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Phosphatase Shp2 exacerbates intestinal inflammation by disrupting macrophage responsiveness to interleukin-10

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Inflammatory cytokines produced by activated macrophages largely contribute to the pathological signs of inflammatory bowel disease (IBD). Interleukin-10 (IL-10) is the predominant anti-inflammatory cytokine in the intestine, and its therapeutic efficacy for IBD has been clinically tested. Nevertheless, how the function of IL-10 is regulated in the intestinal microenvironment remains unknown, which largely hinders the further development of IL-10-based therapeutic strategies. Here, we found that the expression of phosphatase Shp2 was increased in colonic macrophages and blood monocytes from IBD patients compared with those from healthy controls. Shp2 deficiency in macrophages protects mice from colitis and colitis-driven colon cancer. Mechanistically, Shp2 disrupts IL-10–STAT3 signaling and its dependent anti-inflammatory response in human and mouse macrophages. Furthermore, a Shp2-inducing role of TNF- α is unveiled in our study. Collectively, our work identifies Shp2 as a detrimental factor for intestinal immune homeostasis and hopefully will be helpful in the future exploitation of IL-10 immunotherapy for IBD.

Introduction

Inflammatory bowel disease (IBD) is characterized by chronic and recurrent intestinal inflammation with symptoms of abdominal pain, diarrhea, fatigue, or maldigestion. The two major types of IBD are Crohn's disease (CD) and ulcerative colitis (UC), differing mainly in their sites and distribution of inflammation along the digestive tract.

The lumen of the intestine, especially the large intestine is inhabited by $\sim 10^{14}$ commensals with >500 species. These microbes and intestinal lamina propria are merely separated by a single layer of epithelial cells, the breakage of which frequently leads to the invasion of microbes and the subsequent activation of the intestinal immune system, resulting in the initiation of IBD. Thus, it is necessary for the gut to be equipped with various highly specialized immune structures, such as mesenteric LNs (mLNs), Peyer's patches (PPs), intraepithelial lymphocytes (IELs), or the lamina propria (LP) to deal with massive antigenic cues and to sustain an immune equilibrium (Mowat and Agace, 2014). Among intestinal immune cells, macrophages serve as the first line defense against invading microbes through their surface and cytosolic expression of pathogen-associated molecular patterns

(PAMPs). In the steady-state, macrophages maintain intestinal homeostasis through engulfing apoptotic cells, promoting the development of regulatory T cells, or supporting the proliferation of intestinal epithelial progenitors. However, in the pathogenesis of IBD, macrophages directly drive the progression of intestinal inflammation by releasing large amounts of inflammatory mediators, such as TNF- α , IL-6, or IL-1 β . Currently, anti-TNF- α antibodies have been extensively used in the clinical treatment of IBD (Bain and Mowat, 2014). Thus, many studies have investigated the regulatory mechanisms of the PAMP-stimulated proinflammatory activities of intestinal macrophages. However, the most prominent property of intestinal macrophages is that they are subjected to strong deactivation signals, among which the immunomodulatory cytokine IL-10 serves as a predominant "brake" to control the time and extent of colonic inflammation (Shouval et al., 2014). IL-10^{-/-} mice develop spontaneous colitis (Kühn et al., 1993). People with loss-of-function mutations in *IL10*, *IL10RA*, or *IL10RB* (IL-10 receptor gene ILs) receptors are prone to severe early onset enterocolitis (Glocker et al., 2009; Engelhardt and Grimbacher, 2014; Walter, 2014). Owing to its anti-inflamma-

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tory function, recombinant human IL-10 has been used in clinical trials with proven safety in IBD patients (Marlow et al., 2013; Zhou and Sonnenberg, 2018). However, patients have differential responsiveness to IL-10 treatment (van Deventer et al., 1997; Fedorak et al., 2000; Schreiber et al., 2000; Colombel et al., 2001; Braat et al., 2006). The factors determining the anti-inflammatory function of IL-10 in IBD are largely known.

Macrophages are among the cells that express the highest level of IL-10 receptors; several groups have observed that macrophage-restricted deficiency of IL-10 receptors or the downstream STAT3 caused severe spontaneous colonic inflammation (Takeda et al., 1999; Kobayashi et al., 2003; Zigmond et al., 2014), highlighting that appropriate macrophage sensitivity to IL-10 signaling is crucial for controlling the inflammatory status in colon microenvironment. Nevertheless, little attention has been paid to the factors that regulate IL-10–STAT3-mediated macrophage deactivation process during intestinal inflammation, and this lack of understanding hinders the further development of IL-10–based therapeutic strategy for IBD.

As the first identified proto-oncoprotein that belongs to tyrosine phosphatase family, Shp2 has been extensively studied with regard to its function in supporting the malignant behaviors of tumor cells. Recently, emerging evidences have uncovered the crucial roles of Shp2 in immune regulation. For example, loss of Shp2 in CD4⁺ T cells facilitated melanoma growth and metastasis (Zhang et al., 2013). Dendritic cell (DC)–expressed Shp2 promoted Th17 cell-mediated antifungal immunity via activating Syk signaling (Deng et al., 2015). Our group has found that Shp2 deficiency in macrophages aggravated lung fibrosis and age-related emphysema (Tao et al., 2014; Xu et al., 2017). To date, whether and how Shp2 modulate the function of colonic macrophages during intestinal inflammation remain unknown. Here, we report that in the context of colonic inflammation, Shp2 desensitizes mouse and human macrophages to IL-10–STAT3 signaling and its dependent inhibition of inflammatory cytokine production. Macrophage-restricted ablation of Shp2 in mice confers protection against colitis and colitis-driven colorectal cancer. The clinical relevance of macrophage-expressed Shp2 was also examined in IBD patients. Moreover, we found that TNF- α could up-regulate Shp2 expression in macrophages, rendering IL-10 plus anti-TNF- α a potential therapeutic strategy for IBD.

Results

Shp2 deficiency in macrophages protects mice from acute colitis

To study the possible role of macrophage-expressed Shp2 in intestinal inflammation, we crossed *PTPN11^{fllox/flox}* mice with *LysM^{Cre}* mice and confirmed a high efficiency (~80%) of Cre-driven Shp2 deletion in colonic macrophages, but not in colonic DCs (Fig. S1). *LysM^{Cre}–Shp2^{fllox/flox}* mice (Shp2^M KO) and *PTPN11^{fllox/flox}* littermates (Shp2^M WT) were subjected to 2.5% dextran sulfate sodium (DSS) treatment, which led to significantly enhanced Shp2 expression in colonic macrophages (Fig. S2). Of note, Shp2^M KO mice exhibited significantly reduced susceptibility to DSS-induced colitis, as indicated by milder body weight loss, rectal bleeding, diarrhea, and colon shortening, compared with Shp2^M

WT mice (Fig. 1, A and B). Histopathologically, colitic Shp2^M KO mice displayed significantly mitigated mucosal damage than Shp2^M WT mice (Fig. 1 C). Also, the loss of goblet cells was alleviated in inflamed Shp2^M KO colons compared with Shp2^M WT colons (Fig. 1 D). Quantification of bacterial 16S ribosomal RNA in spleens demonstrated decreased bacterial translocation in Shp2^M KO mice (Fig. 1 E). When challenged with 4% DSS, Shp2^M KO mice manifested significantly improved survival compared with Shp2^M WT mice (Fig. 1 F). Although *LysM^{Cre}* transgene is also expressed by neutrophils, the depletion of neutrophils did not apparently change colitis susceptibility, suggesting that neutrophils are unlikely to be accounted for the colitis-protective effect in Shp2^M KO mice (Fig. S2).

To further examine the impact of myeloid Shp2 deficiency on colitis development, we generated *CD11c^{Cre}PTPN11^{fllox/flox}* mice with specific Shp2 deletion in CD11c-expressing cells, which includes most DCs and a proportion of macrophages in colonic lamina propria (CLP). Upon DSS challenge, Shp2^{CD11c} KO mice developed less severe colitis compared with Shp2^{CD11c} WT mice (Fig. S3). Collectively, Shp2 deficiency in macrophages confers protection from colitis in mice.

Shp2 deficiency in macrophages attenuates innate immune-mediated intestinal inflammation

We then evaluated the impact of macrophage Shp2 deficiency on the colonic inflammatory environment. Compared with Shp2^M WT mice, the percentage of Ly6C^{hi}CX3CR1^{int} inflammatory macrophages was significantly lower in the inflamed CLP of Shp2^M KO mice, whereas the percentages of Ly6C^{low}CX3CR1^{hi} resident macrophages, Ly6G⁺ neutrophils, SiglecF⁺ eosinophils, CD4⁺ T cells, and CD8⁺ T cells were similar between the two groups of mice (Fig. 2, A and B).

In terms of cytokine milieu, Shp2 deficiency in macrophages significantly reduced the levels of TNF- α , IL-1 β , and IL-6 in the inflamed colon and lowered the level of TNF- α in the serum (Fig. 2, C and D). Moreover, Shp2^M KO mice had significantly lower levels of these inflammatory cytokines in colonic myeloid cells (Fig. 2 E). In contrast, colonic production of IFN- γ and IL-17A, two T cell–derived inflammatory cytokines, were comparable between the two groups of mice (Fig. 2 F). Also, the levels of Th1-/Th2-/Th17-specific cytokines and transcription factors in mLNs and spleens were similar between the two groups of mice (Fig. 2 G). These observations indicate that the decreased colitis susceptibility in Shp2^M KO mice is mainly a consequence of reduced innate inflammation, rather than T cell–mediated adaptive inflammation.

Shp2 deficiency in macrophages inhibits colon carcinogenesis driven by chronic colitis

Chronic colitis is a major risk factor for the development of colorectal cancer, therefore we extended our studies to chronic colitis-associated colon cancer using AOM/DSS regimen. Shp2^M KO mice developed significantly fewer tumor nodules compared with control mice, whereas the ratio of the number of larger tumor nodules (≥ 2 mm in diameter) to smaller tumor nodules (≤ 2 mm in diameter) was comparable between the two groups of mice, suggesting that loss of Shp2 in macrophages primarily

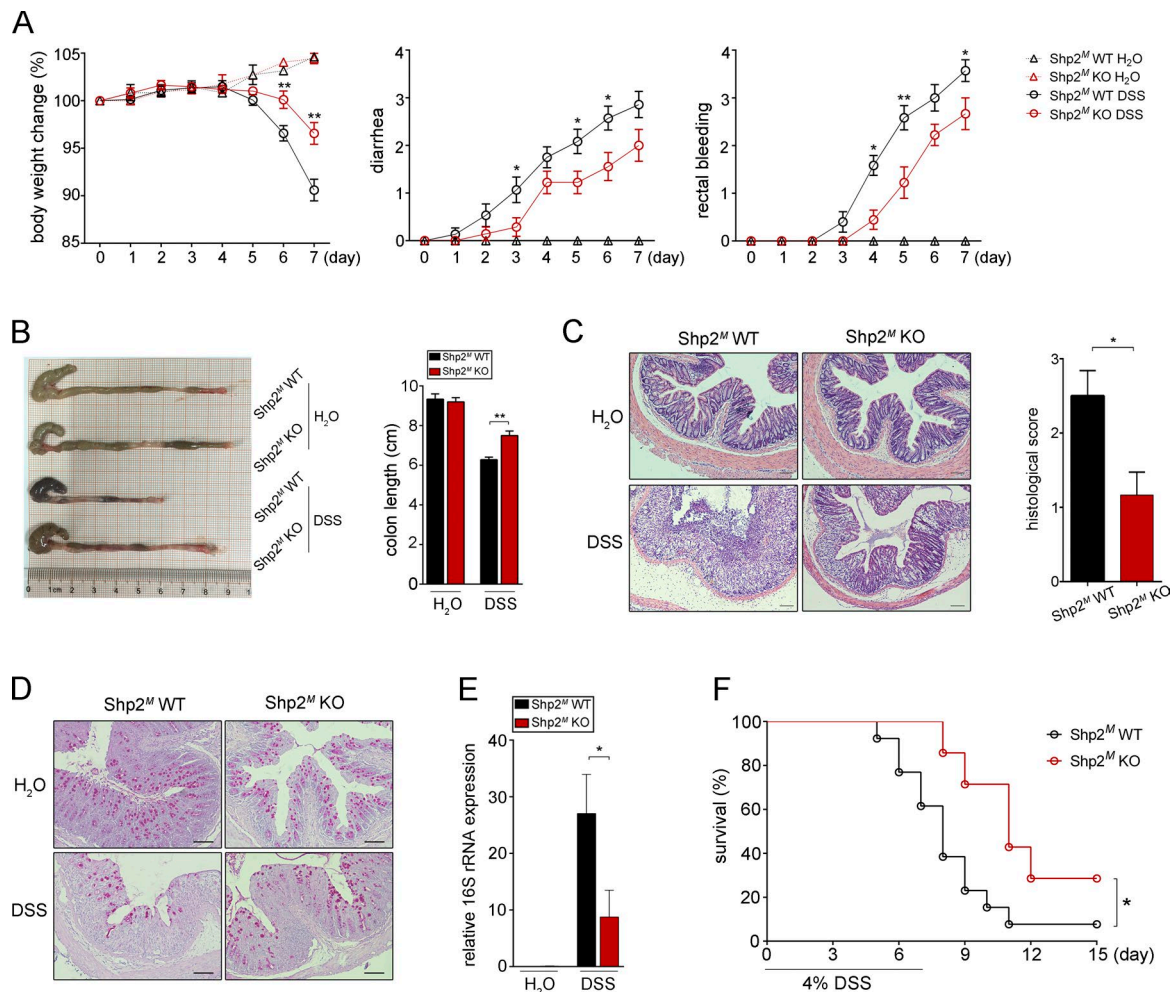


Figure 1. Loss of Shp2 in macrophages decreases the susceptibility of mouse colitis. Mice were challenged with 2.5% DSS in drinking water for 7 d. **(A)** Body weight loss, diarrhea, and rectal bleeding were monitored daily. Data are accumulated from three independent experiments with $n = 4-9$ mice/group in each experiment. **(B)** Colon length was measured at day 7. **(C)** Colon sections were analyzed for histological damage. Bars, 100 μ m. **(D)** Periodic acid-Schiff staining of colon sections. Bars, 100 μ m. **(E)** The expression of bacterial 16S rRNA in spleens was determined by qPCR. **(F)** Mice were challenged with 4% DSS for 7 d, and the survival of mice was monitored. $n = 7-13$. Data are mean \pm SEM and are representative of three independent experiments. *, $P < 0.05$; **, $P < 0.01$; two-tailed unpaired Student's t test (A–C and E) and log-rank test (F).

inhibited inflammation-triggered tumor initiation rather than the growth of existing tumors (Fig. 3, A–C).

Like in acute colitis, Shp2^M KO colons had significantly lower percentages of Ly6ChⁱCX3CR1^{int} inflammatory macrophages as well as Ly6ChⁱCX3CR1⁺ cells (presumably myeloid-derived suppressor cells), and reduced levels of IL-6, IL-1 β , and IL-23, reflecting less severe chronic colitis in Shp2^M KO mice (Fig. 3, D and E). Therefore, macrophage deletion of Shp2 also suppresses colitis-driven colon carcinogenesis.

Loss of Shp2 does not impair PAMP-triggered inflammatory activation of macrophages

The above findings prompted us to suppose that loss of Shp2 in macrophages diminished their proinflammatory activities in response to bacterial stimuli. Unexpectedly, Shp2 deficiency did not obviously impair the capacity of macrophages to express TNF- α , IL-1 β , and IL-6 upon LPS challenge; this was also the case when macrophages were activated by PGN or heat-killed *Escherichia coli* (Fig. 4, A–C). On the other hand, Shp2^M KO macrophages expressed

similar levels of CD80, CD86, MHC-II, and ROS, but a higher level of inducible nitric oxide synthase (iNOS) compared with Shp2^M WT macrophages after LPS stimulation (Fig. 4, D–F). Hence, Shp2 does not have a prominent role in modulating the inflammatory activity of macrophages in response to microbial PAMPs.

Shp2 deficiency promotes IL-10-mediated anti-inflammatory function and signaling in macrophages

We then turned our attention to macrophage-deactivating mechanisms because the function of colonic macrophages was programmed by potent immunosuppressive signals, especially from IL-10. DSS treatment markedly increased colonic IL-10 concentration in both Shp2^M WT and Shp2^M KO mice (Fig. 5 A). Using IL-10-GFP reporter mice, we found that T cells and macrophages accounted for $\sim 90\%$ of colonic IL-10-positive cells, whereas plasmacytoid DCs and B cells contributed minimally to colonic IL-10 (Fig. S5).

Next, we activated macrophages with LPS in the presence of IL-10. As shown in Fig. 5 B, IL-10 strongly suppressed the

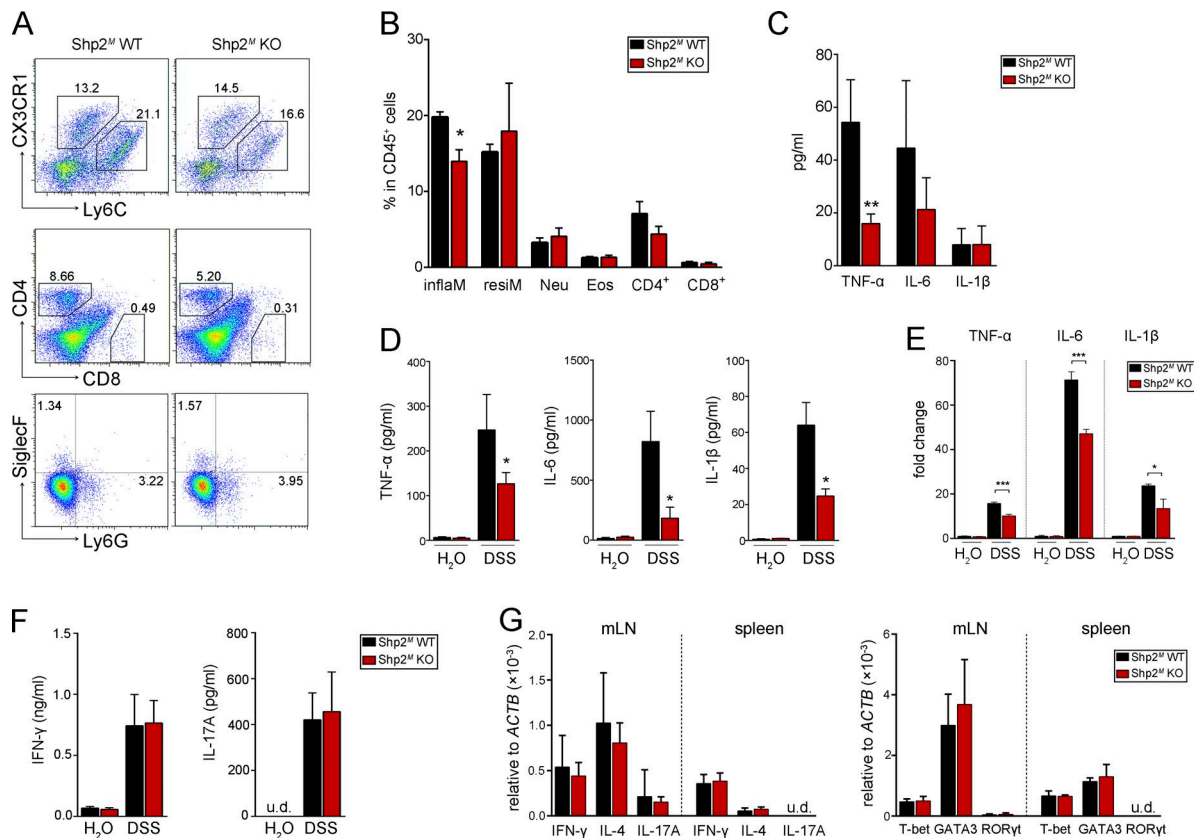


Figure 2. Loss of Shp2 in macrophages attenuates innate intestinal inflammation. Mice were challenged with 2.5% DSS and were sacrificed at day 7. **(A and B)** Single-cell suspensions of CLP were analyzed for the frequencies of different cell populations by flow cytometry (gated on CD45⁺ cells). **(C and D)** The levels of TNF-α, IL-6, and IL-1β in serum (C) and culture supernatant from colon tissues (D) were determined by ELISA. *n* = 5 for DSS cohorts and *n* = 3 for H₂O cohorts. **(E)** Colon tissues were pooled from three mice, CD11b⁺ cells were purified by magnetic-activated cell sorting, and the levels of TNF-α, IL-6, and IL-1β were evaluated by qPCR. **(F)** The levels of IFN-γ and IL-17A in culture supernatant from colon tissues were determined by ELISA. **(G)** The mRNA levels of T cell-related cytokines and transcription factors in mLN and spleen were determined by qPCR. *n* = 4–7. u.d., undetectable. Data are mean ± SEM and are representative of three independent experiments. *, *P* < 0.05; **, *P* < 0.01; ***, *P* < 0.001; two-tailed unpaired Student's *t* test.

production of TNF-α and IL-6 by LPS-activated macrophages. Importantly, the inhibitory efficacy of IL-10 was significantly promoted in Shp2^M KO macrophages. Moreover, Shp2 deficiency enhanced the inhibition of TNF-α at a wide range of IL-10 concentration (Fig. 5 C). In the absence of exogenous IL-10, Shp2^M KO macrophages produced a lower amount of TNF-α than Shp2^M WT macrophages, after receiving long-time LPS stimulation (24 h), but this phenomenon did not occur in the presence of anti-IL-10 antibody, suggesting that Shp2 ablation also potentiated the function of endogenous IL-10 (Fig. 5 B). The stronger IL-10 inhibition of TNF-α was also seen in PGN or *E. coli*-activated Shp2^M KO macrophages (Fig. 5 D). Besides, Shp2 deficiency enhanced IL-10 inhibition of TNF-α mRNA, but not to the extent seen at the protein level, indicating that there were posttranscriptional mechanisms accounting for the function of IL-10 (Fig. 5 E).

Mechanistically, loss of Shp2 in macrophages markedly increased the level of IL-10-induced STAT3 phosphorylation, the central transcription factor downstream of IL-10 receptor. On the other hand, Shp2 deficiency did not affect ERK and p38 signalings in IL-10-treated macrophages (Fig. 5 F). We then examined the modulatory role of Shp2 directly in CLP macrophages (CLPMs). To exclude the effect of autocrine IL-10, CLPMs were

isolated from IL-10^{-/-}Shp2^M KO (DKO) and IL-10^{-/-}Shp2^M WT (control) mice. Shp2 deficiency significantly potentiated IL-10 inhibition of TNF-α and IL-6 in LPS-challenged CLPMs (Fig. 5 G). Besides, IL-10-induced phosphorylation of STAT3 in CLPMs was enhanced by Shp2 deficiency (Fig. 5 H). Consistently, compared with control CLPMs, DKO CLPMs expressed significantly higher level of *SOCS3*, a typical gene downstream of IL-10-STAT3 signaling (Fig. 5 I). Thus, Shp2 serves as a negative regulator of IL-10-STAT3 signaling and its dependent anti-inflammatory function in macrophages.

The colitis-protective effect in Shp2^M KO mice is largely dependent on IL-10

To ascertain if the reduced colitis in Shp2^M KO mice can be explained by the higher macrophage sensitivity to IL-10, we subjected DKO and control mice to 1.5% DSS (IL-10^{-/-} mice develop spontaneous colitis and have high mortality when challenged with 2.5% DSS). DKO mice displayed similar degrees of weight change, diarrhea, rectal bleeding and colon shortening compared with control mice (IL-10^{-/-}Shp2^M WT; Fig. 6, A and B). Furthermore, no significant difference in the levels of inflammatory cytokines was observed between DKO and control colon homogenates (Fig. 6 C).

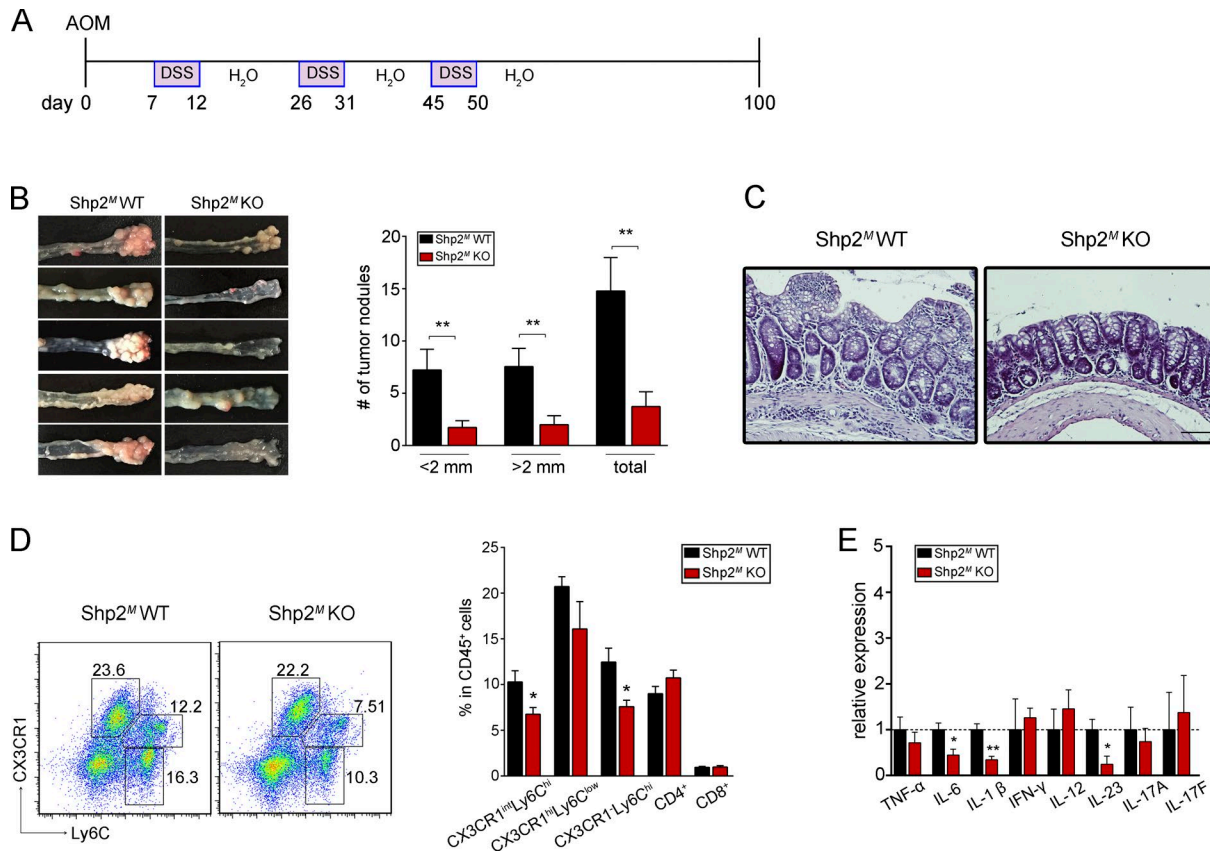


Figure 3. Shp2 deficiency in macrophages inhibits colitis-associated colon cancer. (A) Experimental design of AOM/DSS-induced colon cancer. (B) Colonic tumors were photographed (left) and counted (right). $n = 9-11$. Data are mean \pm SEM and are compiled from two independent experiments. (C) Representative H&E staining of colon tissues. Bar, 100 μ m. (D) The frequencies of different cell populations in CLP were analyzed by flow cytometry. Representative plots showed the myeloid populations. (E) The expression of indicated cytokines in CLP was determined by qPCR. Data are mean \pm SEM and are representative of two independent experiments. *, $P < 0.05$; **, $P < 0.01$; two-tailed unpaired Student's t test.

Because IL-10^{-/-} mice are born with immunodeficiency, which can lead to developmental abnormalities of certain immune cells, we adopted another strategy in which IL-10 was neutralized after DSS treatment. IL-10 neutralization significantly worsened DSS-induced weight loss in Shp2^M KO mice, but not in Shp2^M WT mice, whereas the scores of diarrhea and rectal bleeding were marginally aggravated in both groups of mice (Fig. 6 D). Besides, in contrast to IgG cohorts, Shp2 deficiency in macrophages failed to protect mice from histological damage and colon shortening in anti-IL-10 cohorts (Fig. 6 E). In terms of inflammatory cytokines, anti-IL-10 treatment significantly increased colonic production of TNF- α and IL-6, but not MCP-1 in Shp2^M WT mice. Importantly, IL-10 neutralization caused more prominent elevation of TNF- α , IL-6, and MCP-1 levels in Shp2^M KO mice, and largely abrogated the differential production of these inflammatory cytokines seen in IgG cohorts (Fig. 6 F).

To further determine the role of Shp2 in a more physiological manner, we treated CLPMs with culture supernatant from inflamed colons (coCM). STAT3 phosphorylation was induced to a greater degree in Shp2^M KO CLPMs than in Shp2^M WT CLPMs, and this difference was abrogated by IL-10 neutralization, despite the presence of other STAT3-activating cytokines in the coCM (Fig. 6 G). Therefore, the enhanced sensitivity of macrophages to IL-10 is primarily responsible for the mitigated colitis in Shp2^M KO mice.

Macrophages from IBD patients express increased level of Shp2 which inhibits the function of IL-10

We investigated Shp2 expression in colonic macrophages using clinical IBD specimens. A proportion of CD68⁺ macrophages without high Shp2 expression was seen in colonic mucosa from non-IBD controls. In contrast, the overwhelming majority of macrophages in the inflamed mucosa from CD and UC patients showed a strong colocalization with Shp2^{hi} cells and exhibited high Shp2 fluorescence intensity (Fig. 7 A). Quantitative analysis showed a significant increase in the proportion of Shp2^{hi} macrophages in IBD mucosa compared with healthy mucosa (Fig. 7 B). Likewise, we observed that blood monocytes from IBD patients showed elevated Shp2 expression compared with monocytes from non-IBD controls (Fig. 7 C), and there was a significant positive correlation between the expression levels of Shp2 and TNF- α , as well as IL-6 (Fig. 7 D).

In human THP-1 monocytes and THP-1-differentiated macrophages, Shp2 silencing potentiated IL-10-induced STAT3 phosphorylation as well as IL-10 inhibition of TNF- α (Fig. 7, E-H). Co-immunoprecipitation (Co-IP) analysis showed that STAT3 interacted with full-length Shp2 and the PTP domain of Shp2, whereas its binding to the SH2 domains of Shp2 was almost negligible (Fig. 7 I).

Finally, with the curiosity in seeking the inflammatory stimuli involved in up-regulating Shp2 expression, we found that the level of Shp2 was significantly increased in macrophages treated

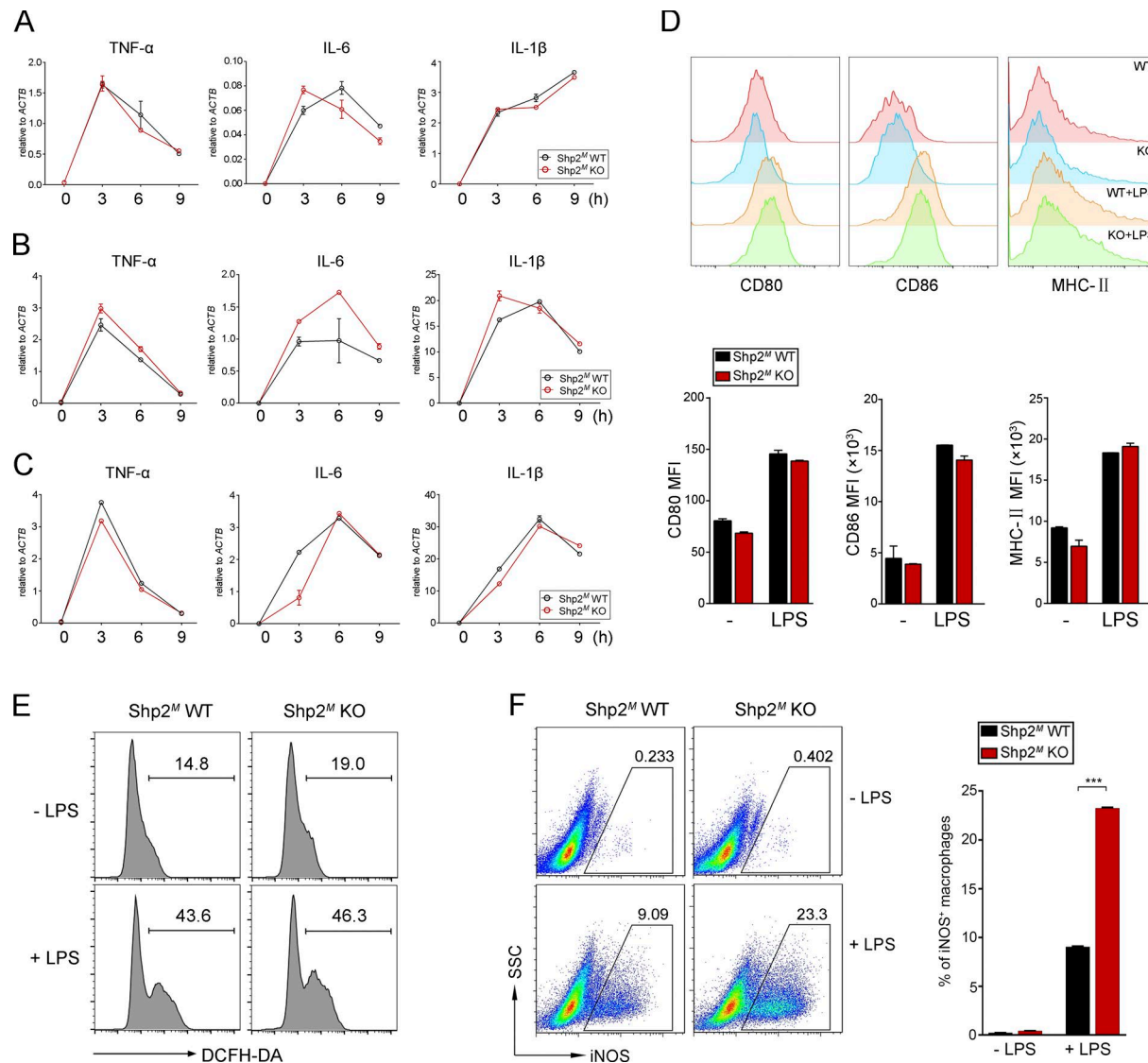


Figure 4. Macrophage production of inflammatory mediators is not obviously affected by Shp2 deficiency. (A–C) Peritoneal macrophages (PMs) from Shp2^{fl} WT and Shp2^{fl} KO mice were stimulated with LPS (100 ng/ml; A), PGN (2 μg/ml; B), or heat-killed *E. coli* (multiplicity of infection = 0.1; C), the expression of TNF-α, IL-6, and IL-1β was determined by qPCR. (D–F) PMs were stimulated with LPS (100 ng/ml), surface expression of CD80, CD86, and MHC-II (D), or intracellular production of ROS (E) or iNOS (F) were detected by flow cytometry. Data are mean ± SEM and are representative of three independent experiments. ***, $P < 0.001$; two-tailed unpaired Student's *t* test.

with TNF-α (Fig. 7, J and K), implying that IL-10 supplementation may achieve higher therapeutic efficacy when used in combination with anti-TNF-α antibodies.

In summary, our study discovers the function of Shp2 in restraining IL-10-dependent control of intestinal inflammation (Fig. 8).

Discussion

The development of IBD is determined by a combination of factors, including genetic predisposition, environmental and dietary factors, and gut microbiota; although, how these factors affect the pathogenesis of IBD is largely unknown, and the relative contribution of each factor to an individual patient may vary greatly. Dysregulation of the intestinal immune system occurs

in nearly all IBD cases, highlighting the importance of appropriate immune regulation in preventing the development of IBD. In addition to its digestive and absorptive functions, the gut can be seen as the largest peripheral immune organ, which contains several highly organized compartments to deal with diverse kinds of luminal antigens. Macrophages, although playing crucial roles in maintaining intestinal homeostasis by their phagocytic capacity, often initiate and worsen the pathological signs of IBD by excessive production of inflammatory cytokines. Many IBD-associated loci identified by genome-wide association studies are involved in regulating the inflammatory behavior of macrophages, e.g., *NOD2*, *ATG16L1*, and *CCL2*. However, we did not observe a prominent role of Shp2 in modulating the immediate production of inflammatory cytokines by macrophages in response to bacterial stimuli, except for iNOS; this result is somewhat contradictory

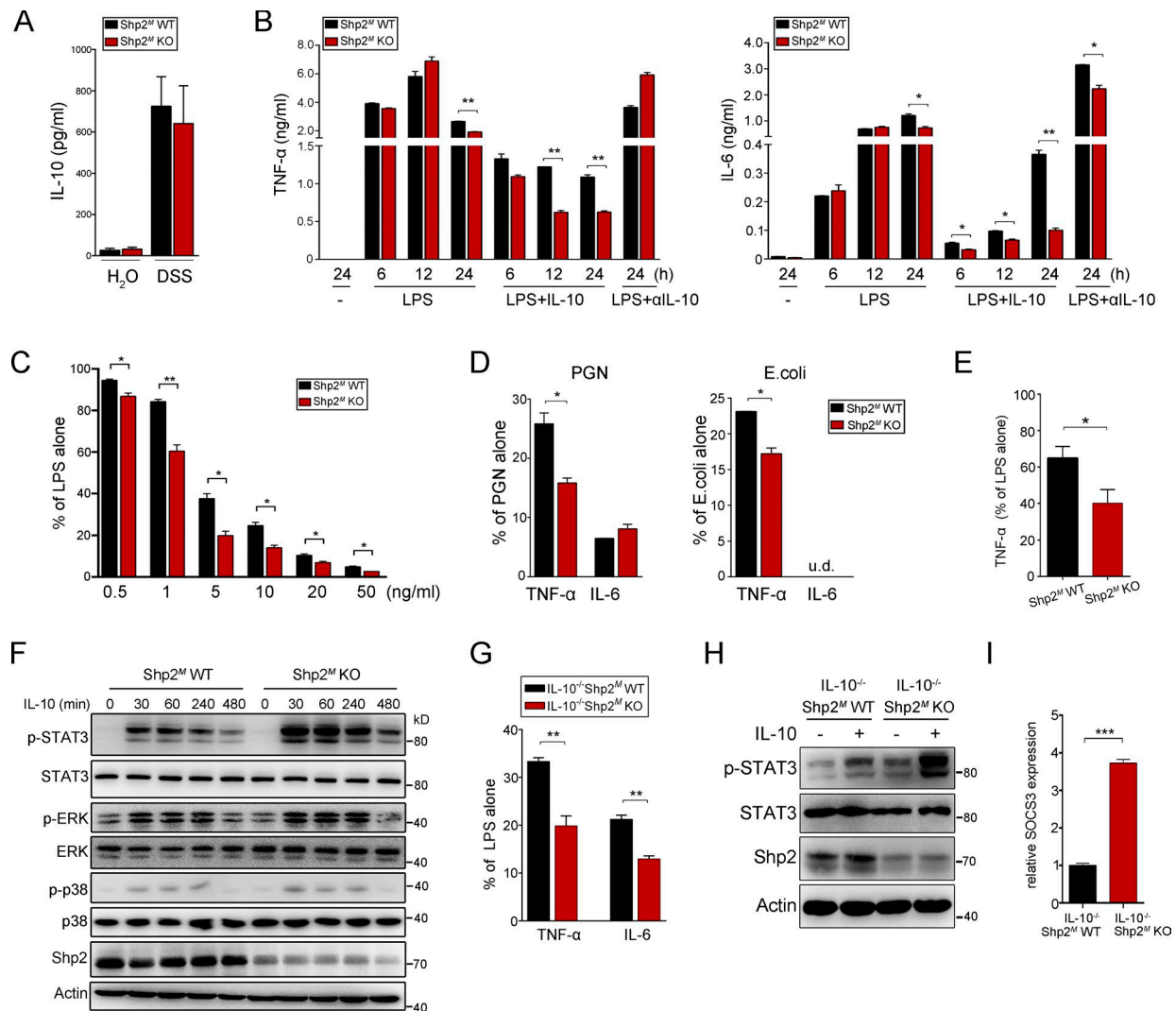


Figure 5. Loss of Shp2 enhances the inhibitory function of IL-10 in macrophages. (A) IL-10 concentration was measured in culture supernatant from colon tissues. (B) Shp2^{WT} and Shp2^{KO} PMs were stimulated with LPS (100 ng/ml) in the absence or presence of IL-10 (10 ng/ml) or anti-IL-10 (αIL-10) for indicated time, the levels of TNF-α and IL-6 in culture supernatant were determined by ELISA. (C) PMs were stimulated with LPS (100 ng/ml) in the absence or presence of indicated concentrations of IL-10 for 12 h; the level of TNF-α in culture supernatant was determined by ELISA. Results were presented as relative cytokine values (value_{LPS + IL-10}/value_{LPS alone}). (D) PMs were stimulated with PGN or *E. coli* in the absence or presence of IL-10 for 12 h, relative TNF-α, and IL-6 levels were determined by ELISA. (E) PMs were stimulated with LPS in the absence or presence of IL-10 for 3 h; relative TNF-α mRNA level was determined by qPCR. (F) PMs were stimulated with IL-10 (10 ng/ml) for indicated time, the phosphorylation of STAT3, ERK, and p38 was determined by Western blot. Data are mean ± SEM and are representative of at least three independent experiments. (G) CLPMs isolated from IL-10^{-/-}Shp2^{WT} and IL-10^{-/-}Shp2^{KO} mice were stimulated with LPS in the absence or presence of IL-10 (5 ng/ml) for 12 h; the levels of TNF-α and IL-6 in culture supernatant were determined by ELISA. (H) CLPMs were stimulated with IL-10 (5 ng/ml) for 30 min; the phosphorylation of STAT3 was determined by Western blot. (I) CLPMs were stimulated with IL-10 (5 ng/ml) for 2 h; the level of SOCS3 was determined by qPCR. Data are mean ± SEM and are representative of two independent experiments. *, *P* < 0.05; **, *P* < 0.01; ***, *P* < 0.001; two-tailed unpaired Student's *t* test.

to a previous report in which Shp2 deficiency increased macrophage production of TNF-α and IL-6. This discrepancy may result from different mice-generating strategies, because they used *Mx^{Cre}PTPN11^{fllox/fllox}* mice that needed continuous poly(I:C) injection to achieve Shp2 ablation (Xu et al., 2012).

Considering that intestinal macrophages have a high incidence of being activated, it is of great significance for the intestinal immune system to evolve strong immune suppression mechanisms. The anti-inflammatory cytokine IL-10 plays a predominant role in this respect (Shouval et al., 2014). Accordingly, IL-10 supplementation therapy has been undertaken in the treatment of IBD

(Marlow et al., 2013). In several trials, improved clinical signs of colitis were observed (van Deventer et al., 1997; Fedorak et al., 2000; Braat et al., 2006), whereas some other studies reported no obvious clinical benefit (Schreiber et al., 2000; Colombel et al., 2001). It seems that individual patients have different responsiveness to IL-10 supplementation, highlighting the need for the development of customized or optimized therapeutic approaches. To this end, clarifying the factors affecting the function of IL-10 and their mechanisms of action will be of great significance. Evidence from animal studies showed that mice with myeloid deficiency of *IL10RA* or *IL10RB* developed severe spontaneous colitis

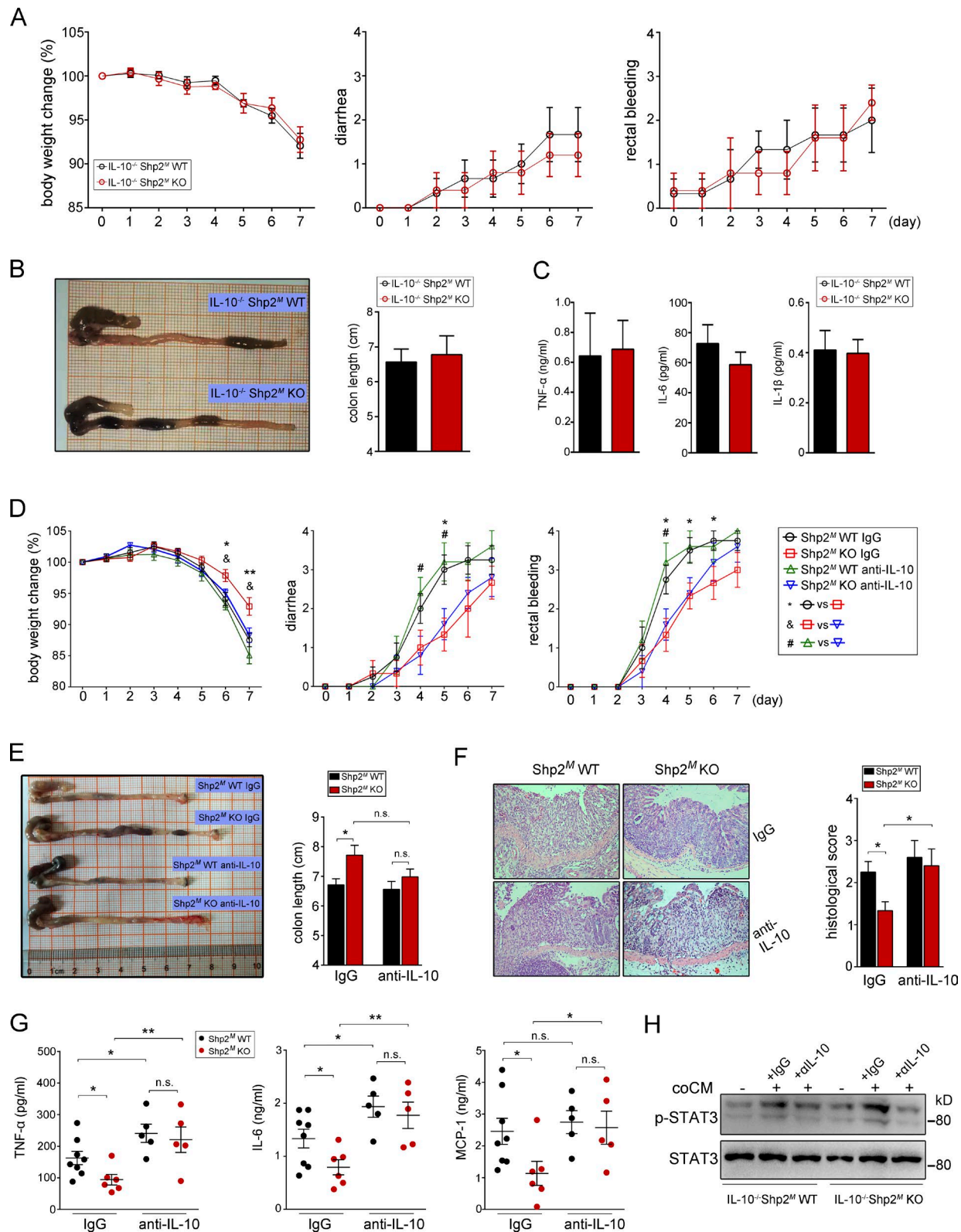


Figure 6. The colitis-protective effect in Shp2^M KO mice is abrogated in the absence of IL-10 function. (A–C) DKO and control mice were fed with 1.5% DSS. **(A)** Body weight loss, diarrhea, and rectal bleeding were monitored daily. **(B)** Colon length was measured at day 7. **(C)** The levels of inflammatory cytokines were measured in colon homogenates by ELISA. $n = 5–6$. **(D–G)** Mice were injected intraperitoneally with 20, 40, and 40 μ g anti-IL-10 or IgG isotype at days 1, 3, and 5 after DSS challenge, respectively. **(D)** Body weight loss, diarrhea, and rectal bleeding were monitored daily. **(E and F)** Colon length and histological changes were measured at day 7. **(G)** The levels of TNF- α , IL-6, and MCP-1 in culture supernatant from colon tissues were measured by ELISA. $n = 5–8$. **(H)** CLPMs were treated with culture supernatant from coCM in the presence of anti-IL-10 or IgG isotype; the phosphorylation of STAT3 was evaluated by Western blot. Data are mean \pm SEM and are representative of two independent experiments. *, &, and #, $P < 0.05$; **, $P < 0.01$; two-tailed unpaired Student's t test. n.s., not significant.

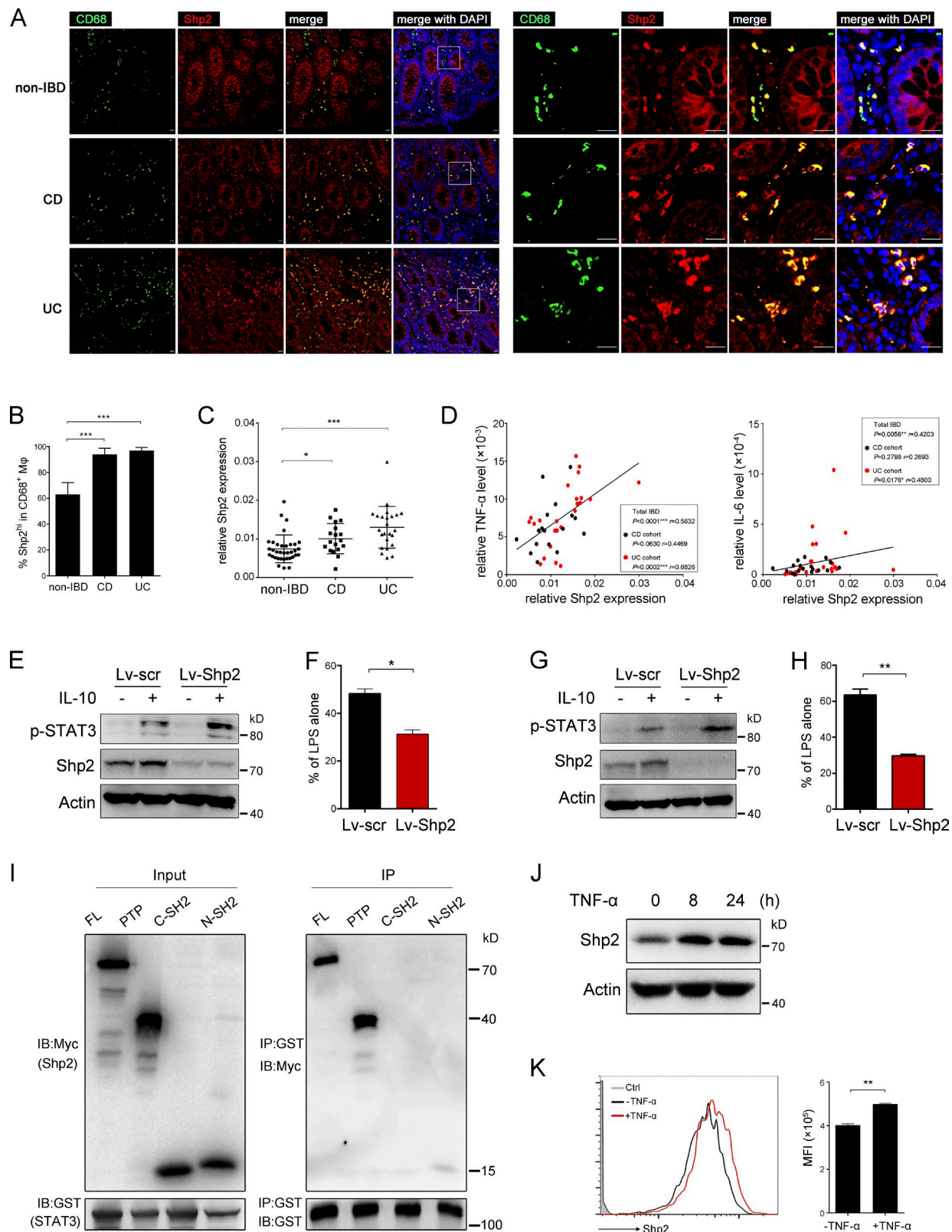


Figure 7. The clinical relevance of macrophage-expressed Shp2 in IBD patients. (A) The expression of Shp2 in CD68⁺ colonic macrophages were examined in mucosal biopsy specimens by immunofluorescence staining. Bars, 20 μ m. Data are representative of three independent experiments. (B) The percentage of CD68⁺Shp2^{hi} cells was quantified. Each group contained three individuals. Data are mean \pm SEM and are compiled from three independent experiments. (C) The expression level of Shp2 in peripheral monocytes was evaluated by qPCR. (D) Association between mRNA levels of Shp2 and TNF- α or IL-6 was analyzed by Spearman's rank correlation test. Data are mean \pm SEM and are from one independent experiment. (E–H) THP-1 monocytes (E and F) or THP-1-differentiated macrophages (G and H) were transduced with Shp2-knockdown lentivirus (Lv-Shp2) or control lentivirus (Lv-scr), followed by stimulation with IL-10 for 30 min. The phosphorylation of STAT3 was determined by Western blot (E and G). IL-10 inhibition of TNF- α was measured by ELISA (F and H). (I) GST-tagged

(Kobayashi et al., 2003; Zigmond et al., 2014), suggesting that the appropriate sensitivity of macrophages to IL-10 is crucial for restraining colonic inflammation. Nevertheless, how the signaling and function of IL-10 are modulated in intestinal macrophages remains poorly investigated. Our work provides new insight into this topic by identifying Shp2 as a negative regulator for IL-10-dependent macrophage deactivation through disrupting the activation of STAT3. In the context of intestinal inflammation, STAT3 plays multifaceted roles in a cell type-dependent manner. STAT3 in CD4⁺ T cells contributes to colitis by supporting pro-inflammatory Th17 response (Durant et al., 2010). In contrast, the expression of STAT3 in intestinal epithelial cells or innate immune cells is colitis protective (Takeda et al., 1999; Pickert et al., 2009). Mice with specific deficiency of STAT3 in myeloid cells develop spontaneous colitis due to the impaired IL-10 signaling (Takeda et al., 1999), although STAT3 is also crucial for the function of some pro-inflammatory cytokines such as IL-6.

In our present work, the *in vivo* effects of IL-10 neutralization are partial and somewhat inconsistent among various colitis parameters, especially in Shp2^M WT mice. This may be because it is difficult to achieve 100% neutralization efficacy *in vivo* by injecting the anti-IL-10 antibody. Another possible explanation is that the macroscopic colitis symptoms are affected by multiple factors, and some of these phenotypes are not regulated or only partially regulated by IL-10. On the other hand, IL-10 neutralization effectively increased the levels of inflammatory cytokines in the colitic mice. Therefore, at least IL-10 is crucial for controlling the production of inflammatory cytokines in the intestine.

In a previous study, *PTPN11* (Shp2-encoding gene) polymorphisms were reported to be associated with UC susceptibility (Narumi et al., 2009). However, the two single-nucleotide polymorphisms are located in the intron region of the *PTPN11* gene, probably these mutations may affect Shp2 expression in an unknown manner. Moreover, genome-wide polymorphisms are relatively weakly informative in demonstrating the cell-specific function of Shp2. Here, we unveil that Shp2 expressed in monocyte/macrophage lineage serves as a pro-inflammatory factor in the intestine. In contrast, mice with Shp2 deficiency in intestinal epithelial cells have abnormal development of intestinal epithelium and disrupted host-microbiota equilibrium (Heuberger et al., 2014; Coulombe et al., 2016). Therefore, although a novel, druggable Shp2 inhibitor with oral availability has recently been developed (Chen et al., 2016), systemic or colonic administration of Shp2 inhibitor does not seem viable. From another perspective, several potential implications are generated from our work: (1) Shp2 may serve as a candidate indicator in estimating the therapeutic efficacy of IL-10, thus allowing for the selection of IL-10-sensitive patients; (2) IL-10 supplementation may synergize with anti-TNF- α therapy (such as infliximab) to achieve higher immunosuppressive effects; and (3) because the ability of Shp2 to inhibit IL-10 function is consistent among bone marrow-derived macrophages (data not shown),

peritoneal macrophages, and CLPMs, our present study may have a broader biological significance and extend to other macrophage-mediated inflammatory disorders in which IL-10 also plays a role in restraining the excessive immune response.

Materials and methods

Mice

LysM^{Cre} mice (C57BL/6 background) were crossed with *PTPN11^{fllox/fllox}* mice (C57BL/6 background) to generate Shp2^M KO mice (*LyM^{Cre}Shp2^{fllox/fllox}*) and Shp2^M WT littermates (*PTPN11^{fllox/fllox}*), as we previously described (Tao et al., 2014; Xu et al., 2017). *CD11c^{Cre}* mice (C57BL/6 background) were crossed with *PTPN11^{fllox/fllox}* mice to generate Shp2^{CD11C} KO mice (*CD11c^{Cre}PTPN11^{fllox/fllox}*) and Shp2^{CD11C} WT littermates (*PTPN11^{fllox/fllox}*). IL-10^{-/-} mice (C57BL/6 background) were provided by L. Yu (Sir Run Run Shaw Hospital, Zhejiang University School of Medicine, Hangzhou, China) and were crossed with Shp2^M KO mice to generate IL-10^{-/-} Shp2^M KO mice (IL-10^{-/-} *LyM^{Cre}PTPN11^{fllox/fllox}*) and IL-10^{-/-} Shp2^M WT littermates (IL-10^{-/-} *PTPN11^{fllox/fllox}*). IL-10-GFP mice (B6.129S6 background) were obtained from The Jackson Laboratories. Mice were housed under specific pathogen-free condition. Age-matched Shp2-conditional KO mice and littermate control mice were used for colitis modeling. Animal experiments were performed according to protocols approved by the Zhejiang University Institutional Animal Care and Use Committee.

Human specimens

Clinical specimens were collected from Sir Run Run Shaw Hospital, Zhejiang University School of Medicine. Experiments involving human participants were approved by the Medical Ethics Committee of Sir Run Run Shaw Hospital.

DSS colitis and AOM/DSS colitis-associated colon cancer

Male mice 10–12 wk old were given 2.5% DSS (160110; MP Bio-medicals) in drinking water for 7 d. (For mice with IL-10^{-/-} background, 6–8-wk-old mice were fed with 1.5% DSS.) Body weight loss, rectal bleeding, and diarrhea were scored daily. Rectal bleeding: 0 = no bleeding, 2 = positive hemocult, and 4 = gross bleeding; diarrhea: 0 = well-formed stools, 2 = soft and pasty stools, and 4 = watery stools.

To establish AOM/DSS-induced colon cancer, mice were intraperitoneally injected with AOM (10 mg/kg weight) at day 0, followed by three cycles of DSS challenge 7 d after AOM administration. In each cycle, mice were given 2.5% DSS-containing water for 5 d followed by normal water for 14 d. At day 100, mice were sacrificed and tumor nodules in distal colons were counted.

ELISA

Cytokine concentrations were quantified by ELISA kits from eBioscience.

STAT3 and myc-tagged Shp2 full length or truncations were overexpressed in HEK293T cells. The interaction between STAT3 and various Shp2 domains was detected by co-IP. (J and K) THP-1-differentiated macrophages were stimulated with TNF- α (20 ng/ml); the level of Shp2 was evaluated by Western blot (J) or flow cytometry (K). Data are mean \pm SEM and are representative of at least two independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; two-tailed unpaired Student's *t* test (B, C, F, H, and K) and Spearman's rank correlation test (D).

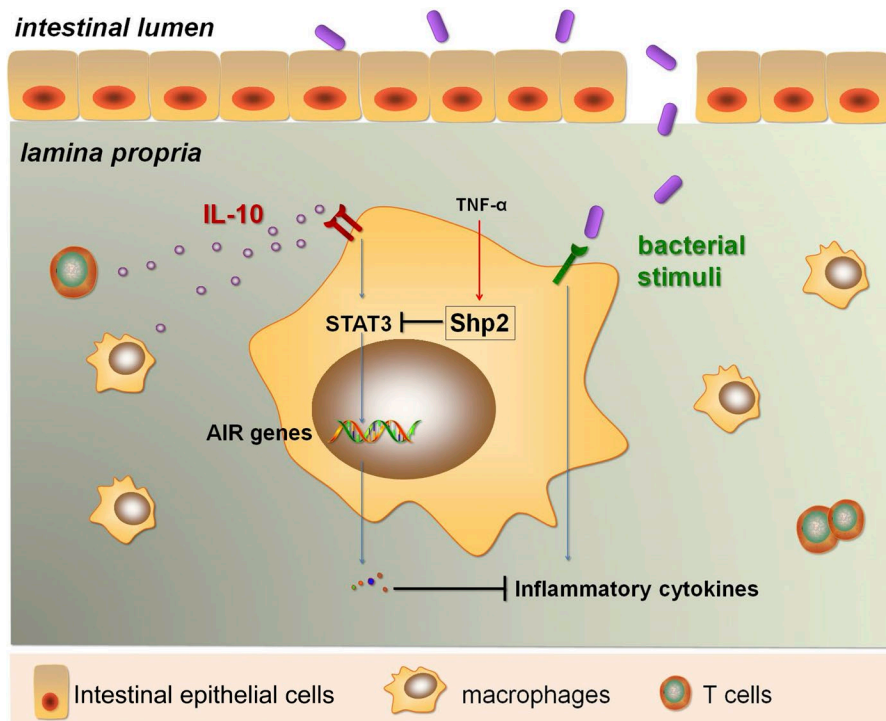


Figure 8. Working model. When colitis occurs, colonic macrophages activated by invading microbes produce inflammatory cytokines, but Shp2 does not play a prominent role during this process. On the other hand, loss of Shp2 potentiates IL-10–STAT3 signaling and its dependent deactivating programs in macrophages, thus decreasing their production of inflammatory cytokines and reducing the severity of colitis. Furthermore, TNF- α has the ability to up-regulate Shp2 expression in macrophages, suggesting that IL-10 supplementation may achieve higher anti-inflammatory efficacy when used in combination with anti-TNF- α antibody.

Western blot

Cells were lysed in 1 \times sodium dodecyl sulfate lysis buffer. Total cell lysates were used for Western blot using antibodies against p-STAT3 (9145; Cell Signaling Technology); STAT3 (sc-482; Santa Cruz); p-ERK (4370; Cell Signaling Technology); ERK (4695; Cell Signaling Technology); p-p38 (9215; Cell Signaling Technology); p38 (9212; Cell Signaling Technology); Shp2 (3397; Cell Signaling Technology); myc (TA150121; Origene); GST (AF2299; Beyotime); and β -actin (AA128; Beyotime). Anti-mouse IgG (7076; Cell Signaling Technology) and anti-rabbit IgG (7074; Cell Signaling Technology) were used for secondary antibodies.

Quantitative PCR (qPCR)

Total RNA was extracted using TRIzol then was reverse transcribed using ReverTraAce qPCR RT kit (Toyobo). qPCR was performed on CFX96 Touch Real-Time PCR Detection System (Bio-Rad) using SYBR Green reagent (Roche). Primer sequences are listed in Table S1.

Isolation of peritoneal macrophages

Mice were intraperitoneally injected with 3% thioglycolate (Merck), and peritoneal cells were harvested by lavaging the peritoneal cavity after 3–4 d. Floating cells were removed by PBS washing, and adherent peritoneal macrophages were cultured in RPMI 1640 containing 10% FBS and 1 \times penicillin/streptomycin at 37°C, followed by treatment with different stimuli according to experimental designs.

Isolation of CLPMs

PBS-flushed colons were longitudinally opened and cut into 5-mm pieces. Colon fragments were incubated with 10 ml HBSS, containing 5 mM EDTA and 1 mM dithiothreitol at 37°C for 50 min with gentle shaking to remove colonic epithelial cells.

The remaining tissues were cut into pieces and digested with HBSS containing 10% FBS, 300 U/ml collagenase IV, and 5 U/ml DNaseI at 37°C for 50 min with gentle shaking. The products were filtered through 70- μ m pore size nylon meshes and washed twice with PBS to obtain single-cell suspensions, which were then labeled with biotin-conjugated anti-CD11b antibody (BioLegend), followed by incubation with anti-biotin microbeads (Miltenyi Biotec). Magnetic-activated cell sorting was performed on a MidiMACS Separator (Miltenyi Biotec) using LS columns (Miltenyi Biotec).

Flow cytometry

Single-cell suspensions were blocked with anti-CD16/CD32 (101320; BioLegend), followed by staining with fluorescence-labeled antibodies against CD45 (103112; BioLegend), CD68 (137005; BioLegend), CD11b (101206; BioLegend), CD4 (116005; BioLegend), CD8 (100705; BioLegend), Ly6C (128005; BioLegend), CX3CR1 (149005; BioLegend) Siglec-F (562068; BD Biosciences), and Ly6G (127606; BioLegend). Staining was performed at 4°C in dark for 20 min. Flow cytometry was run on ACEA NovoCyte Flow Cytometer. Data were analyzed using FlowJo software (Tree Star).

Immunofluorescence staining

Paraffin-embedded mucosal biopsy specimens were cut into sections and stained with FITC-anti-CD68 (134351; Abcam), Alexa Fluor 647-anti-Shp2 (209746; Abcam), and DAPI. Slides were photographed under a Nikon A1R confocal microscope.

Co-IP

HEK293T cells were lysed in Nonidet P 40 reagent containing Protease/Phosphatase Inhibitor Cocktail (Cell Signaling Technology) at 4°C for at least 1 h, and cell lysates were incubated with

appropriate antibody-conjugating SureBeads Protein G Magnetic Beads (Bio-Rad) overnight at 4°C. Co-IP was conducted on a magnetic rack (Bio-Rad).

Culture of colon explants

Approximately 2 cm of colon tissues was opened longitudinally, weighted, washed three times with ice-cold PBS with vortex and cultured in 1 ml DMEM (per 100 mg tissue) containing 10% FBS and 5× penicillin/streptomycin for 16~24 h. The supernatants were centrifuged to remove floating tissue debris, then were analyzed for cytokine concentrations by ELISA.

Histopathology

Distal portion of colons were fixed in 4% paraformaldehyde. Paraffin-embedded, 5-μm-thick sections were used for H&E staining or periodic acid-Schiff staining.

Lentiviral transduction

Lentiviral particles were added to the cultural supernatant of THP-1 cells in the presence of 8 μg/ml polybrene (Santa Cruz). Cell culture plate was centrifuged at 1,300 g for 90 min at 32°C. After 6 h, the supernatant was replaced with fresh RPMI-1640 medium. Following an additional 72 h of culture, THP-1 cells were harvested or were differentiated into macrophages with 100 ng/ml phorbol-12-myristate-13-acetate (Beyotime) for following experiments.

Statistical analysis

Two-tailed unpaired Student's *t* test, log-rank test, and Spearman's rank correlation test were performed using GraphPad Prism; *P* < 0.05 was considered statistically significant.

Online supplemental materials

Fig. S1 analyzes the efficiency of *Cre*-driven *Shp2* deletion in colonic macrophages and DCs. Fig. S2 shows the expression level of *Shp2* in colonic macrophages. Fig. S3 assesses the development of colitis in neutrophil-depleted mice. Fig. S4 compares the severity of colitis between *Shp2*^{CD11c} WT and *Shp2*^{CD11c} KO mice. Fig. S5 analyzes cellular sources of colonic IL-10. Table S1 lists all primer sequences used in this study.

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