm (whole colon sections) and 50 μm (enlarged insets). (C–H) Data are expressed as the mean ± SEM and are representative of at least 3 independent experiments. *P < 0.05, **P < 0.01, and ***P < 0.001, by unpaired, 2-tailed Student’s t test.
expressed genes and also revealed that myeloid Fbxw7 deficiency led to markedly decreased expression of various inflammation-related genes, among which Ccl2 and Ccl7 are the most important (Figure 5B). The decrease in cytokine expression detected in colon tissue from LysM+ Fbxw7fl/fl mice at the later phase of colitis (9 days after DSS treatment) (Figure 5A) might be the consequence of the substantial decrease in inflammatory MPF infiltration. Compared with Fbxw7fl/fl littermates, LysM+ Fbxw7fl/fl mice had markedly lower expression levels of Ccl2 and Ccl7 in the resident macrophage population, whose functions were mainly involved in the recruitment of monocytes (Figure 5C). Quantitative reverse transcription PCR (qRT-PCR) (Supplemental Figure 9, D and E) and ELISA (Figure 5D and Supplemental Figure 9F) further confirmed that myeloid deficiency of Fbxw7 resulted in lower levels of CCL2 and CCL7 as well as the altered expression of their related genes, among which Ccl2 and Ccl7 were decreased in the culture supernatant of CX3CR1 hi resident macrophages recruited fewer monocytes than did macrophages from Fbxw7fl/fl littermates in a Transwell assay (Figure 5E and Supplemental Figure 10C). Furthermore, CCL2 and CCL7 production in monocytes and macrophages from Fbxw7fl/fl mice had markedly lower expression levels of Ccl2 and Ccl7 in the resident macrophage population, whose functions were mainly involved in the recruitment of monocytes (Figure 5C). Quantitative reverse transcription PCR (qRT-PCR) (Supplemental Figure 9, D and E) and ELISA (Figure 5D and Supplemental Figure 9F) further confirmed that myeloid deficiency of Fbxw7 resulted in lower levels of CCL2 and CCL7. We also observed a significant correlation between Fbxw7 and Ccl2 mRNA and Ccl2 mRNA levels in colonic mucosal samples from patients with UC in the GEO database (Supplemental Figure 9G). Collectively, these results suggested that myeloid Fbxw7 deficiency decreases the production of Ccl2 and Ccl7 in resident macrophages.

Monocyte migration from peripheral blood during infection or inflammation requires CCL2/CCL7/CCR2 signaling (31); therefore, we performed a Transwell migration assay to compare the chemoattractive function of resident macrophages from LysM+ Fbxw7fl/fl mice and Fbxw7fl/fl littermates in vitro (Supplemental Figure 10, A and B) and found that LysM+ Fbxw7fl/fl resident macrophages recruited fewer monocytes than did macrophages from Fbxw7fl/fl littermates in a Transwell assay (Figure 5E and Supplemental Figure 10C). Furthermore, CCL2 and CCL7 production decreased in the culture supernatant of CX3CR1hi resident macrophages from LysM+ Fbxw7fl/fl mice compared with macrophages from Fbxw7fl/fl littermates (Figure 5F). Compared with resident macrophages, the culture supernatant of proinflammatory MPs from both LysM+ Fbxw7fl/fl and Fbxw7fl/fl littermate mice showed a weaker ability of the macrophages to recruit monocytes (Figure 5E and Supplemental Figure 10C) with lower levels of chemokines (Figure 5F). Importantly, neutralization of CCL2 and CCL7 abrogated the difference in the monocyte-recruiting ability of Fbxw7fl/fl and LysM+ Fbxw7fl/fl resident macrophages (Figure 5G and Supplemental Figure 10D). This observation suggested that decreased production of CCL2 and CCL7 in LysM+ Fbxw7fl/fl resident macrophages contributed to the reduced recruitment of Ly6Chi monocytes. Furthermore, anti-CCL2 and anti-CCL7 Abs decreased the number of inflammatory MPs that infiltrated into the colon of Fbxw7fl/fl mice (Supplemental Figure 11, A and B) and abrogated the alleviated colitis phenotype of LysM+ Fbxw7fl/fl mice (Figure 5, H and I, and Supplemental Figure 11C, D, and E). Moreover, we adoptively transferred mixed Fbxw7fl/fl and LysM+ Fbxw7fl/fl monocytic Fbxw7fl/fl recipient mice (Supplemental Figure 11E). Flow cytometric analysis revealed a failure of monocyte recruitment in the inflamed colon of LysM+ Fbxw7fl/fl recipient mice when compared with their Fbxw7fl/fl littermates (Figure 5J). Collectively, these data further support the idea that Fbxw7 deficiency decreases the expression of CCL2 and CCL7 in resident macrophages and thereby reduces the accumulation of proinflammatory MPs during colitis progression.

*Fbxw7 interacts with EZH2.* To identify the mechanisms regulating CCL2 and CCL7 production in colon-resident macrophages, we assessed the activation of NF-κB and HIF-1α (32, 33) signaling pathways in BMDMs during the LPS-induced response. We found no potent difference in these signaling pathways between Fbxw7fl/fl and LysM+ Fbxw7fl/fl BMDMs after LPS stimulation (Figure 6, A and B). These results raise the possibility of epigenetic regulation of the Ccl2 and Ccl7 genes in response to inflammatory stimuli. We next assessed changes in histone modifications and found that the modification of H3K27me3 was markedly increased in LysM+ Fbxw7fl/fl BMDMs compared with Fbxw7fl/fl BMDMs after LPS stimulation (Figure 6C). EZH2 was found to be an important enzyme for H3K27me3 (34), and we observed a decrease in the expression of EZH2 protein in BMDMs after LPS challenge (Supplemental Figure 12A). The expression of EZH2 was also decreased in CD11b+ macrophages from CLP mice when compared with macrophages from healthy controls (Supplemental Figure 12B). We speculated that EZH2 protein expression negatively correlates with Fboxw7 levels in CLP macrophages. We found higher EZH2 protein expression levels in Fbxw7-deficient BMDMs than in Fbxw7fl/fl BMDMs after LPS stimulation (Figure 6D). Furthermore, the interaction of endogenous EZH2 and FBXW7 in BMDMs was increased after LPS challenge (Figure 6E). We consistently found that FBXW7 colocalized with EZH2 upon LPS stimulation in BMDMs (Figure 6F).

FBXW7 recognizes and binds to substrates through a stretch of eight WD40 repeat domains (34), and the binding consensus motif (T/S)PXX(S/T/D/E) has been identified in several FBXW7 substrates, including c-Myc, cyclin E1, and c-Jun (36–38). Protein motif analysis showed that 2 domains in the EZH2 protein sequence might be the FBXW7 binding motif (Figure 6G). To determine whether those amino acid sequences were required for FBXW7 recognition and ubiquitination, we constructed 3 mutants of EZH2, in which serine residues at positions 261, 265, and/or 367, and 371 were replaced with glycine residues. The results showed that mutant EZH2 (261, 265A) and mutant EZH2 (367, 371A) bound to FBXW7 less efficiently than did EZH2, and that mutant EZH2 (261, 265, 367, and 371) could not bind to FBXW7 (Figure 6H). These results strongly suggest that the serine at positions 261, 265, 367, and 371 of EZH2 was critical for its interaction with FBXW7.

*FBXW7 mediates the degradation and ubiquitination of EZH2.* We confirmed that EZH2 was the target of FBXW7 in macrophages, as there was no significant difference between Fbxw7fl/fl and LysM+ Fbxw7fl/fl BMDMs in terms of Ez2 mRNA expression levels upon LPS stimulation (Supplemental Figure 12C). However, we found that the decrease in EZH2 protein expression after LPS stimulation (Supplemental Figure 12A) was completely blocked by the proteasome inhibitor MG132 (Figure 7A). A cycloheximide chase assay showed that FBXW7 deficiency extended the half-life of endogenous EZH2 protein in BMDMs (Figure 7, B and C). These data indicated that FBXW7 might induce EZH2 degradation through proteasomes. Exogenously expressed EZH2 was degraded by overexpressed FBXW7 in HEK293 cells, which was also abrogated by MG132 (Figure 7D).
Figure 5. Fbxw7 deficiency downregulates CCL2 and CCL7 in resident macrophages. (A) Colon tissue was cultured overnight, and cytokines in the supernatant were measured by ELISA (n = 5). (B) Heatmap showing expression changes of inflammation-related genes in BMDMs from LysM+ Fbxw7fl/fl mice and Fbxw7fl/fl littermates. The expression profile is accompanied by a colored bar indicating the standardized log2 intensities. BMDMs were isolated from LysM+ Fbxw7fl/fl mice and Fbxw7fl/fl littermates and stimulated with LPS (100 ng/mL) for the transcriptomic assay. (C) CD11b+ CX3CR1hi resident macrophages and CD11b+ CX3CR1int proinflammatory MPhs were sorted from CLP of Fbxw7fl/fl (WT) and LysM+ Fbxw7fl/fl (KO) mice (n = 5) after 5 days of DSS or H2O administration, and gene expression levels were quantified by gene-specific amplification and qRT-PCR. The ratio of Ccl2 and Ccl7 expression in CX3CR1hi resident macrophages to Ccl2 and Ccl7 expression in CX3CR1int MPhs is shown. (D) Colonic tissues of WT and KO mice treated with DSS were cultured overnight, CCL2 and CCL7 expression in the supernatant was measured by ELISA (n = 5). (E) In a Transwell assay, the number of monocytes in the lower chamber recruited by supernatant of CLP-derived CX3CR1hi resident macrophages and CX3CR1int proinflammatory MPhs was counted by flow cytometry (n = 5). (F) Concentration of CCL2 and CCL7 in the culture medium of CX3CR1hi macrophages and CX3CR1int MPhs (n = 5). (G) Number of monocytes in the lower chamber recruited by supernatant of CLP-derived CX3CR1hi resident macrophages and CX3CR1int proinflammatory MPhs was counted by flow cytometry (n = 5). (H) Gross morphology of colons from Fbxw7fl/fl and LysM+ Fbxw7fl/fl mice, with or without anti-CCL2 and anti-CCL7 Ab treatment (n = 5). (I) Body weight changes were assessed daily (n = 5). (J) The percentages of labeled WT and KO monocytes gated on CD45+Ly6Chi living cells from DSS-challenged recipient mice (n = 5) were analyzed by flow cytometry. Graph shows a summary of the percentages of labeled WT monocytes recruited to the CLP of WT and KO recipient mice on day 5 or 9 after DSS treatment. Data are presented as the mean ± SD and are representative of 3 independent experiments. *P < 0.05, **P < 0.01, and ***P < 0.001, by unpaired, 2-tailed Student’s t test.
ubiquitination of EZH2 was mediated by FBXW7 in a dose-dependent manner (Figure 7F). FBXW7 could induce K48-linked polyubiquitination of EZH2 (Figure 7G). Furthermore, FBXW7 deficiency in macrophages almost canceled K48-linked polyubiquitination of endogenous EZH2 compared with WT. However, FBXW7 could not decrease the expression of mutant EZH2 (position 4A) (Figure 7E). Collectively, these results indicated that EZH2 could be degraded by FBXW7 via the ubiquitin-proteasome system. Our study to determine whether FBXW7 functions as an E3 ubiquitin ligase of EZH2 revealed that the ubiquitination of EZH2 was mediated by FBXW7 in a dose-dependent manner (Figure 7F). FBXW7 could induce K48-linked polyubiquitination of EZH2 (Figure 7G). Furthermore, FBXW7 deficiency in macrophages almost canceled K48-linked polyubiquitination of endogenous EZH2 compared with WT.
mal promoter of the Ccl2 gene in a clock gene BMAL1–dependent manner (39). We hypothesized that FBXW7 increases the expression of CCL2 and CCL7 chemokines by decreasing the protein levels of EZH2. 

Ezh2 was knocked down by Ezh2 siRNA (siEzh2) in BMDMs, siEzh2 reduced expression of EZH2 and H3K27me3 

macrophages (Figure 7H). Together, these data demonstrate that FBXW7 promotes K48-linked polyubiquitination and proteasome degradation of EZH2 in macrophages.

EZH2 inhibits macrophage Ccl2 and Ccl7 expression by H3K27me3 modification. EZH2 has been shown to be recruited to the proximal promoter of the Ccl2 gene in a clock gene BMAL1–dependent manner (39). We hypothesized that FBXW7 increases the expression of CCL2 and CCL7 chemokines by decreasing the protein levels of EZH2. Ezh2 was knocked down by Ezh2 siRNA (siEzh2) in BMDMs, siEzh2 reduced expression of EZH2 and H3K27me3.
The GFP-pAAV-shFbxw7 vector was verified in C57BL/6 mice by immunofluorescence (Supplemental Figure 13A) and qRT-PCR (Supplemental Figure 13B). AAV-shFbxw7 treatment decreased mucosal ulcerations in Il10−/− colitis mice (Figure 9, A and B). In the DSS-induced colitis model, AAV-shFbxw7–treated C57BL/6 mice had less body weight loss, a lower DAI (Figure 9C), and an alleviated colon phenotype and shortening (Figure 9, D and E) when compared with AAV-shNC mice. Moreover, AAV-shFbxw7–treated mice showed significantly decreased expression of Ccl2 and Ccl7 in colon tissues, but significant change in expression of Ccr2 and Cx3cl1 (Figure 9F). Furthermore, AAV-shFbxw7 treatment significantly improved the survival of mice with colitis (Figure 9G) compared with AAV-shNC–treated mice. Altogether, these results suggest that AAV-mediated colonic Fbxw7 silencing protects mice with colitis against inflammation and disease progression by downregulating Ccl2 and Ccl7 expression.

Discussion

The E3 ubiquitin ligase FBXW7 is mutated in approximately 10% of human colon cancers; it acts as a tumor suppressor in several tissues and targets multiple transcriptional activators and proto-oncogenes for ubiquitin-mediated degradation (41). However, the role of FBXW7 in inflammation and the immune response remains unknown. In this study, we revealed a mechanism for the mediation of IBD progression, whereby FBXW7 promotes Ccl2 and Ccl7 expression.
Furthermore, it is highly probable that the disappearance of the difference in colitis severity between Fbxw7 fl/fl and LysM+ Fbxw7 fl/fl mice was due to the elimination of macrophages. Altered profiles of inflammatory molecules and inflammation-related signaling pathways involving cytokines, chemokines, inflammasomes, antimicrobial peptides, and neuropeptides are all involved in the pathogenesis of CD and UC (3). The degree of local inflammation and tissue damage in UC and CD is dependent on the local expression of specific chemokines within IBD tissues. GWAS have pointed to several IBD susceptibility loci that contain genes that encode proteins involved in chemokine signaling, including CC-chemokine receptor 6 (CCR6), CCL2, and CCL13.

A recent study has reported that treatment of intestinal crypts with supernatants of commensal-specific T cells from patients with CD induced higher protein secretion of the chemokines CXCL8 and CXCL1 and prompted a predominant neutrophil and Th17 recruitment to the intestinal epithelium (43). The expression of Il17c mRNA has been shown to correlate with CCL20 in the inflamed expression through the degradation of EZH2 in CX3CR1hi resident macrophages, which results in increased recruitment of CX3CR1int MPhs and amplification of intestinal inflammation. We believe our work provides new insights into the molecular and cellular networks of the intestinal inflammatory microenvironment.

A constant balance between resident macrophages and inflammatory MPhs is critical for maintaining homeostasis in a healthy gut and ensuring protective immunity when required. Interestingly, we noticed a more marked reduction of CD11b+CX3CR1hi resident macrophages compared with CD11b+CX3CR1int inflammatory MPhs after liposome injection. This preferential ability may be due to the greater phagocytic activity of CD11b+CX3CR1hi resident macrophages (42), which are key players in the capture and destruction of invading pathogens, as well as in the clearance of apoptotic or senescent cells. Our findings suggested that the increased susceptibility to DSS-induced colitis of both Fbxw7 fl/fl and LysM+ Fbxw7 fl/fl mice after liposome treatment was related to the depletion of resident macrophages in the colon.

Figure 9. Decreased expression of Fbxw7 relieves experimental colitis. (A) H&E-stained images of colon sections from 12-week-old Il10−/− mice injected with AAV weekly from 5 weeks until 9 weeks of age (n = 7) and their (B) histopathological colitis scores. Scale bars: 50 μm. (C) Body weight changes and DAI for healthy controls and C57BL/6 mice with DSS-induced colitis were assessed daily (n = 5). (D) Gross morphology of colons from healthy mice and mice with colitis sacrificed on day 9. (E) Colon length measurements (n = 7). (F) Colon tissue from mice sacrificed on day 7 was subjected to qRT-PCR to determine mRNA expression levels of chemokines and their ligands (n ≥6). Data are presented as the mean ± SD and are representative of at least 3 independent experiments. *P < 0.05, **P < 0.01, and ***P < 0.001, by unpaired, 2-tailed Student’s t test (B, C, and F). (G) AAV-shNC- and AAV-shFb-treated mice were administered 4% DSS to induce acute colitis. Survival of mice was monitored until day 15 (n = 5). ***P < 0.001, by log-rank test. Data are representative of 3 independent experiments.
CCL2 and CCL7 produced by resident macrophages from reported in tumorigenesis (53), but it remains largely unknown relationship between FBXW7 and epigenetic modifiers has been expression in resident macrophages upon DSS challenge. The interaction mediates the function of FBXW7 in reducing EZH2 is essential for the interaction of EZH2 with FBXW7 and that this provide evidence that threonine at positions 261 and 367 of EZH2 has been shown to affect TNF-α signaling and promote the inflammatory response in intestinal epithelial cells in colitis (52). We have demonstrated that expression of the cytokines IL-6 and IFN-γ in primary macrophages during the innate immune response through demethylation of H3K27me3 at the Il16 and Ifnb promoters (50). A selective H3K27 demethylase inhibitor was also demonstrated to reduce LPS-induced proinflammatory cytokine production by human primary macrophages (51). EZH2, acting as a catalytic subunit of PRC2, is critical in mediating the formation of H3K27me3, which is associated with transcriptional gene suppression (34). Ezh2 deficiency has been shown to affect TNF-α signaling and promote the inflammatory response in intestinal epithelial cells in colitis (52). We provide evidence that threonine at positions 261 and 367 of EZH2 is essential for the interaction of EZH2 with FBXW7 and that this interaction mediates the function of FBXW7 in reducing EZH2 expression in resident macrophages upon DSS challenge. The relationship between FBXW7 and epigenetic modifiers has been reported in tumorigenesis (53), but it remains largely unknown in inflammation. Here, we have demonstrated that expression of CCL2 and CCL7 produced by resident macrophages from LysM-Fbxw7fl/fl mice was lower than that in macrophages from Fbxw7fl/fl littermates and that a selective inhibitor of H3K27me3 in macrophages had the effect of promoting Ccl2 and Ccl7 expression.

In summary, we identified a critical role of FBXW7 in the regulation of colitis by inducing Ccl2 and Ccl7 expression in resident macrophages and promoting the accumulation of proinflammatory MPhs. FBXW7/EZH2/CCL2/CCL7 is an important pathway for shaping the status and activity of colon-resident macrophages and inflammatory MPhs in the colitis setting. Our results indicate that FBXW7 may be a predictive marker of IBD severity and a potential target for IBD treatment, since Fbxw7 siRNA had a significant therapeutic effect in our colitis mouse model.

Methods

**Mice and reagents.** Fbxw7fl/fl mice (on a C57BL/6 background) were obtained from The Jackson Laboratory. LysM-Cre mice on a C57BL/6 background were provided by Ximei Wu (Zhejiang University School of Medicine, Hangzhou, China). Iii10−/− mice were provided by Yanmei Han (National Key Laboratory of Medical Immunology and Institute of Immunology, Second Military Medical University, Shanghai, China). All mice were bred at the Zhejiang University Laboratory Animal Center under specific pathogen-free conditions. C57BL/6 mice were purchased from the Model Animal Research Center of Nanjing University (Nanjing, China).

Mice were born and bred at the same facility and kept on the same rack in an animal housing room that was maintained under specific pathogen-free conditions with a controlled temperature (22°C) and photoperiod (12-hour light/12-hour dark cycle) and unrestricted access to standard mouse chow and water, except for mice used in some experiments described herein. The cohousing experiments were conducted with weaned 3-week-old Fbxw7fl/fl mice that were randomly cohoused with age-matched LysM-Fbxw7fl/fl mice at a 1:1 ratio for 7 weeks prior to DSS colitis induction, which was performed during the cohousing period.

Abs against the HA tag (sc-805), Myc tag (sc-40; sc-789), Flag tag (sc-807), GAPDH (sc-130619), and ubiquitin (sc-271289) were obtained from Santa Cruz Biotechnology. Abs specific for p-p65 (product no. 3033), p65 (product no. 8242), EZH2 (product no. 3147), and HIF-1α (product no. 36169) were from Cell Signaling Technology. Lys48-specific linked polyubiquitin Ab (product no. 05-1307) was from MilliporeSigma. Abs against FBXW7 were obtained from Abcam (ab12292) and Thermo Fisher Scientific (40-1500). Abs against H3K36me3 (ab9050), H3K9me3 (ab8989), H3K4me3 (ab8580), H3K27me3 (ab6002), and H3 (ab1791) were from purchased from Abcam. GM132 (M8699), CHX (C4859), and Flag-M2 Magnetic Beads (product no. M8823) were from MilliporeSigma. ELISA kits for mouse IL-6 (BMS603-0), TNF-α (BMS607-3), CCL2 (BMS6005), and CCL7 (BMS6006) were purchased from eBioscience.

**Human peripheral blood samples.** A total of 68 peripheral blood samples were collected from patients with IBD hospitalized in Sir Run Shaw Hospital (Zhejiang University School of Medicine, China) from May 11, 2017 to August 1, 2017, and from 50 healthy adults as a control group. The diagnosis and IBD severity evaluation were made according to the 2012 Expert Consensus Document on the Diagnosis and Treatment of IBD formulated by the Chinese Society for Gastroenterology (CSGE) (54). The severity of CD was determined using the Best CD activity index (CDAI) calculator (55). The severity of UC disease was determined by the Mayo Score with composite indices (56). Basic information concerning the patients, including age and sex, is summarized in Supplemental Table 1. Monocytes were isolated using a human monocyte isolation kit (Haoyang Biological Technology Co.) according to the manufacturer’s recommendations.

**Randomization method.** The following covariates for mice were controlled: age, sex, littermate status, and age (8–10 weeks). Adult male mice and their littermates were used. Under such conditions, the mice were randomly assigned to experimental groups. For studies using media collected from stimulated cells or tissues, samples were randomly allocated to ELISA plate wells prior to measurement. Random fields were analyzed in confocal H&E-stained and immunohistochemical images.

**Fecal microbiome analysis.** LysM-Fbxw7fl/fl mice and Fbxw7fl/fl littermates (8–to-10-week-old male or female C57BL/6J mice) were fed in different cages. The mice were kept in the same environment with the other experimental mice. Freshly formed feces were collected from the cage and weighed. Bacterial DNA was extracted from the stool.
Supernatants were collected and kept frozen until assessment. CCL2 and CCL7 levels in the supernatant were detected by conventional double-sandwich ELISA (BD Biosciences).}

**Migration assay.** Sorted splenic Ly6C\(^{hi}\) monocytes (1 × 10\(^4\)) from Fbxw7\(^{fl/fl}\) and LysM\(^{−/−}\) mice were added into the upper compartment of the Transwell chambers (24-well plates, 8-mm pores, BD Biosciences) in 100 μL serum-free medium. Sorted CX3CR1\(^{hi}\) resident M1Ps (5 × 10\(^5\)) from CLP of Fbxw7\(^{fl/fl}\) and LysM\(^{−/−}\) mice were fixed with 10% formalin and stained with DAPI.

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shown to result in a partial reduction of macrophages (63). Controls included i.p. injections of 200 μL PBS.

Preparation of mouse neutrophils from peripheral blood. Mouse blood (350 ± 50 μL per animal) was collected by tail bleeding into HBSS-EDTA (HBSS without calcium, magnesium, phenol red, and sodium bicarbonate; pH 7.2, 15 mM EDTA, BSA, 1%). Neutrophils were isolated using Percoll-based density gradient centrifugation (64) (350,000 ± 40,000 cells were obtained per mouse, 97% of which were neutrophils).

Neutrophil depletion. Every 48 hours on days -1, 1, 3, and 5 during DSS treatment, mice were i.p. injected with 400 μg anti-Ly6G mAb (BioLegend, 1A8) or rat IgG2a isotype control mAb (BioLegend, RTK2758) diluted in sterile PBS. For confirmation of depletion during the colitis experiments, colons from anti-Ly6G-treated and isotype control–treated mice in health or with colitis were harvested for each independent experiment and analyzed by flow cytometry for a reduction in CD45+CD11b+Gr-1+ cells.

Immunohistochemical and immunofluorescence staining. Colon tissues were fixed in 4% formalin, and immunohistochemical and immunofluorescence staining was performed for human and mouse colon sections at the Histomorphology Platform of Zhejiang University, following the manufacturer’s standard protocol (reagents purchased from Beijing Zhongshan Jinqiao Biotechnology Company). Human colon specimens were scored using Constantine’s protocol (66), under high magnification, and integrated staining intensity and the percentage of positive cells were scored.

Colitis analysis. Colons were collected immediately after the mice were sacrificed. The entire colon (from the cecum to the anus) was removed and measured, and colon lengths were reported as described previously (66). The distal colon was removed, fixed in 10% formalin, and the tissue sections were paraffin embedded and stained with H&E for light microscopic examination to assess colon injury and inflammation. The degree of colitis was scored without any prior knowledge of the experimental groups as follows (67): degree of epithelial regeneration (scale of 0–3), distorsion/branching (scale of 0–3), inflammation (scale of 0–3) and crypt damage (scale of 0–4), percentage of area with inflammation (scale of 0–4) and crypt damage (scale of 0–4), and depth of inflammation (scale of 0–3). The total score ranged from 0 points (no colitis) to 24 points (severe colitis).

In TNBS-induced colitis, the DAI (scale of 0 to 4) was monitored daily and used to assess the severity of colitis on the basis of weight loss, blood in the stool, and stool consistency according to described previously methods (68).

Plasmid constructs and transfection. Recombinant vectors encoding mouse Ezh2 were created by PCR-based amplification of complementary DNA from murine BM cells, followed by subcloning into the Pcmv-Tag2B eukaryotic expression vector (Invitrogen, Thermo Fisher Scientific). All constructs were confirmed by DNA sequencing. The plasmids were transfected into HEK293T cells with JetPrime (Polyplus). Primary macrophages were transfected with siRNA through INTERFERin Reagent (Polyplus) according to the standard protocol. BMDMs were transfected with siRNA using INTERFERin Reagent (Polyplus) according to the standard protocol. The Ezh2 siRNA sequences were 5’-GCAAAGCTGGTGTGCAG-3′, 5’-GCAAAGCTGGTGTGCAG-3′, and 5’-CACAAAGCTGGTGTGCAG-3′ (Life Technologies, Thermo Fisher Scientific).

Immunoprecipitation and immunoblot analyses. SDS-PAGE and immunoblot analyses were performed as described previously (25). BMDMs were immunoprecipitated using anti-FBXW7 plus protein A/G agarose. The proteins were then separated using SDS-PAGE and subjected to immunoblot analysis with anti-EZH2 or anti-FBXW7 Abs.

ChIP analysis. ChIP analysis was done using H3K27me3 (Abcam, ab6002), as described previously (69), and the process was performed according to the manufacturer’s instructions for the ChIP Assay Kit (Cell Signaling Technology). Fold enrichment was quantified using qRT-PCR and calculated as a percentage of input chromatin (percentage of input).

RNA-Seq. Total RNA from BMDMs (1 × 10⁶) was extracted using TRIzol (Takara). Preparation of the library and transcriptomic sequencing were carried out using the Illumina HiSeq ×Ten (Novogene Bioinformatics Technology). Mapping of 100-bp paired-end reads to genes was done using HTSeq software (version 0.6.0), and fragments per kilobase of transcript per million fragments mapped (FPKM) were also analyzed. RNA-Seq data are available in the NCBI’s Gene Expression Omnibus (GEO) repository (GEO GSE125242).

pAAV-EGFP administration. Mice were fasted overnight and pretreated with 20 mM N-acetyl-L-cysteine (NAC) (MilliporeSigma) as described previously (70) and then anesthetized with chloral hydrate by i.p. injection. The colon was washed with an intrarectal injection of 100 μL of 20 mM NAC using a stainless-steel straight, round-tipped microsyringe and allowed to drain for 15 minutes, and this step was repeated twice. Next, mice were reanesthetized, and 5 × 10¹⁰ physical particles of pAAV-EGFP in 100 μL PBS were administered by enema, and tail-vein injection of pAAV-EGFP was given once a week for 3 weeks. The transgene plasmids used were p-AAV-CMV-EGFP and p-AAV-shFbxw7 (U6-shNC/shFbxw7-mir30arm) and were generated by Shanghai SunBio Medical Biotechnology.

Statistics. Statistical analysis was performed with GraphPad Prism software. All data are expressed as the mean ± SEM or SD. All experiments were repeated independently at least 3 times. Statistical significance between 2 experimental groups was determined using an unpaired, 2-tailed Student’s t test. Differences with a P value of less than 0.05 were considered statistically significant. Three or more groups were compared with ANOVA. Multiple comparisons between variables were assessed by 1-way ANOVA with Tukey’s multiple comparisons test. For the mouse survival study, Kaplan-Meier survival curves were generated, and a log-rank test (Mantel-Cox) was used to determine statistical significance.

Study approval. Written patient consent was provided, and ethics approval for the use of human samples was granted by the Medical Ethics Committee of Zhejiang University School of Medicine before harvesting of human tissue and blood samples. All animal research was performed under a protocol approved by the Medical Experimental Animal Care Commission of Zhejiang University.

Author contributions. QW, LL, and JH designed the research. JH, YS, GL, YL, XT, TP, ZJ, and LL performed experiments and acquired and analyzed data. PX and QC provided clinical specimens. JH, LL, and QW wrote the manuscript. XC supervised the study. All authors read and approved the final manuscript.
Acknowledgments

This work was supported by grants from the National Natural Science Foundation of China (81771699, 31870907, 81715124); the National Program on Key Basic Research Project (2014CB542101); and the Natural Science Foundation of Zhejiang Province (Z19H100001).

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