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

Hisako Kayama[a,b,c,1,2], Masako Kohyama[d,e,1], Daisuke Okuzaki[f], Daisuke Motooka[f], Soumik Barman[a,b,c], Ryu Okumura[a,b,c], Masato Muneta[a], Katsuaki Hoshino[g], Izumi Sasaki[h], Wataru Ise[i], Hiroshi Matsuno[j], Junichi Nishimura[j], Tomohiro Kurosaki[i], Shota Nakamura[f], Hisashi Arase[d,e], Tsuneyasu Kaisho[h,k,l,2], and Kiyoshi Takeda[a,b,c,2]

[a]Department of Microbiology and Immunology, Graduate School of Medicine, Osaka University, 565-0871 Osaka, Japan; [b]The World Premier International Research Center Initiative (WPI) Immunology Frontier Research Center, Osaka University, 565-0871 Osaka, Japan; [c]Core Research for Evolutional Science and Technology, Japan Agency for Medical Research and Development, 100-0004 Tokyo, Japan; [d]Laboratory of Immunochemistry, Immunology Frontier Research Center, Osaka University, 565-0871 Osaka, Japan; [e]Department of Immunochemistry, Research Institute for Microbial Diseases, Osaka University, 565-0871 Osaka, Japan; [f]Genome Information Research Center, Research Institute for Microbial Diseases, Osaka University, 565-0871 Osaka, Japan; [g]Department of Immunology, Faculty of Medicine, Kagawa University, 761-0793 Kagawa, Japan; [h]Department of Immunology, Institute of Advanced Medicine, Wakayama Medical University, 641-8509 Wakayama, Japan; [i]Laboratory of Lymphocyte Differentiation, Immunology Frontier Research Center, Osaka University, 565-0871 Osaka, Japan; [j]Department of Gastroenterological Surgery, Graduate School of Medicine, Osaka University, 565-0871 Osaka, Japan; [k]Laboratory for Immune Regulation, WPI Immunology Frontier Research Center, Osaka University, 565-0871 Osaka, Japan; and [l]Japan Laboratory for Inflammatory Regulation, Research Center for Allergy and Immunology RIKEN Center for Integrative Medical Science (IMS-RCAI), 230-0045 Kanagawa, Japan

Edited by Kenneth M. Murphy, Washington University, St. Louis, MO, and approved July 10, 2018 (received for review May 17, 2018)

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 CX_3CR1^{high} Mϕs. Intestinal CX_3CR1^{high} Mϕs highly expressed Spi-C in a heme-dependent manner, and myeloid lineage-specific *Spic*-deficient (*Lyz2-cre*; $Spic^{flox/flox}$) 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 CX_3CR1^{high} Mϕs to prevent colitis. LPS-induced production of IL-6 and IL-1α, but not IL-10 and TNF-α, by large intestinal Mϕs from *Lyz2-cre*; $Spic^{flox/flox}$ mice was markedly enhanced. The interaction of Spi-C with IRF5 was linked to disruption of the IRF5-NF-κB p65 complex formation, thereby abrogating recruitment of IRF5 and NF-κB p65 to the *Il6* and *Il1a* promoters. Collectively, these results demonstrate that heme-mediated Spi-C is a key molecule for the noninflammatory signature of intestinal Mϕs by suppressing the induction of a subset of TLR-inducible genes through binding to IRF5.

Spi-C | intestinal macrophage | inflammation | heme | IRF5

Tissue-resident macrophages (Mϕs) play a key role in innate immune surveillance against tissue-invading pathogens. In addition, they have been characterized as anti-inflammatory Mϕs responsible for maintaining tissue homeostasis and resolving inflammatory responses (1). For example, intestinal resident CX_3CR1^{high} Mϕs derived from $Ly6C^{high}$ $CCR2^{+}$ monocytes after birth (2, 3) mediate innate immune defense against intestinal microorganisms by exerting high phagocytic activity (4). To maintain mucosal tolerance, noninflammatory gene expression patterns in intestinal CX_3CR1^{high} Mϕs are precisely controlled through IL-10 receptor signaling-dependent mechanisms (5, 6). Intestinal CX_3CR1^{high} Mϕs in *Il10ra*-deficient mice displayed the elevated expression of Toll-like receptor (TLR)-dependent proinflammatory mediators such as *Il12b*, *Il23a*, and *Nos2*, while a subset of TLR-inducible genes such as *Il6* and *Tnf* was unchanged (5). Accordingly, large intestinal CX_3CR1^{high} Mϕs from $Il10^{-/-}$ mice did not show increased IL-6 production (7). Thus, the activity of intestinal CX_3CR1^{high} Mϕs 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

Significance

Following hemorrhage in damaged tissues, hemoglobin induces macrophages (Mϕs) possessing ability to protect against tissue inflammation. Hemorrhage-appearing 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 CX_3CR1^{high} Mϕs. We found that the *Spic* 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 CX_3CR1^{high} Mϕs through inhibition of IRF5-NF-κB 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.

Author contributions: H.K., M.K., D.O., D.M., R.O., S.N., H.A., and K.T. designed research; H.A., T. Kaisho, and K.T. supervised the study; H.K., K.H., and S.N. performed research; K.H., I.S., W.I., T. Kurosaki, and T. Kaisho contributed new reagents/analytic tools; H.K., D.O., D.M., S.B., R.O., M.M., H.M., J.N., and S.N. analyzed data; and H.K., M.K., and K.T. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

Data deposition: The data reported in this paper have been deposited in the Gene Expression Omnibus (GEO) database, www.ncbi.nlm.nih.gov/geo (accession nos. GSE100804 and GSE100805).

[1]H.K. and M.K. contributed equally to this work.

[2]To whom correspondence may be addressed. Email: kayama@ongene.med.osaka-u.ac.jp, tkaisho@wakayama-med.ac.jp, or ktakeda@ongene.med.osaka-u.ac.jp.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1808426115/-/DCSupplemental.

Published online July 30, 2018.

colitis that 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 *Spic* 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 genes 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 CX_3CR1^{high} Mϕs. Unlike other tissue-resident Mϕs, transcription factors that regulate the differentiation and function of CX_3CR1^{high} Mϕs in the large intestine are poorly understood. Thus, to identify transcription factors specifically expressed in CX_3CR1^{high} Mϕs among large intestinal innate myeloid cells, we comprehensively analyzed gene expression profiles in CX_3CR1^{high} Mϕs, CX_3CR1^- CD11b$^+$ CD11c$^+$ cells, CD11b$^-$ CD11c^{high} DCs, and CD11b$^+$ CD11c$^-$ cells (*SI Appendix*, Table S1). Among four subsets, CX_3CR1^{high} Mϕs highly expressed *Spic*. *Spic*$^{igfp/igfp}$ 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. 1*A*). In the colon, Spi-C–expressing CD11b$^+$ CD11c$^{+/-}$ cells highly expressed CX_3CR1 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 CX_3CR1^{high} 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 CX_3CR1^{high} Mϕs, myeloid lineage cell-specific Spi-C–deficient (*Lyz2-cre*; *Spic*$^{flox/flox}$) mice were generated (*SI Appendix*, Fig. S2 *A–C*). The expression of *Spic* mRNA was markedly reduced in large intestinal CX_3CR1^{high} Mϕs in *Lyz2-cre*; *Spic*$^{flox/flox}$ mice compared with *Spic*$^{flox/flox}$ mice (*SI Appendix*, Fig. S2*D*). However, the frequency of CX_3CR1^{high} Mϕs was not altered in the large intestinal lamina propria (*SI Appendix*, Fig. S2*E*). 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 *Spic*$^{flox/flox}$ and *Lyz2-cre*; *Spic*$^{flox/flox}$ mice (*SI Appendix*, Fig. S2 *F* and *G*). In the spleen of *Lyz2-cre*; *Spic*$^{flox/flox}$ mice, the number of RPMϕs was normal (*SI Appendix*, Fig. S3*A*). In addition, *Spic* expression was not reduced in RPMϕs isolated from *Lyz2-cre*; *Spic*$^{flox/flox}$ mice (*SI Appendix*, Fig. S3*B*). This might be due to the lower expression level of *Lyz2* in RPMϕs than intestinal in CX_3CR1^{high} Mϕs (*SI Appendix*, Fig. S3*C*).

We investigated whether Spi-C in CX_3CR1^{high} Mϕs regulates intestinal inflammation by orally administering 1.5% DSS. Compared with *Spic*$^{flox/flox}$ mice, *Lyz2-cre*; *Spic*$^{flox/flox}$ 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 *Lyz2-cre*; *Spic*$^{flox/flox}$ mice following DSS administration compared with *Spic*$^{flox/flox}$ mice (Fig. 1*F*). These findings indicate that Spi-C in intestinal CX_3CR1^{high} Mϕs plays an important role in the suppression of intestinal inflammation.

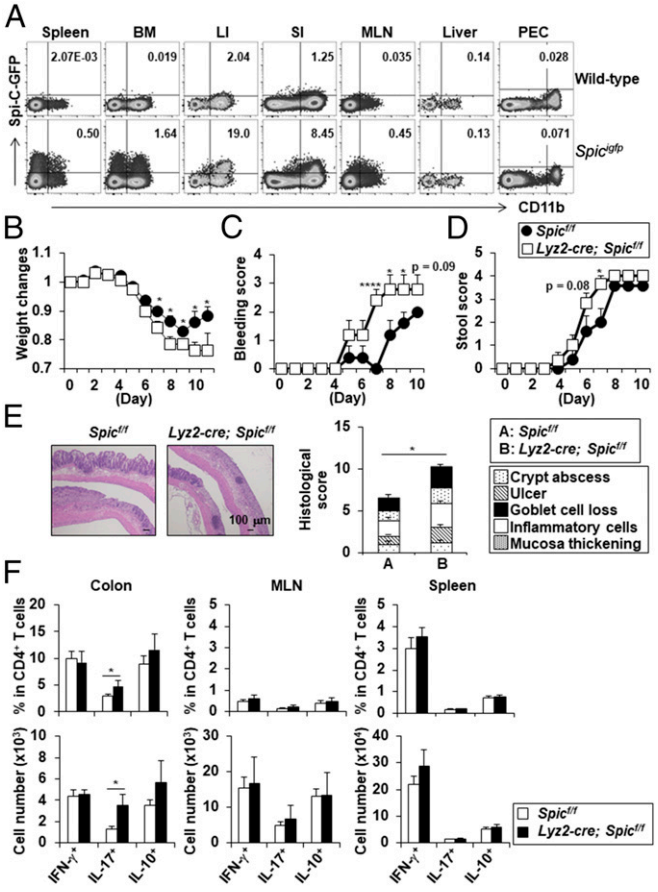

Fig. 1. *Lyz2-cre*; *Spic*$^{flox/flox}$ mice showed exacerbated intestinal inflammation following DSS administration. (*A*) Flow cytometric plots of Spi-C-EGFP– and CD11b-expressing cells. The cells from the spleen, bone marrow (BM), large intestine (LI), small intestine (SI), mesenteric lymph nodes (MLN), liver, and peritoneal cavity (PEC) from wild-type and *Spic*$^{IRES\text{-}GFP/GFP}$ mice were stained with anti-CD45 antibody, anti-CD11b antibody, and 7-AAD. All data are representative of two independent experiments. (*B–F*) *Spic*$^{flox/flox}$ and *Lyz2-cre*; *Spic*$^{flox/flox}$ mice were administered with 1.5% DSS for 7 d. (*B*) Weight changes of *Spic*$^{flox/flox}$ (n = 9) and *Lyz2-cre*; *Spic*$^{flox/flox}$ (n = 8) mice. *P < 0.05. (*C* and *D*) Bleeding score (*C*) and stool score (*D*) of mice administered 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 *Spic*$^{flox/flox}$ (n = 9) and *Lyz2-cre*; *Spic*$^{flox/flox}$ (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 *Spic*$^{flox/flox}$ (n = 7) and *Lyz2-cre*; *Spic*$^{flox/flox}$ (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 *Spic* 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. S4*A*). 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. S4*B*), thus indicating that intestinal Mϕs have a chance to be exposed to heme in the intestine. Therefore, we examined whether the induction of *Spic* expression in CX_3CR1^{high} Mϕs by heme mediates the suppression of intestinal inflammation. The i.p. injection of hemin (a Fe^{3+} oxidization product of heme) up-regulated *Spic* expression as well as iron/heme metabolism-related genes such as *Hmox1*, *Slc40a1*, and *Blvrb* in large intestinal CD11b$^+$ CD11c$^+$ cells (*SI Appendix*, Fig. S4*C*). Among CD11b$^+$ innate myeloid

cells in the large intestinal lamina propria of hemin-injected mice, CX_3CR1^{high} Mϕs most highly expressed *Spic* (*SI Appendix*, Fig. S4*D*). We next examined the therapeutic effect of heme on DSS-induced colitis in $Spic^{flox/flox}$ and *Lyz2-cre*; $Spic^{flox/flox}$ mice (Fig. 2). Without hemin pretreatment, *Lyz2-cre*; $Spic^{flox/flox}$ mice exhibited severe clinical parameters such as weight loss, stool bleeding, stool consistency, and colon shortening (Fig. 2 *A*–*D*), which was associated with worsened colonic histopathology (Fig. 2*E*), compared with those in $Spic^{flox/flox}$ mice. In $Spic^{flox/flox}$ mice, hemin treatment reduced all of the clinical parameters with a marked amelioration of intestinal pathology, as reported previously (23). In contrast, *Lyz2-cre*; $Spic^{flox/flox}$ mice suffered from profound weight loss with severe stool bleeding and shortening of the colon even after hemin treatment, although a partial suppression of the weight loss and amelioration of stool consistency were observed. Moreover, histological analysis did not show a hemin-mediated remediation of pathological changes in *Lyz2-cre*; $Spic^{flox/flox}$ mice. In accordance with the reduced severity of colitis in the colons of $Spic^{flox/flox}$ mice, but not of *Lyz2-cre*; $Spic^{flox/flox}$ mice, the number of colitogenic Th1 and Th17 cells after DSS administration was markedly decreased by hemin pretreatment (Fig. 2*F*). Without pretreatment with hemin, production of IL-6 and IL-17 in the colon was slightly increased in *Lyz2-cre*; $Spic^{flox/flox}$ mice compared with that in $Spic^{flox/flox}$ mice (*SI Appendix*, Fig. S5). In $Spic^{flox/flox}$ mice pretreated with hemin, 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 $Spic^{flox/flox}$ mice. In contrast, *Lyz2-cre*; $Spic^{flox/flox}$ mice did not show the hemin-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 Spi-C is required for the heme-dependent suppression of intestinal inflammation.

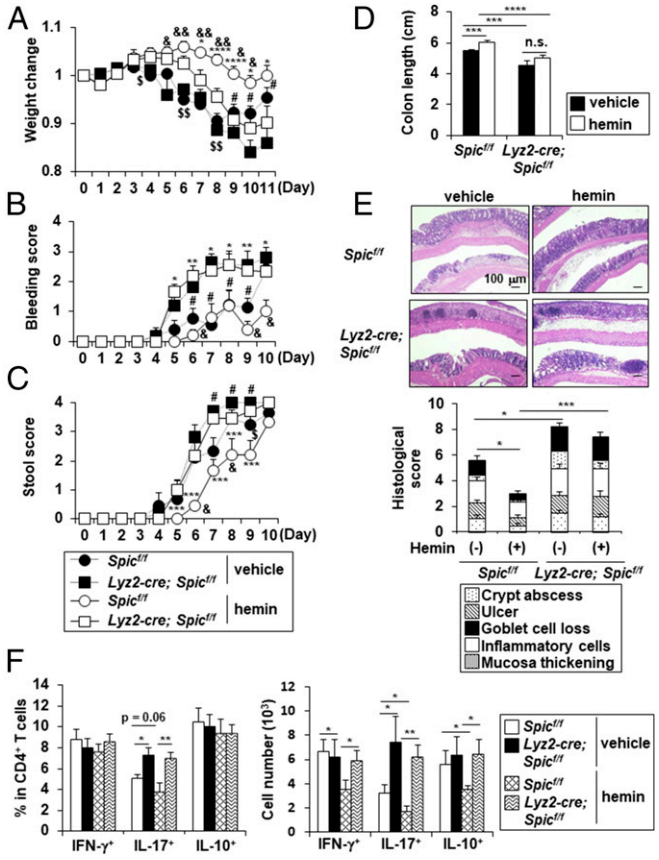

Fig. 2. Minimal therapeutic effects of hemin on symptoms of DSS-induced colitis in *Lyz2-cre*; $Spic^{flox/flox}$ mice. $Spic^{flox/flox}$ and *Lyz2-cre*; $Spic^{flox/flox}$ mice intraperitoneally injected with 1 mg hemin or vehicle on days −2 and −1 of DSS administration and administered with 1.5% DSS for 7 d (n = 8–9 per group) (*A*, *E*, and *F*) or 11 d (n = 5–8 per group) (*B*–*D*). (*A*) Weight changes. $^{*}P < 0.05$, $^{****}P < 0.001$, $^{\#}P < 0.05$, $^{\$}P < 0.05$, $^{\$\$}P < 0.01$, $^{\&}P < 0.05$, $^{\&\&}P < 2 \times 10^{-5}$. (*B* and *C*) Bleeding score (*B*) and stool score (*C*). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.005$, $^{\#}P < 0.05$, $^{\$}P < 0.05$, $^{\&}P < 0.05$, $^{*}P$ denotes significance between heme-treated control and *Spic* mutant mice, $^{\#}P$ denotes significance between vehicle-injected control and *Spic* mutant mice, $^{\$}P$ denotes significance between vehicle-injected and heme-injected *Spic* mutant mice, and $^{\&}P$ denotes significance between vehicle-injected and heme-injected control mice (*A*–*C*). (*D*) Colon length. $^{***}P < 0.005$, $^{****}P < 0.001$. (*E*) Representative colon sections (*Upper*) and histological score (*Bottom*) of the colons. $^{*}P < 0.05$, $^{***}P < 0.005$. (*F*) Frequency (*Left*) and number (*Right*) of IFN-γ–, IL-17–, and IL-10–producing $CD4^+$ T cells. $^{*}P < 0.05$, $^{**}P < 0.01$.

Decreased Expression of a Subset of TLR-Dependent Genes by Heme-Inducible Spi-C. To determine how Spi-C controls the function of intestinal CX_3CR1^{high} Mϕs, we investigated the overall gene expression patterns in BMDMϕs prepared from $Spic^{flox/flox}$ and *Lyz2-cre*; $Spic^{flox/flox}$ mice. BMDMϕs were stimulated with or without LPS following hemin treatment and used for RNA-seq analysis. In $Spic^{-/-}$ BMDMϕs stimulated with LPS, 85 genes were up-regulated compared with LPS-stimulated $Spic^{flox/flox}$ BMDMϕs (*SI Appendix*, Table S2). We focused on proinflammatory cytokine genes such as *Il6* and *Il1a* 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*, *Il1a*, and *Tnf* in hemin-pretreated Mϕs derived from peripheral blood monocytes (PB-MO) isolated from $Spic^{flox/flox}$ and *Lyz2-cre*; $Spic^{flox/flox}$ mice using quantitative RT-PCR (Fig. 3*A*). The expression of *Il6* and *Il1a*, but not *Tnf*, was markedly increased in $Spic^{-/-}$ PB-MOMϕs compared with control cells (Fig. 3*A*). To confirm the effects of Spi-C on LPS-inducible gene expression, we generated RAW264.7 cells stably expressing Spi-C (Fig. 3 *B* and *C*) and analyzed the expression of *Il6*, *Il1a*, and *Tnf* (Fig. 3*D*). Spi-C–expressing RAW264.7 cells showed a marked decrease in the expression of *Il6* and *Il1a*, 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 hemin-treated $Spic^{-/-}$ PB-MOMϕs in response to LPS was augmented compared with control cells (Fig. 3*E*). These findings indicate that the heme-mediated expression of Spi-C negatively regulates the expression of a subset of LPS-inducible genes.

Enhanced Production of IL-6 and IL-1α by $Spic^{-/-}$ Intestinal CX_3CR1^{high} Mϕs. We analyzed the role of Spi-C in the regulation of TLR-induced proinflammatory cytokine production by intestinal myeloid cells. Among intestinal $CD11b^+$ innate myeloid cells, CX_3CR1^{high} Mϕs and $CX_3CR1^{int/-}$ 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 Spi-C deficiency in intestinal CX_3CR1^{high} Mϕs 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 $CX_3CR1^{int/-}$ cells from control or mutant mice, indicating that Spi-C suppresses a subset of TLR4-inducible genes including *Il6* and *Il1a* in intestinal CX_3CR1^{high} Mϕs.

Dietary Iron Mediates the Induction of Spi-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 CX_3CR1^{high} Mϕs. C57BL/6J mice fed with an AIN93G (control)

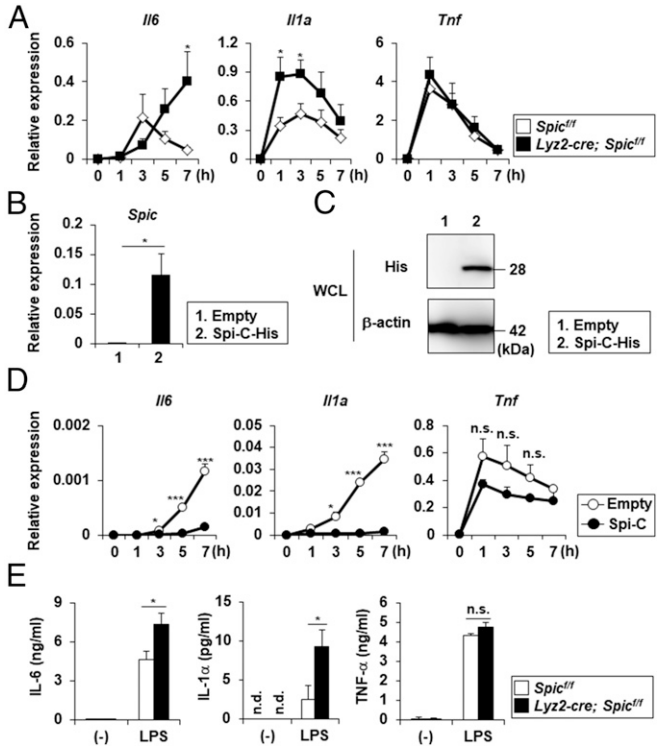

Fig. 3. Expression of a subset of LPS-inducible genes up-regulated in *Spic*$^{-/-}$ Mϕs. (*A*) PB-MOMϕs prepared from control and *Spic* mutant mice were stimulated with LPS following pretreatment with hemin for 18 h and analyzed for the expression of *Il6*, *Il1a*, and *Tnf*. Graphs show the mean ± SEM of three independent experiments. *$P < 0.05$. (*B*) Expression of *Spic* in RAW264.7 cells stably expressing Spi-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 Spi-C-His stably expressing RAW264.7 cells and control cells. Data are representative of two independent experiments. (*D*) RAW264.7 cells stably expressing Spi-C-His and control cells stimulated with LPS were analyzed for the expression of *Il6*, *Il1a*, 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-MOMϕs. Graphs show the mean ± SEM from four independent experiments. *$P < 0.05$. n.d., not detected; n.s., not significant.

or iron-reduced (ΔFe) diet were analyzed for the expression of *Spic* in intestinal CX_3CR1^{high} Mϕs. Mice fed with the ΔFe diet had a lower concentration of blood hemoglobin compared with the control diet (*SI Appendix*, Fig. S6*A*). In addition, ΔFe-diet–fed mice exhibited a decreased concentration of iron in the serum, spleen, and colon (*SI Appendix*, Fig. S6*B*). In this context, expression of *Spic*, *Hmox1*, *Slc40a1*, and *Blvrb* was reduced in CX_3CR1^{high} Mϕs isolated from the colon of mice given the ΔFe diet (*SI Appendix*, Fig. S6*C*). Bacterial composition of feces analyzed by 16S rRNA 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. S6*D*). 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 CX_3CR1^{high} Mϕs, we compared the expression of *Il6* and *Tnf* in CX_3CR1^{high} Mϕs stimulated with or without LPS (*SI Appendix*, Fig. S6 *E*–*G*). In large intestinal CX_3CR1^{high} Mϕs from ΔFe-diet–fed mice, the LPS-induced expression of *Il6*, but not of *Tnf*, was markedly increased (*SI Appendix*, Fig. S6*E*). Accordingly, IL-6 production in response to LPS by CX_3CR1^{high} Mϕs was enhanced in mice fed with the ΔFe diet compared with the control diet, whereas TNF-α was normally produced (*SI Appendix*, Fig. S6*F*). In contrast, expression of *Il6* was not increased by the ΔFe diet in *Lyz2-cre*; *Spic*$^{flox/flox}$ mice (*SI Appendix*, Fig. S6*G*). Administration of hemin reduced *Il6* expression in CX_3CR1^{high} Mϕs of ΔFe-diet–fed *Spic*$^{flox/flox}$ mice, but not *Lyz2-cre*; *Spic*$^{flox/flox}$ mice (*SI Appendix*, Fig. S6*G*). Thus, the reduced *Spic* expression caused by the iron deficiency is associated with the enhanced *Il6* expression in CX_3CR1^{high} Mϕs.

Spi-C–Mediated Repression of a Subset of LPS-Inducible Genes by Association with IRF5. Because dietary iron-mediated Spi-C expression is implicated in the down-regulation of *Il6* expression in CX_3CR1^{high} Mϕs, we examined how Spi-C impacts on the transcriptional initiation of its target genes in Mϕs. Ingenuity Pathway Analysis displayed the upstream regulators of 85 genes up-regulated in *Spic*$^{-/-}$ BMMϕs (*SI Appendix*, Table S2). Among the top five predicted upstream regulators, the IRF family members such as IRF3, IRF7, and IRF5 were included (Fig. 5*A*). Therefore, we analyzed the association of Spi-C with IRF3, IRF5, and IRF7 by a coimmunoprecipitation assay (Fig. 5*B*). Among the three molecules, Spi-C interacted with IRF5 and IRF7, but not IRF3. A recent study demonstrated that IRF5-NF-κB p65 complex formation is indispensable for driving the expression of a subset of LPS-inducible genes (32). Therefore, we examined whether Spi-C altered the association of IRF5 and NF-κB p65 (Fig. 5*C*). NF-κB p65 bound to IRF5 as previously reported (32), but not to Spi-C. However, in the presence of Spi-C, formation of the IRF5-NF-κB p65 complex was markedly reduced. In contrast to IRF5, IRF7 did not bind to NF-κB p65 (Fig. 5*D*). IRF5 recruitment to the NF-κB–binding site in the promoter of its target genes is guided by NF-κB p65 (32). Therefore, to investigate the effects of the Spi-C–mediated disruption of IRF5-NF-κB p65 complex formation on transcriptional initiation of *Il6* in response to LPS, we analyzed the recruitment of NF-κB p65 and IRF5 to the NF-κB site of the *Il6* promoter in the RAW264.7 cells stably expressing Spi-C-His used in Figs. 3 and 5 *E*–*G*. Chromatin immunoprecipitation assay showed that Spi-C did not bind to the *Il6* promoter even after LPS stimulation (Fig. 5*E*). In control cells, the LPS-dependent recruitment of IRF5 and NF-κB p65 was observed at the NF-κB site of the *Il6* promoter (Fig. 5 *F* and *G*). However, neither IRF5

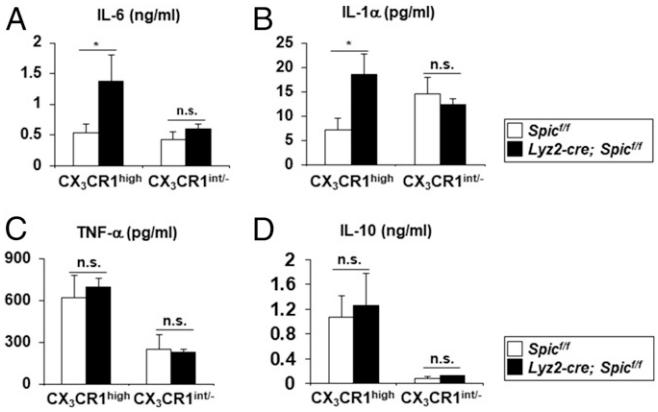

Fig. 4. Increased production of IL-6 and IL-1α by intestinal CX_3CR1^{high} Mϕs of *Lyz2-cre*; *Spic*$^{flox/flox}$ mice. (*A*–*D*) LPS-induced production of IL-6 (*A*), IL-1α (*B*), TNF-α (*C*), and IL-10 (*D*) by large intestinal CX_3CR1^{high} Mϕs and $CX_3CR1^{intermediate\ (int)/-}$ cells. Mean values ± SEM from four independent experiments are shown. n.s., not significant. *$P < 0.05$.

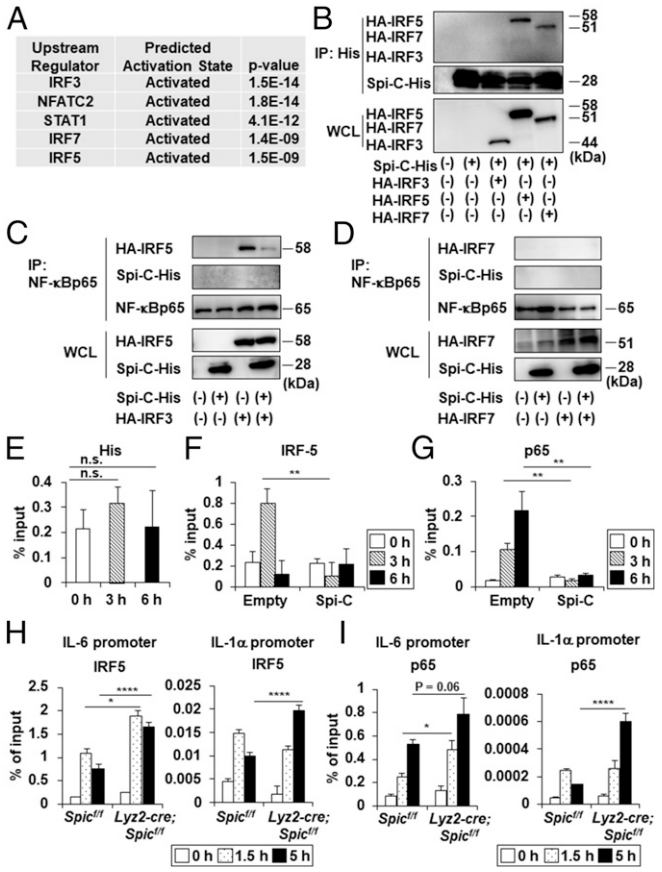

Fig. 5. Spi-C suppresses the expression of a subset of TLR4-dependent genes by interacting with IRF5. (*A*) Enrichment analysis of the upstream transcriptional regulators of genes elevated in *Spic*$^{-/-}$ BMDMϕs. (*B–D*) Coimmunoprecipitation (IP) assay with anti-His (*B*) and anti–NF-κB p65 (*C* and *D*) antibodies for immunoprecipitation and the indicated antibodies for immunoblotting. (*E–G*) ChIP assay for the *Il6* promoter of the NF-κB–binding site was performed with anti-His (*E*), anti-IRF5 (*F*), and anti–NF-κB p65 (*G*) antibodies. All graphs show the mean ± SD. Data are representative of two independent experiments. $^{**}P < 0.01$. n.s., not significant. (*H* and *I*) ChIP assay for the NF-κB site of the *Il6* promoter and *Il1a* promoter was performed with anti-IRF5 (*H*) and anti–NF-κB p65 (*I*) antibodies using control and *Spic*$^{-/-}$ BMDMϕs stimulated with LPS following pretreatment with hemin for 18 h. Graphs show the means ± SD. Data are representative of two independent experiments. $^{*}P < 0.05$, $^{****}P < 0.001$.

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*$^{-/-}$ BMDMϕ (Fig. 5 *H* and *I*). BMDMϕ prepared from *Spic*$^{flox/flox}$ and *Lyz2-cre*; *Spic*$^{flox/flox}$ mice was pretreated with hemin for 18 h and stimulated with LPS. In *Spic*$^{-/-}$ BMMϕ, the recruitment of IRF5 to the *Il6* promoter was increased after 1.5 and 5 h of LPS stimulation compared with control cells (Fig. 5*H*). In addition, elevated IRF5 recruitment to the *Il1a* promoter was observed 5 h after LPS stimulation in *Spic*$^{-/-}$ cells (Fig. 5*H*), and significantly enhanced NF-κB p65 recruitment to the promoters of *Il6* and *Il1a* was found in *Spic*$^{-/-}$ BMMϕ relative to that found in control cells (Fig. 5*I*). Furthermore, the recruitment of IRF5 to the promoters of *Il6* and *Il1a*, but not of *Tnf*, in large intestinal CX_3CR1^{high} Mϕs was increased in *Lyz2-cre*; *Spic*$^{flox/flox}$ mice relative to that in *Spic*$^{flox/flox}$ 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 CX_3CR1^{high} Mϕs negatively regulates the transcription of a subset of TLR-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 CX_3CR1^{high} Mϕs residing in the large intestine and small intestine. Although a previous study reported that a lack of *Spic* resulted in the absence of splenic RPMϕs (18), intestinal CX_3CR1^{high} Mϕs developed normally in *Lyz2-cre*; *Spic*$^{flox/flox}$ 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 RPMϕs and intestinal CX_3CR1^{high} Mϕs in the future.

In the colon of hemin-injected mice, *Spic* expression was increased in CX_3CR1^{high} Mϕ compared with that in $Ly6C^{+}$ monocytes, which give rise to CX_3CR1^{high} Mϕs in the intestinal lamina propria, indicating that the induction of Spi-C in CX_3CR1^{high} Mϕs by hemin might take place locally in the intestine. Intestinal lamina propria CX_3CR1^{high} Mϕs extend their dendrites into the lumen, where hemoglobin and iron are present even in the steady state. In addition, CX_3CR1^{high} Mϕs 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 CX_3CR1^{high} Mϕs. In the current study, we verified that systemic heme supplementation greatly remedied DSS-induced colitis in *Spic*$^{flox/flox}$ mice and suppressed *Il6* expression in CX_3CR1^{high} Mϕs in ΔFe-diet–fed mice. In *Lyz2-cre*; *Spic*$^{flox/flox}$ mice, hemin treatment partially prevented severe weight loss during the early phase of DSS-induced colitis and only modestly suppressed expression of *Il6* in ΔFe-diet–fed *Lyz2-cre*; *Spic*$^{flox/flox}$ 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 Mϕ 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 Mϕs by local hemolysis 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 of 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*$^{-/-}$ BMMϕs. 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 *Irf5*$^{-/-}$ Mϕs. 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*$^{-/-}$ large intestinal CX_3CR1^{high} Mϕs, 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 Mϕs 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-1α (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 Mϕs may represent a therapeutic approach for inflammatory bowel diseases.

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*$^{igfp/igfp}$ 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. BMDMϕs stimulated with 100 ng/mL LPS for the indicated periods following pretreatment with 40 μM hemin for 18 h and intestinal CX_3CR1^{high} Mϕs were used for ChIP assay according to a previously described protocol (43). *Il6*- and *Il1a*-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.

ACKNOWLEDGMENTS. We thank T. Kondo and Y. Magota for their technical assistance, C. Hidaka for secretarial assistance, and J. L. Croxford, PhD (Edanz Group: www.edanzediting.com/ac) for editing a draft of this manuscript. This work was supported by grants from the Ministry of Education, Culture, Sports, Science and Technology of Japan; the Japan Agency for Medical Research and Development (K.T.); a Grant-in-Aid for Young Scientists (B) (26860330); the Takeda Science Foundation; the Mochida Memorial Foundation for Medical and Pharmaceutical Research; the Japan Intractable Diseases (Nanbyo) Research Foundation; and the Astellas Foundation for Research on Metabolic Disorders (H.K.).

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