Heme ameliorates dextran sodium sulfate-induced colitis through providing intestinal macrophages with noninflammatory profiles

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The local environment is crucial for shaping the identities of tissue-resident macrophages (MΦs). When hemorrhage occurs in damaged tissues, hemoglobin induces differentiation of anti-inflammatory MΦs with reparative function. Mucosal bleeding is one of the pathological features of inflammatory bowel diseases. However, the heme-mediated mechanism modulating activation of intestinal innate immune cells remains poorly understood. Here, we show that heme regulates gut homeostasis through induction of Spi-C in intestinal Cx3CR1-/- MΦs. Intestinal Cx3CR1-/- MΦs highly expressed Spi-C in a heme-dependent manner, and myeloid lineage-specific Spic-deficient (Ly22-cre; SpiCfl/fl) mice showed severe intestinal inflammation with an increased number of Th17 cells during dextran sodium sulfate-induced colitis. Spi-C down-regulated the expression of a subset of Toll-like receptor (TLR)-inducible genes in intestinal Cx3CR1-/- MΦs to prevent colitis. LPS-primed production of IL-6 and IL-1α, but not IL-10 and TNF-α, by large intestinal MΦs from Ly22-cre; Spicfl/fl mice was markedly enhanced. The interaction of Spi-C with IRF5 was linked to disruption of the IRFS-NF-kB p65 complex formation, thereby abrogating recruitment of IRF5 and NF-kB p65 to the Il6 and Il1a promoters. Collectively, these results demonstrate that heme-mediated Spi-C is a key molecule for the non-inflammatory signature of intestinal MΦs, which might be modulated by an unknown IL-10-independent mechanism.

In addition to precursor heterogeneity, local environmental factors are implicated in the diversity of tissue-resident MΦs by inducing tissue-specific transcription factors as well as epigenetic modifications, leading to unique transcriptional profiles (8, 9). When hemorrhage occurs in damaged tissues, hemoglobin provides either an anti-inflammatory feature or a restorative function for infiltrating MΦs through a scavenger receptor such as CD163 (10–15). Among the colonoscopic features, bleeding within the mucosa is commonly seen in patients with ulcerative colitis through providing intestinal macrophages with noninflammatory profiles.

Significance

Following hemorrhage in damaged tissues, hemoglobin induces macrophages (MΦs) possessing ability to protect against tissue inflammation. Hemorrhage-causing mucosa is observed in patients with inflammatory bowel disease. However, heme-mediated modulation of intestinal MΦ activity remains poorly understood. Here, we provide evidence that Spi-C induced by heme is a key molecule for providing noninflammatory gene expression patterns of intestinal Cx3CR1-/- MΦs. We found that the Spi deficiency in intestinal MΦs resulted in increased sensitivity to dextran sodium sulfate-induced colitis. Heme-mediated Spi-C inhibited a subset of LPS-induced genes such as Il6 and Il1a by intestinal Cx3CR1-/- MΦs through inhibition of IRFS-NF-kB p65 complex formation. These results reveal a mechanism modulating the noninflammatory phenotype of intestinal MΦs and may help identify targets for therapy of intestinal inflammation.

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coitus is a form of inflammatory bowel disease (16). However, whether heme evokes the noninflammatory profiles in Mϕs, thereby contributing to mucosal healing, is unclear.

Spi-C, which belongs to the Spi subfamily of Ets transcription factors including PU.1, Spi-B, and Spi-D (17), was reported to regulate the differentiation of splenic red pulp Mϕs (RPMϕs) and F4/80+ VCAM+ bone marrow Mϕs (BMMϕs) (18, 19) and the development of B lymphocytes (20–22). In addition, erythrocyte-derived heme is a tissue factor that drives the expression of Spi-C during the development of RPMϕs and F4/80+ VCAM+ BMMϕs (19). However, the function of Spi-C in the regulation of innate immune responses remains poorly understood.

In this study, we identified the mechanism by which heme-mediated Spi-C negatively regulates a subset of TLR-inducible gene expression profiles in intestinal Mϕs through disruption of IRF5-NF-κB p65 complex formation to provide noninflammatory profiles for intestinal Mϕs, thereby maintaining intestinal homeostasis.

Results

Expression of Spi-C in Intestinal CX3CR1high Mϕs. Unlike other tissue-resident Mϕs, transcription factors that regulate the differentiation and function of CX3CR1high Mϕs in the large intestine are poorly understood. Thus, to identify transcription factors specifically expressed in CX3CR1high Mϕs among large intestinal innate myeloid cells, we comprehensively analyzed gene expression profiles in CX3CR1high Mϕs, CX3CR1− CD11b+ CD11c+ cells, CD11b− CD11c+high DCs, and CD11b+ CD11c− cells (SI Appendix, Table S1). Among four subsets, CX3CR1high Mϕs highly expressed Spic. SpiCflox/flox mice showed that a subpopulation of CD11b+ innate myeloid cells in the colon, small intestine, and mesenteric lymph nodes (MLN) expressed Spi-C, as did those in the spleen and bone marrow (19) (Fig. 1A). In the colon, Spi-C–expressing CD11b+ CD11c+high cells highly expressed CX3CR1 in both a steady state and a dextran sodium sulfate (DSS)-induced inflammatory state (SI Appendix, Fig. S1), suggesting that Spi-C is selectively expressed in CX3CR1high Mϕs among large intestinal innate myeloid cells.

Exacerbated Colitis in Myeloid Lineage Cell-Specific Spi-C-Deficient Mice. To assess the physiological role of Spi-C in intestinal CX3CR1high Mϕs, myeloid lineage cell-specific Spi-C-deficient (Ly2-2-cre; Spicflox/flox) mice were generated (SI Appendix, Fig. S2 A–C). The expression of Spic mRNA was markedly reduced in large intestinal CX3CR1high Mϕs in Ly2-2-cre; Spicflox/flox mice compared with Spicflox/flox mice (SI Appendix, Fig. S2D). However, the frequency of CX3CR1high Mϕs was not altered in the large intestinal lamina propria (SI Appendix, Fig. S2E). In the steady state, there was no difference in IL-17+, IFN-γ+, and IL-10–producing CD4+ T cells as well as in Foxp3+ Treg cells in the colon, MLN, and spleen between Spicflox/flox and Ly2-2-cre; Spicflox/flox mice (SI Appendix, Fig. S2 F and G). In the spleen of Ly2-2-cre; Spicflox/flox mice, the number of RPMϕs was normal (SI Appendix, Fig. S3A). In addition, Spi-C expression was not reduced in RPMϕs isolated from Ly2-2-cre; Spicflox/flox mice (SI Appendix, Fig. S3B). This might be due to the lower expression level of Ly2 in RPMϕs than intestinal CX3CR1high Mϕs (SI Appendix, Fig. S3C).

We investigated whether Spi-C in CX3CR1high Mϕs regulates intestinal inflammation by orally administering 1.5% DSS. Compared with Spicflox/flox mice, Ly2-2-cre; Spicflox/flox mice showed greater weight loss, more profound bleeding in the stools, slightly increased pasty stools, and more severe intestinal pathologies (Fig. 1 B–E). In addition, the number of IL-17–producing CD4+ T cells was increased in the colon, but not in the MLN and spleen of Ly2-2-cre; Spicflox/flox mice following DSS administration compared with Spicflox/flox mice (Fig. 1F). These findings indicate that Spi-C in intestinal CX3CR1high Mϕs plays an important role in the suppression of intestinal inflammation.

Fig. 1. Ly2-2-cre; Spicflox/flox mice showed exacerbated intestinal inflammation following DSS administration. (A) Flow cytometric plots of Spi-C, EGFP–, and CD11b-expressing cells. The cells from the spleen, bone marrow (BM), large intestine (LI), small intestine (SI), mesenteric lymph nodes (MLN), liver, and peritoneal cavity (PEC) from wild-type and SpiCRES–GFPϕs mice were stained with anti-CD45 antibody, anti-CD11b antibody, and 7-AAD. All data are representative of two independent experiments. (B–F) SpiCRES–GFPϕ and Ly2-2-cre; Spicflox/flox mice were administered with 1.5% DSS for 7 d. (B) Weight changes of Spicflox/flox (n = 9) and Ly2-2-cre; Spicflox/flox (n = 8) mice. *P < 0.05. (C and D) Bleeding score (C) and stool score (D) of mice admininstered with 1.5% DSS for 10 d (n = 5 per group). **P < 0.05, ****P < 0.001. Mean ±SEM from two independent experiments are shown. E H&E staining and histopathological score of Spicflox/flox (n = 9) and Ly2-2-cre; Spicflox/flox (n = 8) mice. *P < 0.05. (F) The frequency (Upper) and number (bottom) of IFN-γ–, IL-17–, and IL-10–producing CD4+ T cells from Spicflox/flox (n = 7) and Ly2-2-cre; Spicflox/flox (n = 4) mice. *P < 0.05. All graphs show mean ± SEM from two independent experiments.

Attenuation of DSS-Induced Colitis by Heme Is Spi-C-Dependent. A previous study identified heme as an inducer of Spi expression in Mϕs (19). Heme-associated iron was present in the intestinal lamina propria as well as the spleen in both a steady state and a DSS-induced inflammatory condition (SI Appendix, Fig. S4A). In addition, hemoglobin and iron were detected in the intestinal lumen in the steady state, and their concentrations were increased after DSS administration (SI Appendix, Fig. S4B), thus indicating that intestinal Mϕs have a chance to be exposed to heme in the intestine. Therefore, we examined whether the induction of Spi expression in CX3CR1high Mϕs by heme mediates the suppression of intestinal inflammation. The i.p. injection of hemin (a Fe3+ oxidation product of heme) up-regulated Spi expression as well as iron/heme metabolism-related genes such as Hmox1, Slc40a1, and Bvhr in large intestinal CD11b+ CD11c+ cells (SI Appendix, Fig. S4C). Among CD11b+ innate myeloid cell types, Mϕs expressed the highest levels of Spi-C. As shown in the splenic Mϕs, heme-induced Spi-C expression was strongly induced, whereas in other cell types, Spi-C expression was only weakly up-regulated. However, by confocal microscopy, we observed that Spi-C–positive cells were mainly localized in the lamina propria, indicating that Spi-C is specifically expressed in intestinal Mϕs.
cells in the large intestinal lamina propria of hemin-injected mice, Cx3CR1/P FMs most highly expressed Spic (SI Appendix, Fig. S4D). We next examined the therapeutic effect of heme on DSS-induced colitis in Spic<sup>+/+</sup> and Lyz2-cre; Spic<sup>+/+-</sup> mice (Fig. 2). Without heme pretreatment, Lyz2-cre; Spic<sup>+/+-</sup> mice exhibited severe clinical parameters such as weight loss, stool bleeding, stool consistency, and colon shortening (Fig. 2A–D), which was associated with worsened colonic histopathology (Fig. 2E), compared with those in Spic<sup>+/+</sup> mice. In Spic<sup>+/+-</sup> mice, heme treatment reduced all of the clinical parameters with a marked amelioration of intestinal pathology, as reported previously (23). In contrast, Lyz2-cre; Spic<sup>+/+-</sup> mice suffered from profound weight loss with severe stool bleeding and shortening of the colon even after heme treatment, although a partial suppression of the weight loss and amelioration of stool consistency were observed. Moreover, histological analysis did not show a heme-mediated remediation of pathological changes in Lyz2-cre; Spic<sup>+/+-</sup> mice. In accordance with the reduced severity of colitis in the colons of Spic<sup>+/+</sup> mice, but not of Lyz2-cre; Spic<sup>+/+-</sup> mice, the number of colitogenic Th1 and Th17 cells after DSS administration was markedly decreased by heme pretreatment (Fig. 2F). Without pretreatment with heme, production of IL-6 and IL-17 in the colon was slightly increased in Lyz2-cre; Spic<sup>+/+-</sup> mice compared with that in Spic<sup>+/+</sup> mice (SI Appendix, Fig. S5). In Spic<sup>+/+-</sup> mice pretreated with heme, production of proinflammatory cytokines including IL-6, IL-17, IFN-γ, and IL-1α in the colon was greatly reduced compared with that in untreated Spic<sup>+/+</sup> mice. In contrast, Lyz2-cre; Spic<sup>+/+-</sup> mice did not show the heme-mediated suppression of IL-17, IFN-γ, and IL-1α production in the colons, although a modest reduction of IL-6 production was induced. These findings suggest that Sp-C is required for the heme-dependent suppression of intestinal inflammation.

**Decreased Expression of a Subset of TLR-Dependent Genes by Heme-Inducible Sp-C.** To determine how Sp-C controls the function of intestinal Cx3CR1/P FMs, we investigated the overall gene expression patterns in BMDMs prepared from Spic<sup>+/+-</sup> and Lyz2-cre; Spic<sup>+/+-</sup> mice. BMDMs were stimulated with or without LPS following heme treatment and used for RNA-seq analysis. In Spic<sup>+/+</sup> BMDMs stimulated with LPS, 85 genes were up-regulated compared with LPS-stimulated Spic<sup>+/+-</sup> BMDMs (SI Appendix, Table S2). We focused on proinflammatory cytokine genes such as Il6 and Il1α among these genes because they were reported to promote intestinal inflammation (24–28). To confirm the RNA-seq results, we analyzed the LPS-induced expression of Il6, Il1α, and Tnf in heme-pretreated FMs derived from peripheral blood monocytes (PB-MO) isolated from Spic<sup>+/+</sup> and Lyz2-cre; Spic<sup>+/+-</sup> mice using quantitative RT-PCR (Fig. 3A). The expression of Il6 and Il1α, but not Tnf, was markedly increased in Spic<sup>+/+</sup> PB-MOMs compared with control cells (Fig. 3A). To confirm the effects of Sp-C on LPS-inducible gene expression, we generated RAW264.7 cells stably expressing Sp-C (Fig. 3B and C) and analyzed the expression of Il6, Il1α, and Tnf (Fig. 3D). Sp-C-expressing RAW264.7 cells showed a marked decrease in the expression of Il6 and Il1α, but not of Tnf, following LPS stimulation. In accordance with the mRNA expression patterns, the production of IL-6 and IL-1α, but not of TNF-α, by heme-treated Spic<sup>+/+-</sup> PB-MOMs in response to LPS was augmented compared with control cells (Fig. 3E). These findings indicate that the heme-mediated expression of Sp-C negatively regulates the expression of a subset of LPS-inducible genes.

**Enhanced Production of IL-6 and IL-1α by Spic<sup>-/-</sup> Intestinal CX3CR1/P FMs.** We analyzed the role of Sp-C in the regulation of TLR-induced proinflammatory cytokine production by intestinal myeloid cells. Among intestinal CD11b<sup>+</sup> innate myeloid cells, CX3CR1/P FMs and CX3CR1<sup>int</sup>-cells from the large intestinal lamina propria were cultured in the presence of LPS and analyzed for the production of IL-6, IL-1α, TNF-α, and IL-10 (Fig. 4). The Sp-C deficiency in intestinal CX3CR1/P FMs led to increased production of IL-6 and IL-1α, but not of TNF-α and IL-10, while there was no difference in the production of all cytokines by CX3CR1<sup>int</sup>-cells from control or mutant mice, indicating that Sp-C suppresses a subset of TLR4-inducible genes including Il6 and Il1α in intestinal CX3CR1/P FMs.

**Dietary Iron Mediates the Induction of Sp-C in Intestinal Innate Myeloid Cells.** We next attempted to identify factors driving Spic expression in intestinal innate myeloid cells. Heme-mediated degradation of the transcription repressor Bach1 elicits the expression of Spic as well as Hmox1, Slc40a1, and Blvrb (19, 29). Previous studies have shown that a reduced intake of dietary iron decreased hemoglobin levels in vivo (30, 31). Thus, we examined the effect of dietary iron on Spic expression in large intestinal CX3CR1/P FMs. C57BL/6J mice fed with an AIN93G (control)
or iron-reduced (ΔFe) diet were analyzed for the expression of Spic in intestinal Cx3CR1<sup>high</sup> Mφs. Mice fed with the ΔFe diet had a lower concentration of blood hemoglobin compared with the control diet (SI Appendix, Fig. S6A). In addition, ΔFe-diet-fed mice exhibited a decreased concentration of iron in the serum, spleen, and colon (SI Appendix, Fig. S6B). In this context, expression of Hmox1, Slec40a1, and Bbvb was reduced in Cx3CR1<sup>high</sup> Mφs isolated from the colon of mice given the ΔFe diet (SI Appendix, Fig. S6C). Bacterial composition of feces analyzed by 16S rDNA gene sequencing was not altered at the phylum level between mice fed with the AIN93G and ΔFe diet, although a minor Proteobacteria population was slightly increased in the iron deficiency (SI Appendix, Fig. S6D). Thus, the iron deficiency did not alter the bacterial composition at the phylum level in the colon, indicating that the reduced Spic expression in the ΔFe-diet–fed mice was not induced by the altered composition of microbiota. To assess the impact of the decline in iron/hemoglobin levels by the ΔFe diet on the TLR-dependent production of proinflammatory cytokines by intestinal Cx3CR1<sup>high</sup> Mφs, we compared the expression of Ifn-α and TNF-α in Cx3CR1<sup>high</sup> Mφs with or without LPS (SI Appendix, Fig. S6 E–G). In large intestinal Cx3CR1<sup>high</sup> Mφs from ΔFe-diet–fed mice, the LPS-induced expression of Ifn-α, but not of TNF-α, was markedly increased (SI Appendix, Fig. S6E). Accordingly, IL-6 production in response to LPS by Cx3CR1<sup>high</sup> Mφs was enhanced in mice fed with the ΔFe diet compared with the control diet, whereas TNF-α was normally produced (SI Appendix, Fig. S6F). In contrast, expression of Ifn-α was not increased by the ΔFe diet in Lys2-cre; Spic<sup>ΔCR1<sup>lox/lox</sup></sup> mice (SI Appendix, Fig. S6G). Administration of hemin reduced ΔFe expression in Cx3CR1<sup>high</sup> Mφs of ΔFe-diet–fed Spic<sup>ΔCR1<sup>lox/lox</sup></sup> mice, but not Lys2-cre; Spic<sup>ΔCR1<sup>lox/lox</sup></sup> mice (SI Appendix, Fig. S6G). Thus, the reduced Spic expression caused by the iron deficiency is associated with the enhanced Ifn-α expression in Cx3CR1<sup>high</sup> Mφs.

**Fig. 3.** Expression of a subset of LPS-inducible genes up-regulated in Spic<sup>ΔCR1<sup>lox/lox</sup></sup> Mφs. (A) PB-MOMs prepared from control and Spic mutant mice were stimulated with LPS following pretreatment with hemin for 18 h and analyzed for the expression of Il6, Il1α, and Tnf. Graphs show the mean ± SEM of three independent experiments. *P < 0.05. (B) Expression of Spic in RAW264.7 cells stably expressing Spic-C-His and control cells. Graphs show the mean ± SD. Data are representative of four independent experiments. *P < 0.05. (C) Western blot analysis with anti-His and anti-β-actin antibodies using Spic-C-His stably expressing RAW264.7 cells and control cells. Data are representative of two independent experiments. (D) RAW264.7 cells stably expressing Spic-C-His and control cells stimulated with LPS were analyzed for the expression of Il6, Il1α, and Tnf. Graphs show the mean ± SD. Data are representative of two independent experiments. *P < 0.05, ***P < 0.005. n.s., not significant. (E) Production of IL-6, IL-1α, and TNF-α in response to LPS by hemin-pretreated PB-MOMs. Graphs show the mean ± SEM from four independent experiments. *P < 0.05. n.d., not detected; n.s., not significant.

**Fig. 4.** Increased production of IL-6 and IL-1α by intestinal Cx3CR1<sup>high</sup> Mφs of Lys2-cre; Spic<sup>ΔCR1<sup>lox/lox</sup></sup> mice. (A–D) LPS-induced production of IL-6 (A), IL-1α (B), TNF-α (C), and IL-10 (D) by large intestinal Cx3CR1<sup>high</sup> Mφs and Cx3CR1<sup>intermediate</sup> (intm)<sup>−</sup> cells. Mean values ± SEM from four independent experiments are shown. n.s., not significant. *P < 0.05.
nor NF-κB p65 was recruited to the Il6 promoter of Spi-C–expressing RAW264.7 cells (Fig. 5 F and G). We analyzed the LPS-induced recruitment of IRF5 and NF-κB p65 to the promoters of Spi-C target genes in SpiC<sup>Cre</sup> BMDM<sub>ϕ</sub> (Fig. 5 H and I). BMDM<sub>ϕ</sub> prepared from SpiC<sup>Δlox/Δlox</sup> and Lyz2-cre; SpiC<sup>Δlox/Δlox</sup> mice was pretreated with hemin for 18 h and stimulated with LPS. In SpiC<sup>Cre</sup> BMDMs, the recruitment of IRF5 to the Il6 promoter was increased after 1.5 and 5 h of LPS stimulation compared with control cells (Fig. 5H). In addition, elevated IRF5 recruitment to the Il1a promoter was observed 5 h after LPS stimulation in SpiC<sup>Cre</sup> cells (Fig. 5H), and significantly enhanced NF-κB p65 recruitment to the promoters of Il6 and Il1a was found in SpiC<sup>Cre</sup> BMDM<sub>ϕ</sub> relative to that found in control cells (Fig. 5I). Furthermore, the recruitment of IRF5 to the promoters of Il6 and Il1a, but not of Tnf, in large intestinal CxCR1<sup>Cre</sup> M<sub>ϕ</sub>s was increased in Lyz2-cre; SpiC<sup>Δlox/Δlox</sup> mice relative to that in SpiC<sup>Δlox/Δlox</sup> mice during DSS-induced colitis (SI Appendix, Fig. S7). These findings suggest that Spi-C suppresses the expression of a subset of LPS-inducible genes such as Il6 and Il1a by disrupting formation of the IRF5-NF-κB p65 complex through direct binding to IRF5.

Discussion

In this study, we showed that induction of Spi-C by the dietary iron-mediated heme in intestinal CxCR1<sup>Cre</sup> M<sub>ϕ</sub>s negatively regulates the transcription of a subset of TLR4-inducible genes through IRF5 binding and subsequent disruption of IRF5-NF-κB p65 complex formation, leading to the prevention of intestinal inflammation.

We demonstrated that Spi-C is expressed in CxCR1<sup>Cre</sup> M<sub>ϕ</sub>s residing in the large intestine and small intestine. Although a previous study reported that a lack of SpiC resulted in the absence of splenic RPM<sub>ϕ</sub>s (18), intestinal CxCR1<sup>Cre</sup> M<sub>ϕ</sub>s developed normally in Lyz2-cre; SpiC<sup>Δlox/Δlox</sup> mice. Thus, it would be important to determine if the reason for this discrepancy is associated with either a differential expression level of SpiC or distinct progenitors between RPM<sub>ϕ</sub>s and intestinal CxCR1<sup>Cre</sup> M<sub>ϕ</sub>s in the future.

In the colon of hemin-injected mice, SpiC expression was increased in CxCR1<sup>Cre</sup> M<sub>ϕ</sub>s compared with that in Lyz6C<sup>ϕ</sup> monocytes, which give rise to CxCR1<sup>Cre</sup> M<sub>ϕ</sub>s in the intestinal lamina propria, indicating that the induction of Spi-C in CxCR1<sup>Cre</sup> M<sub>ϕ</sub>s by hemin might take place locally in the intestine. Intestinal lamina propria CxCR1<sup>Cre</sup> M<sub>ϕ</sub>s extend their dendrites into the lumen, where hemoglobin and iron are present even in the steady state. In addition, CxCR1<sup>Cre</sup> M<sub>ϕ</sub>s express CD163 (33, 34), which scavenges hemoglobin (35). Therefore, endocytosis of the luminal hemoglobin-haptoglobin complex through CD163 may induce the expression of SpiC in intestinal CxCR1<sup>Cre</sup> M<sub>ϕ</sub>s. In the current study, we verified that systemic heme supplementation greatly remedied DSS-induced colitis in SpiC<sup>Δlox/Δlox</sup> mice and suppressed Il6 expression in CxCR1<sup>Cre</sup> M<sub>ϕ</sub>s in aFe-diet–fed mice. In Lyz2-cre; SpiC<sup>Δlox/Δlox</sup> mice, hemin treatment partially prevented severe weight loss during the early phase of DSS-induced colitis and only modestly suppressed expression of Il6 in Fe-diet–fed Lyz2-cre; SpiC<sup>Δlox/Δlox</sup> mice. Therefore, systemic heme repletion might abrogate intestinal inflammation in at least two ways: a Spi-C–dependent mechanism and a heme oxynase-1–dependent mechanism as previously reported (23).

Previous studies have defined that the hemoglobin-mediated modulation of M<sub>ϕ</sub> phenotypes is implicated in reduction of tissue injury in the brain (10–12, 36). Mucosal bleeding is a common symptom of inflammatory bowel diseases such as ulcerative colitis. Therefore, it would be interesting to analyze whether induction of SPI-C in human intestinal M<sub>ϕ</sub>s by local hemoysis is implicated in their acquisition of noninflammatory features, thereby associating them with mucosal healing.

The LPS-mediated recruitment of IRF5 to the NF-κB–binding site in the promoters of its target genes was aided by NF-κB p65 complex formation, leading to the prevention of intestinal tissue injury in the brain (10–12, 36). Mucosal bleeding is a common symptom of inflammatory bowel diseases such as ulcerative colitis. Therefore, it would be interesting to analyze whether induction of SPI-C in human intestinal M<sub>ϕ</sub>s by local hemoysis is implicated in their acquisition of noninflammatory features, thereby associating them with mucosal healing.

In the colon of hemin-injected mice, SpiC expression was increased in CxCR1<sup>Cre</sup> M<sub>ϕ</sub>s compared with that in Lyz6C<sup>ϕ</sup> monocytes, which give rise to CxCR1<sup>Cre</sup> M<sub>ϕ</sub>s in the intestinal lamina propria, indicating that the induction of Spi-C in CxCR1<sup>Cre</sup> M<sub>ϕ</sub>s by hemin might take place locally in the intestine. Intestinal lamina propria CxCR1<sup>Cre</sup> M<sub>ϕ</sub>s extend their dendrites into the lumen, where hemoglobin and iron are present even in the steady state. In addition, CxCR1<sup>Cre</sup> M<sub>ϕ</sub>s express CD163 (33, 34), which scavenges hemoglobin (35). Therefore, endocytosis of the luminal hemoglobin-haptoglobin complex through CD163 may induce the expression of SpiC in intestinal CxCR1<sup>Cre</sup> M<sub>ϕ</sub>s. In the current study, we verified that systemic heme supplementation greatly remedied DSS-induced colitis in SpiC<sup>Δlox/Δlox</sup> mice and suppressed Il6 expression in CxCR1<sup>Cre</sup> M<sub>ϕ</sub>s in aFe-diet–fed mice. In Lyz2-cre; SpiC<sup>Δlox/Δlox</sup> mice, hemin treatment partially prevented severe weight loss during the early phase of DSS-induced colitis and only modestly suppressed expression of Il6 in Fe-diet–fed Lyz2-cre; SpiC<sup>Δlox/Δlox</sup> mice. Therefore, systemic heme repletion might abrogate intestinal inflammation in at least two ways: a Spi-C–dependent mechanism and a heme oxynase-1–dependent mechanism as previously reported (23).

Previous studies have defined that the hemoglobin-mediated modulation of M<sub>ϕ</sub> phenotypes is implicated in reduction of tissue injury in the brain (10–12, 36). Mucosal bleeding is a common symptom of inflammatory bowel diseases such as ulcerative colitis. Therefore, it would be interesting to analyze whether induction of SPI-C in human intestinal M<sub>ϕ</sub>s by local hemoysis is implicated in their acquisition of noninflammatory features, thereby associating them with mucosal healing.

The LPS-mediated recruitment of IRF5 to the NF-κB–binding site in the promoters of its target genes was aided by NF-κB p65 (32). However, whether IRF5 mediates the persistent occupancy of NF-κB p65 in these promoters is unclear. In the present study, we observed a reduced recruitment of NF-κB p65 as well as IRF5 to the Il6 promoter in Spi-C stably expressing RAW264.7 cells stimulated with LPS. In addition, the recruitment of NF-κB p65 and IRF5 to the promoters of Il6 and Il1a was enhanced in SpiC<sup>Cre</sup> BMDMs. These findings suggest that IRF5 may regulate NF-κB p65 occupancy at the promoters of its target genes. Therefore, it is important to analyze whether the LPS-induced recruitment of NF-κB p65 to the promoters of IRF5 target genes is altered in Il6–/– M<sub>ϕ</sub>s. TNF–α is an IRF5–dependent gene as are IL-6 and IL-1α (32). However, the IRF5 recruitment to the Tnf promoter remained normal in SpiC<sup>Δlox/Δlox</sup> large intestinal CxCR1<sup>Cre</sup> M<sub>ϕ</sub>s, suggesting that proper IRF5 occupancy at the Tnf promoter is controlled by a Spi-C–independent mechanism. Therefore, it would be important to determine why Spi-C is selectively required for modulation of a subset of IRF5–dependent genes in the future.

Collectively, our results indicate that heme-induced Spi-C in intestinal M<sub>ϕ</sub>s regulates the transcriptional initiation of a subset of TLR4-inducible genes including Il6 and Il1a to attenuate...
intestinal inflammation. Previous studies demonstrated that treatment with neutralizing IL-6 mAbs improved DSS-induced colitis (37, 38), whereas Il6−/− mice administrated with DSS showed severe intestinal pathology with reduced epithelial cell proliferation (39). Therefore, it appears that the IL-6-signaling pathway contributes to the pathogenesis of intestinal inflammation while exerting its homeostatic function by regulating intestinal epithelial integrity. In addition to IL-6, some of the Spi-C target genes such as Il-10 (26–28), TREM-1 (40), CD38 (41), and CXCL11 (42) have been reported to mediate intestinal inflammation. Thus, the development of the method that controls Spi-C expression in human intestinal 

Materials and Methods

Detailed information on the materials, methods, and associated references can be found in SI Appendix, SI Materials and Methods.

Mice. C57BL/6J mice were purchased from Japan SLC. SpiC+/+ mice were generated as described previously (19). All of the mice were maintained under specific pathogen-free conditions. All animal experiments were conducted in accordance with the guidelines of the Animal Care and Use Committee of Osaka University.

ChIP Assay. BMDMs stimulated with 100 ng/mL LPS for the indicated periods following pretreatment with 40 μM hemin for 18 h and intestinal CXCR4+/+ Mφs were used for ChIP assay according to a previously described protocol (43). Il6 and Il1α-specific primers were designed to include the NF-κB-binding site.

Statistical Analysis. Differences between the control and experimental groups were evaluated by Student’s t test. Differences where P < 0.05 were considered statistically significant.

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