腸炎惹起性サイトカイン関連シグナル伝達分子 cIAPs の発現は、IRF4 の活性化と逆相関する

正 木 翔

博士学位論文

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Doctoral Dissertation

Expression levels of cellular inhibitor of apoptosis proteins and colitogenic cytokines are inversely correlated with the activation of interferon regulatory factor 4

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論博

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Research Article

Expression levels of cellular inhibitor of apoptosis proteins and colitogenic cytokines are inversely correlated with the activation of interferon regulatory factor 4

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Abstract

Cellular inhibitors of apoptosis proteins 1 (cIAP1) and 2 (cIAP2) are involved in signaling pathways mediated by Toll-like receptors (TLRs) and tumor necrosis factor (TNF)-α. Excessive activation of TLRs and TNF-α underlies the immunopathogenesis of Crohn's disease (CD) and ulcerative colitis (UC). However, the roles played by cIAP1 and cIAP2 in the development of CD and UC remain poorly understood. In this study, we attempted to clarify the molecular link between cIAP1/cIAP2 and colonic inflammation. Human monocyte-derived dendritic cells (DCs) treated with siRNAs specific for cIAP1 or cIAP2 exhibited reduced pro-inflammatory cytokine responses upon stimulation with TLR ligands. Expression of cIAP1 and cIAP2 in human DCs was suppressed in the presence of interferon regulatory factor 4 (IRF4). This effect was associated with inhibition of cIAP1 and cIAP2 polyubiquitination. To verify these *in vitro* findings, we created mice overexpressing IRF4 in DCs and showed that these mice were resistant to trinitrobenzene sulfonic acid-induced colitis as compared with wild-type mice; these effects were accompanied by reduced expression levels of cIAP1 and cIAP2. Pro-inflammatory cytokine production by mesenteric lymph node cells upon stimulation with TLR ligands was reduced in mice with DC-specific IRF4 overexpression as compared with that in wild-type mice. Finally, in clinical samples of the colonic mucosa from patients with CD, there was a negative relationship between the percentage of $IRF4^+$ DCs and percentages of $cIAP1^+$ or $cIAP2^+$ lamina propria mononuclear cells. These data suggest that the colitogenic roles of cIAP1 and cIAP2 are negatively regulated by IRF4.

Keywords: cellular inhibitor of apoptosis protein 1, cellular inhibitor of apoptosis protein 2, colitis, interferon regulatory factor 4, Toll-like receptor

Abbreviations: CD, Crohn's disease; CIAP, cellular inhibitor of apoptosis protein; DC, dendritic cell; ELISA, enzyme-linked immunosorbent assay; HA, human influenza hemagglutinin; HEK293, human embryonic kidney 293; IB, immunoblotting; IFN, interferon; IL, interleukin; IP, immunoprecipitation; IRAK-M, IL-1 receptor-associated kinase M; IRF4, interferon regulatory factor 4; LPMC, lamina propria mononuclear cell; LPS, lipopolysaccharide; MDP, muramyl dipeptide; MLN, mesenteric lymph node; NF-κB, nuclear factor-κB; NOD2, nucleotide-binding oligomerization domain 2; PAM, Pam3CSK4; RIPK2, receptor-interacting serine/threonine kinase 2; Tg, transgenic; TLR, Toll-like receptor; TNBS, trinitrobenzene sulfonic acid; TNF, tumor necrosis factor; TRAF6, TNF receptor-associated factor 6; Ub, ubiquitin; UC, ulcerative colitis

Introduction

Although sensing of commensal bacteria by Toll-like receptors (TLRs) is critical for maintaining intestinal homeostasis, excessive activation of TLRs leads to colitis [1, 2]. This notion is fully supported by the fact that spontaneous development of colitis in interleukin (IL)-10-deficient mice was abrogated in the presence of myeloid cell-specific deletion of myeloid differentiation factor 88, a critical downstream signaling molecule of multiple TLRs [3]. To avoid colitogenic consequences of TLR activation, TLR signaling is tightly controlled by negative regulators [4]. IL-1 receptor-associated kinase M (IRAK-M) and interferon (IFN) regulatory factor 4 (IRF4) are prototypical negative regulators of TLR signaling pathways [4]. Given that excessive TLR activation underlies the immunopathogenesis of colitis, it is likely that dysfunction of IRAK-M or IRF4 drives colitis. In fact, IRAK-M-deficient mice exhibited severe colitis in response to dextran sodium sulfate treatment due to the excessive activation of TLRs [5]. More importantly, recent studies have highlighted protective roles played by IRAK-M and IRF4 in Crohn's disease (CD) in the presence of nucleotide-binding oligomerization domain 2 (*NOD2*) gene mutations related to CD [6–10]. CD-associated *NOD2* mutations are typically loss-of-function mutations and are associated with defective responses to the NOD2 ligand, muramyl dipeptide (MDP), a small peptide derived from bacterial cell wall components. We and others have shown that sensing of MDP by NOD2 expressed in macrophages or dendritic cells (DCs) induces the expression of IRAK-M

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and IRF4, which, in turn, inhibit TLR activation, maintaining intestinal homeostasis [6–10]. In contrast, macrophages or DCs bearing CD-associated *NOD2* mutations cannot control TLR-mediated pro-inflammatory cytokine responses upon exposure to commensal bacteria, because these loss-of-function mutations prevent the induction of IRAK-M or IRF4 expression. Thus, it is clear that negative regulators of TLRs play indispensable roles in the maintenance of intestinal homeostasis.

Cellular inhibitors of apoptosis proteins 1 (cIAP1) and 2 (cIAP2) are involved in signaling pathways mediated by TLRs, tumor necrosis factor (TNF)-α, and IL-1β [11–13]. In our previous studies, we found that inhibition of cIAP1 and cIAP2 by AT406, a pan-IAP inhibitor, attenuated colitis triggered by the administration of trinitrobenzene sulfonic acid (TNBS) [14]. Consistent with this finding, the colitogenic roles played by cIAP1 and cIAP2 have been reported in dextran sodium sulfate-induced colitis [15]. In addition, the colonic mucosa of patients with CD and ulcerative colitis (UC) is characterized by enhanced expression of *BIRC2* and *BIRC3* mRNAs, encoding cIAP1 and cIAP2, respectively, as shown in our previous studies [14]. Thus, the activation of cIAP1 and cIAP2 is involved in the development of CD and UC. However, the molecular mechanisms underlying the activation of cIAP1 and cIAP2, leading to the development of colitis, are poorly understood.

In this study, we evaluated whether the colitogenic pro-inflammatory cytokine responses mediated by cIAP1 and cIAP2 were associated with the activation of IRF4.

Methods

Preparation of human monocyte-derived DCs

Human monocyte-derived DCs obtained from healthy controls were prepared as previously described [8]. Ethical permission for this study was granted by the Review Boards of Kyoto University and Kindai University. AT406 was used to inhibit cIAP1 and cIAP2. Human DCs (n = 4, 1.5 × 10^6 cells/mL) were treated with 0.35 μM AT406 for 4 h and then stimulated with MDP (25 μg/mL; InvivoGen, San Diego, CA, USA), Pam3CSK4 (PAM, 10 μg/mL; InvivoGen), and lipopolysaccharide (LPS; 1 μg/mL; InvivoGen) for another 24 h in complete RPMI medium. In some experiments, human DCs (1.9 × 10^6 cells/mL, n = 4) were transfected with 50 nM control siRNA (Qiagen, Hilden, Germany), *BIRC2* 7 siRNA (Qiagen, referred to as *BIRC2* siRNA#1), *BIRC2* 8 siRNA (Qiagen, *BIRC2* siRNA#2), *BIRC3* 7 siRNA (Qiagen, *BIRC3* siRNA#1), or *BIRC3* 8 siRNA (Qiagen, *BIRC3* siRNA#2). siRNA transfection into human DCs was performed using a human DC nucleofector kit (Lonza Japan, Sagamihara, Japan) as previously described [8]. The day after transfection, human DCs were stimulated with MDP, PAM, and LPS for 24 h, as described above.

Activation of nuclear factor (NF)-κB was assessed using a transcription assay kit obtained from Active Motif (Carlsbad, CA, USA), as previously described [7, 8]. Human DCs (1.9 × 10^6 cells/mL, n = 2) were transfected with 50 nM control siRNA, *BIRC2* siRNA#1 (Qiagen), or *BIRC3* siRNA#1 (Qiagen). The day after transfection, human DCs were stimulated with MDP, PAM, and LPS for 1 h, as described above. Nuclear extracts were prepared using a nuclear extraction kit (Active Motif).

Overexpression experiments

The human monocytic cell line THP-1 was purchased from American Tissue Culture Collection (ATCC; Manassas, VA, USA). A vector encoding FLAG-tagged IRF4 (1 μg) was transfected into THP-1 cells (1.0 × 10^6 cells/mL) using Nucleofector (Lonza). The day after transfection, human DCs were stimulated with MDP, PAM, and LPS for 24 h, as previously described.

Ubiquitination experiments

Vectors encoding human influenza hemagglutinin (HA)-tagged wild-type ubiquitin (Ub), HA-tagged lysine 48 (K48)-linked Ub, HA-tagged K63-linked Ub, cMyc-DDK-tagged cIAP1, cMyc-DDK-tagged cIAP2, and FLAG-tagged IRF4 (each 1 μg) [7, 8, 16] were used to transfect human embryonic kidney 293 (HEK293) cells (ATCC; 1.0 × 10^6 cells/mL) using Fugene 6 (Promega, Fitchburg, WI, USA). After 1 or 2 days of transfection, cell lysates were subjected to immunoprecipitation (IP) and immunoblotting (IB).

IP and IB

Cell lysates were immunoprecipitated with anti-cMyc beads (Sigma–Aldrich, St. Louis, MO, USA) followed by IB with an anti-HA antibody (Sigma–Aldrich) to evaluate polyubiquitination. Anti-FLAG (Sigma-Aldrich) and anti-actin antibodies (Santa Cruz Biotechnology, Dallas, TX, USA) were used for IB. The IP and IB experiments were performed as described previously [7, 8]. Expression levels of cIAP1 and cIAP2 in human DCs and THP-1 cells were analyzed using anti-cIAP1 and anti-cIAP2 primary antibodies (Cell Signaling Technology, Danvers, MA, USA). Expression levels of IRF4 in human DCs and THP-1 cells were assessed using anti-IRF4 (Cell Signaling Technology) and anti-FLAG antibodies, respectively.

Generation of mice with DC-specific IRF4 overexpression

The mouse CD11c Tet-on vector was kindly provided by Dr. Atsushi Kitani (Mucosal Immunity Section, National Institute of Allergy and Infectious Diseases, National Institutes of Health). FLAG-tagged IRF4 cDNA was inserted at *Eco*RI sites upstream of the CD11c promoter. Finally, a 7,597-base *Not*I-*Xho*I fragment of the CD11c promoter linked to the FLAG-IRF4 cDNA was microinjected to construct CD11c-FLAG IRF4 transgenic (Tg) mice. The DC-specific IRF4 $Tg^{+/-}$ C57BL/6J mouse founders were crossed with C57BL/6J mice for four generations. The genotype of mouse offspring was determined by polymerase chain reaction screening using the following primers: forward, 5′-TGCTAAGTGTCATAGTGGTGGTAGA-3′ and reverse, 5′-AGAGGGAAAAAGATCTCAGTGGTAT-3′.

TNBS-induced colitis

TNBS was used to induce colitis as previously described [7, 8, 14]. Briefly, wild-type mice (n = 13) and CD11c-FLAG IRF4 $Tg^{+/-}$ mice (n = 23) were intrarectally administered TNBS (3.75 mg; Sigma–Aldrich) in 50% ethanol. On day 4, the mice were sacrificed. Pathological scores for TNBS-induced colitis were calculated according to previous reports [7, 8, 14]. Mesenteric lymph node (MLN) cells (2 × 10^6 cells/mL) were stimulated with anti-CD3 antibodies (5 μg/mL; eBioscience, San Diego, CA, USA), MDP (25 μg/mL), PAM (10 μg/mL),

LPS (1 μg/mL), and flagellin (5 μg/mL; InvivoGen) for 60 h. Animal experiments were approved by the Review Board of the Kyoto University and adhered to the ARRIVE guidelines.

Immunohistochemical and immunofluorescence analyses

Immunohistochemical analyses were performed as described previously [17]. Deparaffinized colon tissue sections (wild-type: $n = 5$; CD11c-FLAG IRF4 $Tg^{+/-}$: $n = 14$) were incubated with antibodies targeting phosphorylated IκBα (Cell Signaling Technology), CD3 (Abcam, Cambridge, UK), or CD11b (Abcam) and visualized using the DAKO Envision System (DAKO Japan, Tokyo, Japan). The expression of cIAP1 and cIAP2 in mouse colonic sections was visualized by immunofluorescence analysis. Deparaffinized colon tissue sections (wild-type: $n = 5$; CD11c-FLAG IRF4 $Tg^{+/-}$: $n = 14$) were incubated with goat anti-cIAP1(Santa Cruz Biotechnology) or goat anti-cIAP2 antibodies (Santa Cruz Biotechnology) and visualized with Alexa Fluor 546-conjugated anti-goat IgG antibodies (Invitrogen, Carlsbad, CA, USA). To visualize FLAG-tagged IRF4 expression, deparaffinized colon tissue samples were incubated with mouse anti-FLAG antibodies (Cell Signaling Technology) in combination with rabbit anti-CD3 antibodies (Cell Signaling Technology) or rabbit anti-CD11c antibodies (Cell Signaling Technology) and then visualized with Alexa Fluor 488- or 546-conjugated anti-mouse or rabbit IgG antibodies (Invitrogen). In some experiments, surgical specimens from patients with CD were used as previously described [14, 16]. Deparaffinized human sections were incubated with rabbit anti-cIAP1 (Abcam), rabbit anti-cIAP2 (Abcam), mouse anti-IRF4 (DAKO Japan), or rabbit anti-CD11c antibodies (Abcam). Alexa Fluor 488- or 546-conjugated anti-mouse or -rabbit IgG antibodies (Invitrogen) were used as the secondary antibody. Photomicrographs were captured using immunofluorescence microscopy (Keyence Biozero 8100; Osaka, Japan).

Enzyme-linked immunosorbent assay (ELISA)

Concentrations of cytokines (human TNF-α, human IL-12p40, mouse IL-6, mouse IL-12p40, and mouse IFN-γ) in the culture supernatants were measured using ELISA kits from eBioscience.

Statistical analysis

Two-tailed Student's *t*-tests were used to evaluate the significance of observed differences. Statistical analyses were performed using GraphPad Prism (GraphPad Software Inc., San Diego, CA, USA). Results with *P* values less than 0.05 were considered statistically significant.

Results

cIAP1 and cIAP2 are necessary for TLR-mediated pro-inflammatory cytokine responses

To verify the involvement of cIAP1 and cIAP2 in signaling pathways mediated by NOD2 and TLRs, human monocyte-derived DCs were treated with the pan-cIAP inhibitor AT406 [14] followed by stimulation with the NOD2 ligand MDP, the TLR2 ligand PAM, and the TLR4 ligand LPS. As shown in Fig. 1A, the addition of AT406 to the culture medium markedly reduced the production of TNF-α and IL-12p40 upon the exposure to NOD2, TLR2, and TLR4 ligands. As an alternative approach, human monocyte-derived DCs were transfected with two different siRNAs specific to *BIRC2* or *BIRC3* to knockdown the expression of cIAP1 or cIAP2. As shown in Fig. 1B, siRNA knockdown of *BIRC2* or *BIRC3* decreased the production of IL-12p40 by DCs upon stimulation with TLR2 and TLR4 ligands as compared with that in DCs transfected with control siRNA. In addition, IL-12p40 production upon exposure to the NOD2 ligand MDP was suppressed by the knockdown of cIAP2 expression, although the result was not significant.

The production of TNF-α and IL-12p40 requires nuclear translocation of the NF-κB subunit [7, 8, 10]. As shown in Fig. 1C, transactivation of the NF-κB subunits p65 and c-Rel upon stimulation with LPS was significantly suppressed in DCs transfected with *BIRC2*- or *BIRC3*-specific siRNAs compared with that observed in DCs transfected with control siRNA. Thus, these experiments utilizing inhibitors and siRNAs specific to cIAP1 and cIAP2 strongly suggest that cIAP1 and cIAP2 mediate pro-inflammatory cytokine responses through TLR2 and TLR4.

Expression of cIAP1 and cIAP2 is reduced in the presence of IRF4

Because we found that activation of cIAP1 and cIAP2 was necessary for the production of pro-inflammatory cytokines mediated by TLRs, we next explored the molecular mechanisms regulating the expression of cIAP1 and cIAP2. IRF4 is a well-established negative regulator of TLR-mediated signaling pathways [18, 19]. IRF4 downregulates polyubiquitination of TNF receptor-associated factor 6 (TRAF6) and receptor-interacting serine/threonine kinase 2 (RIPK2) to control TLR-mediated innate immune responses [7, 8, 10]. Therefore, we hypothesized that IRF4 may negatively regulate pro-inflammatory responses mediated by cIAP1 and cIAP2 through the downregulation of cIAP activation. To verify our hypothesis, we prepared human monocyte-derived DCs from four healthy volunteers and then stimulated human DCs with LPS or MDP to induce IRF4 expression. As shown in Fig. 2A, IRF4 expression was markedly induced by stimulation with LPS or MDP as compared with that in unstimulated cells. Human DCs expressed cIAP1 and cIAP2 under physiological conditions. However, the expression levels of cIAP1 and cIAP2 were markedly reduced after stimulation with LPS or MDP, suggesting that the presence of IRF4 was associated with reduced cIAP1 and cIAP2 expression in human DCs. Thus, exposure of human DCs to LPS and MDP led to marked reductions in cIAP1 and cIAP2 expression, which was inversely correlated with that of IRF4.

The human monocytic cell line THP-1 lacks functional IRF4 [7, 8]. THP-1 cells were therefore transfected with a FLAG-tagged IRF4 vector or a control vector. As in the case of human DCs, transfection of FLAG-tagged IRF4 resulted in marked reductions in cIAP1 and cIAP2 expression (Fig. 2B). Induction of IRF4 expression and reduction of cIAP1 and cIAP2 expression decreased TNF-α and IL-12p40 production upon stimulation with TLR2 and TLR4 ligands (Fig. 2C). These data suggest that the presence of IRF4 inhibits the expression of cIAP1 and cIAP2 in human DCs and THP-1 cells.

IRF4 negatively regulates the polyubiquitination of cIAP1 and cIAP2

Given that IRF4 inhibits polyubiquitination of TRAF6 and RIPK2 [7, 8, 10], we examined whether IRF4 blocked

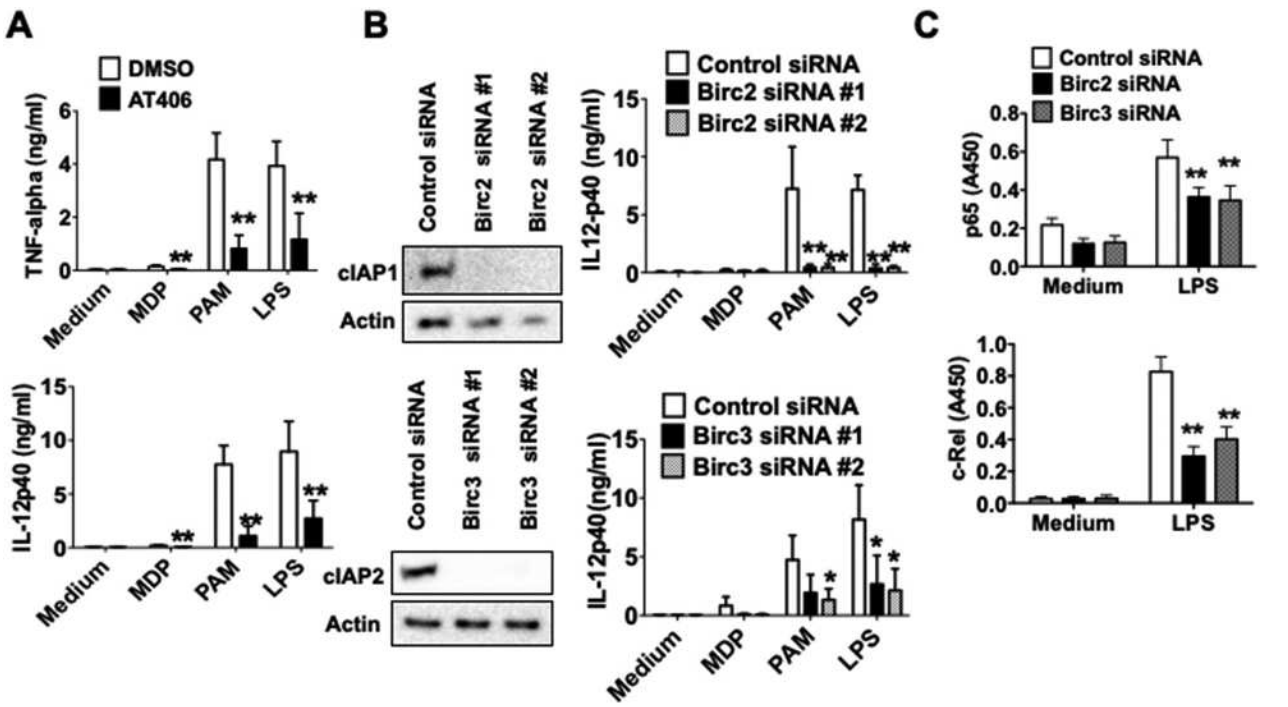

Figure 1: Pharmacological inhibition and siRNA-mediated knockdown of cellular inhibitor of apoptosis proteins 1 and 2 (cIAP1 and cIAP2) reduces pro-inflammatory cytokine responses in human dendritic cells (DCs) upon the exposure to TLR ligands. (A) Human monocyte-derived DCs ($n = 4$, 1.5×10^6 cells/mL) were treated with 0.35 μM AT406 or DMSO for 4 h and then stimulated with muramyl dipeptide (MDP, 25 μg/mL), Pam3CSK4 (PAM, 10 μg/mL), and lipopolysaccharide (LPS, 1 μg/mL) for another 24 h. Culture supernatants were subjected to enzyme-linked immunosorbent assays (ELISAs). (B) Human monocyte-derived DCs (1.9×10^6 cells/mL, $n = 4$) were transfected with 50 nM control siRNA, *BIRC2* siRNA#1, *BIRC2* siRNA#2, *BIRC3* siRNA#1, or *BIRC3* siRNA#2. The next day, DCs were stimulated with MDP, PAM, and LPS for 24 h. Culture supernatants were subjected to ELISA. Knockdown of cIAP1 and cIAP2 expression was confirmed by immunoblotting. (C) Nuclear extracts were prepared from human monocyte-derived DCs (n = 2) transfected with control siRNA, *BIRC2* siRNA#1, or *BIRC3* siRNA#1. The next day, DCs were stimulated with LPS for 1 h, and nuclear extracts were then prepared. Activation of NF-κB was analyzed using TransFactor assays. The results are expressed as means ± standard deviations. $*P < 0.05$, $**P < 0.01$ versus DCs treated with DMSO or control siRNA, as assessed by two-tailed Student's t-tests.

the polyubiquitination of cIAP1 and cIAP2. To this end, HEK293 cells were transfected with wild-type HA-tagged Ub, cMyc-DDK-tagged cIAP1, cMyc-DDK-tagged cIAP2, and FLAG-tagged IRF4. As shown in Fig. 3A, IP with anti-cMyc antibodies followed by IB with anti-HA antibodies revealed that polyubiquitination of cIAP1 and cIAP2 was markedly reduced in the presence of IRF4.

K48-linked polyubiquitination induces degradation of ubiquitinated proteins, whereas K63-linked polyubiquitination leads to their activation [20]. We next examined which type of polyubiquitination was affected by IRF4. HEK293 cells were transfected with HA-tagged K63-linked Ub, K48-linked HA-tagged Ub, FLAG-tagged IRF4, and cMyc-DDK-tagged cIAP1. IP with anti-cMyc antibodies followed by IB with anti-HA antibodies showed that transfection with FLAG-tagged IRF4 inhibited K63- and K48-linked polyubiquitination of cIAP1 (Fig. 3B). Similarly, transfection with FLAG-tagged IRF4 inhibited K63- and K48-linked polyubiquitination of cIAP2 (Fig. 3C). Thus, these overexpression experiments suggest that IRF4 negatively regulates polyubiquitination of cIAP1 and cIAP2. This was not consistent with a previous study showing that K63- and K48-linked polyubiquitination leads to the activation and degradation of target proteins, respectively [20]. This discrepancy can be partially explained by the recent finding that Ub chains branched at K48 and K63 are abundant in mammalian cells and that K48-K63 branched Ub chains are involved in NF-κB signaling [21].

Generation of mice with DC-specific IRF4 overexpression

IRF4 expressed in DCs functions as a negative regulator of TLR-mediated signaling pathways [18, 19]. Having confirmed that IRF4 downregulates pro-inflammatory cytokine responses through cIAP1 and cIAP2 *in vitro*, we next examined the *in vivo* relevance of these findings. To this end, we created mice expressing IRF4 under the control of the CD11c promoter. FLAG-tagged mouse IRF4 cDNA was inserted into the *Eco*RI sites upstream of the mouse CD11c promoter (Fig. 4A). Mice heterozygous for the DC-specific IRF4 overexpressing transgene (CD11c-FLAG IRF4 $Tg^{+/-}$) were born at a Mendelian ratio. Histopathologic analyses using colonic tissue samples revealed that there were no major abnormalities in the colon of CD11c-FLAG IRF4 $Tg^{+/-}$ mice and wild-type mice (Supplementary Fig. 1). Thus, DC-specific IRF4 expression did not affect colon pathology at a steady state.

Overexpression of FLAG-tagged IRF4 was confirmed by immunoblotting using splenic $CD11c^+$ cells (Fig. 4B). FLAG-tagged IRF4 expression was observed in splenic $CD11c^+$ cells obtained from male and female CD11c-FLAG IRF4 $Tg^{+/-}$ mice. Immunofluorescence analysis revealed that FLAG-tagged IRF4 was expressed in $CD11c^+$ DCs in the colonic mucosa of CD11c-FLAG IRF4 $Tg^{+/-}$ mice, but not wild-type mice (Supplementary Fig. 2). In contrast, $CD3^+$ T cells in the colonic mucosa of CD11c-FLAG IRF4 $Tg^{+/-}$ mice did not

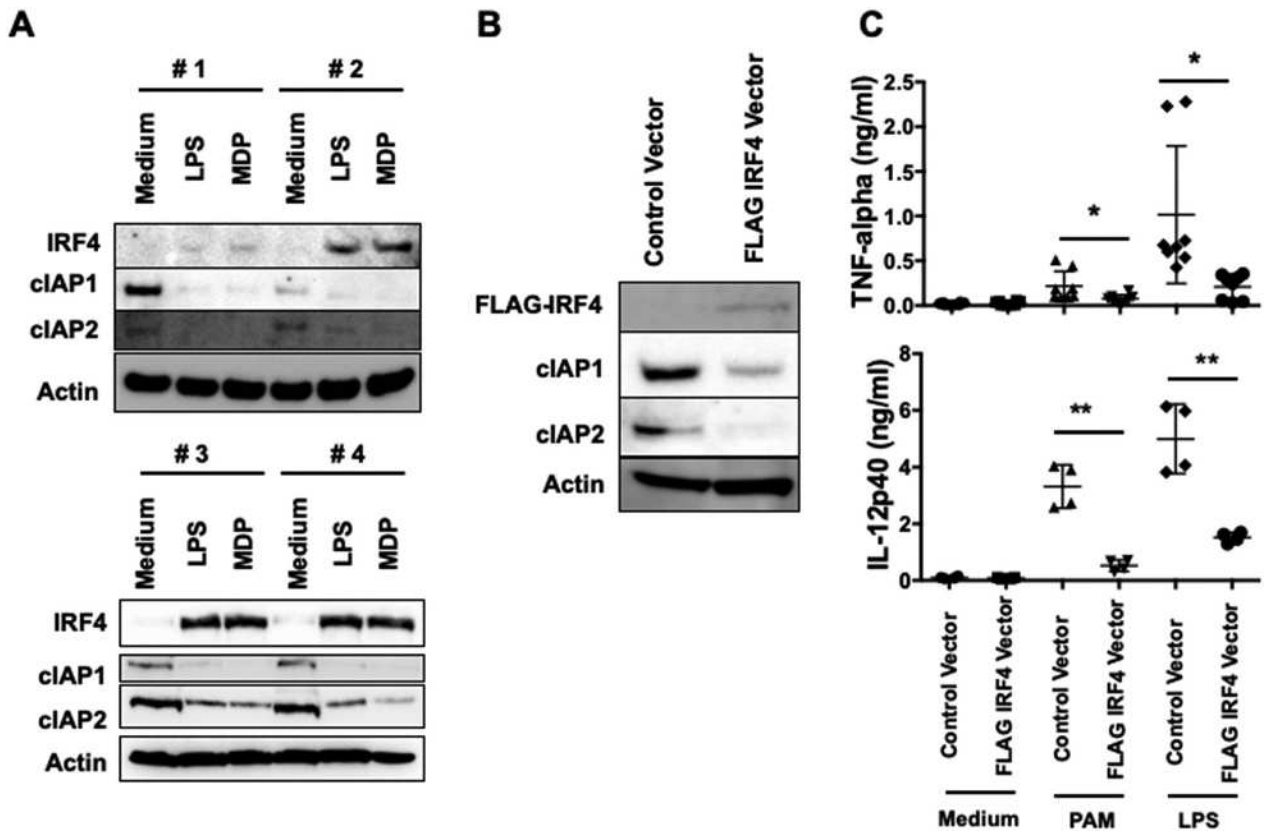

Figure 2: Expression levels of cIAP1 and cIAP2 are reduced in the presence of interferon regulatory factor 4 (IRF4). (A) Human monocyte-derived DCs ($n = 4$) were stimulated with 25 μg/mL MDP and 1μg/mL LPS for 24 h. Expression levels of IRF4, cIAP1, and cIAP2 were analyzed by immunoblotting. (B, C) THP-1 cells (1×10^6 cells/mL) were transfected with control vector (1 μg) or FLAG IRF4 vector (1 μg) and then stimulated with 10μg/mL PAM and 1μg/mL LPS for 24 h. Culture supernatants were subjected to ELISA for measurement of TNF-α and IL-12p40 levels. Expression levels of FLAG-tagged IRF4, cIAP1, and cIAP2 24 h after transfection (B). THP-1 cells were transfected with IRF4 vector or control vector. Cytokine production was measured in duplicate assays. Individual data (eight TNF-α and four IL-12p40 data in two experiments) are shown. Results are expressed as means ± standard deviations. $*P < 0.05$, $**P < 0.01$ versus THP-1 cells treated with control vector, as assessed by two-tailed Student's t-tests.

express FLAG-tagged IRF4 (Supplementary Fig. 2). These immunofluorescence and IB studies strongly suggest that overexpression of IRF4 is limited to CD11c$^+$ DCs.

CD11c-FLAG IRF4 Tg$^{+/-}$ mice are resistant to TNBS-induced colitis

We next addressed whether CD11c-FLAG IRF4 Tg$^{+/-}$ mice were protected from experimental colitis. Wild-type mice and CD11c-FLAG IRF4 Tg$^{+/-}$ mice received an intrarectal administration of TNBS in 50% ethanol. As shown in Fig. 5A, significant differences in body weight were observed. Wild-type mice lost body weight in response to the intrarectal challenge with TNBS. In contrast, little body weight loss was observed in CD11c-FLAG IRF4 Tg$^{+/-}$ mice. Pathological examinations revealed destruction of crypt architecture accompanied by the infiltration of immune cells into the lamina propria in wild-type mice, but not in CD11c-FLAG IRF4 Tg$^{+/-}$ mice (Fig. 5B). The TNBS colitis score was significantly lower in CD11c-FLAG IRF4 Tg$^{+/-}$ mice than in wild-type mice according to semiquantitative pathological analysis using a scoring system (Fig. 5C).

Expression of cIAP1 and cIAP2 was reciprocally regulated by the IRF4 level in our *in vitro* experiments. As shown in Fig. 5D, the expression levels of cIAP1 and cIAP2 were much higher in wild-type mice than in CD11c-FLAG IRF4 Tg$^{+/-}$ mice. Semiquantitative analyses of cIAP expression were performed by counting cells positive for cIAP1 and cIAP2. The number of cells positive for cIAP1 or cIAP2 was much lower in the lamina propria mononuclear cells (LPMCs) of CD11c-FLAG IRF4 Tg$^{+/-}$ mice than in those of wild-type mice (Fig. 5E). The number of cells positive for cIAP1 or cIAP2 was also lower in the epithelial cells (ECs) of CD11c-FLAG IRF4 Tg$^{+/-}$ mice than in those of wild-type mice. Thus, CD11c-FLAG IRF4 Tg$^{+/-}$ mice were resistant to TNBS-induced colitis, and this effect was accompanied by the downregulation of cIAP1 and cIAP2 expression.

Suppression of pro-inflammatory cytokine responses in CD11c-FLAG IRF4 Tg$^{+/-}$ mice

Pro-inflammatory cytokine responses mediated by the activation of NF-κB underlie the immunopathogenesis of TNBS-induced colitis [7, 8, 14]. As shown in Fig. 6A, the number of LPMCs positive for phospho-IκBα was is much greater in wild-type mice than in CD11c-FLAG IRF4 Tg$^{+/-}$ mice. Immunohistochemical analyses also revealed greater accumulation of CD11b$^+$ myeloid cells and CD3$^+$ T cells in the lamina propria of wild-type mice as compared with that in CD11c-FLAG IRF4 Tg$^{+/-}$ mice. Semiquantitative analyses were performed by counting cells positive for phospho-IκBα, CD11b, or CD3. As shown in Fig. 6B, the number of cells positive

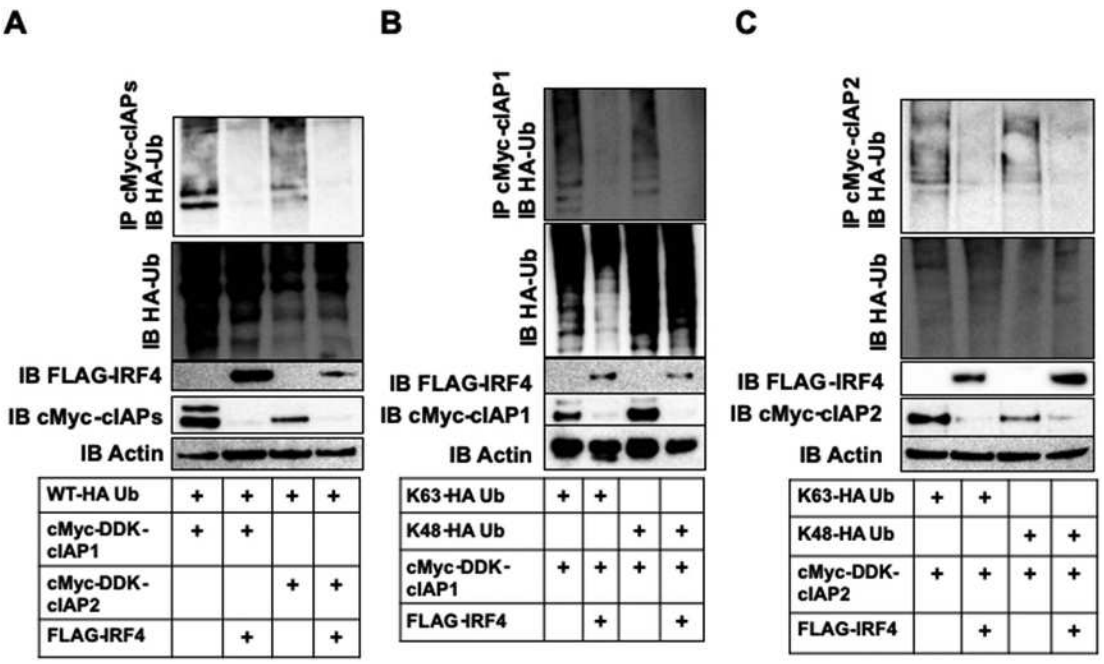

Figure 3: IRF4 suppresses the polyubiquitination of cIAP1 and cIAP2. (A–C) Human embryonic kidney 293 (HEK293) cells (1 × 10^6 cells/mL) were transfected with vectors (each 1 μg) encoding human influenza hemagglutinin (HA)-tagged wild-type ubiquitin (Ub), HA-tagged lysine 48 (K48)-linked Ub, HA-tagged K63-linked Ub, cMyc-DDK-tagged cIAP1, cMyc-DDK-tagged cIAP2, or FLAG-tagged IRF4. Polyubiquitinated cIAP1 and cIAP2 were immunoprecipitated with anti-cMyc beads followed by immunoblotting with anti-HA antibodies.

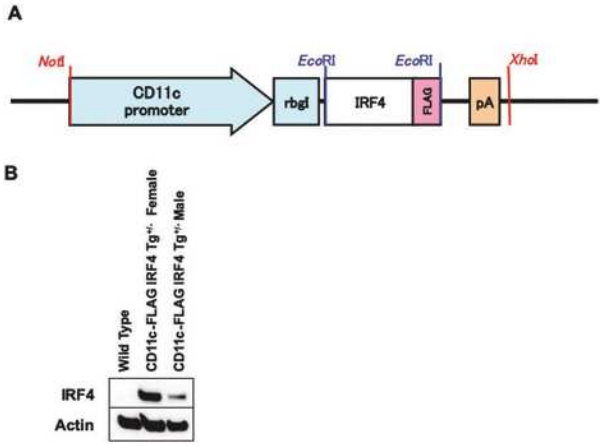

Figure 4: Generation of mice with DC-specific IRF4 overexpression. (A) The transgene construct to generate transgenic mice consisted of FLAG IRF4 cDNA under the control of the CD11c promoter. (B) Splenic CD11c$^+$ cells were isolated from wild-type mice and CD11c-FLAG IRF4 transgenic (Tg)$^{+/-}$ mice. Protein extracts were isolated from these cells and subjected to immunoblotting using anti-FLAG antibodies.

for phospho-IκBα, CD11b, or CD3 was significantly lower in CD11c-FLAG IRF4 Tg$^{+/-}$ mice than in wild-type mice.

We then measured pro-inflammatory cytokine production by MLN cells from mice challenged with TNBS. MLN cells were stimulated with MDP, PAM, LPS, and flagellin to examine the production of IL-6 and IL-12p40. The production of IL-6 and IL-12p40 was markedly suppressed in MLN cells from CD11c-FLAG IRF4 Tg$^{+/-}$ mice compared with that in wild-type mice (Fig. 6C). In addition, IFN-γ production by MLN cells was lower in CD11c-FLAG IRF4 Tg$^{+/-}$ mice than in wild-type mice. Taken together, these data suggested that CD11c-FLAG IRF4 Tg$^{+/-}$ mice were resistant to TNBS-induced colitis through the downregulation of pro-inflammatory cytokine responses and cIAP1 and cIAP2 expression. Moreover, downregulation of pro-inflammatory cytokine responses may mediate reduced expression of cIAP1 and cIAP2 in the ECs of CD11c-FLAG IRF4 Tg$^{+/-}$ mice because the expression of cIAP1 and cIAP2 has been shown to be regulated by pro-inflammatory cytokine responses [14].

Expression of cIAP1 and cIAP2 in patients with CD

Finally, we addressed the clinical relevance of the negative regulation of cIAP1 and cIAP2 expression by IRF4. For this purpose, we utilized surgical specimens obtained from patients with CD [14, 16]. The percentage of IRF4$^+$ cells in total CD11c$^+$ DCs was negatively correlated with expression levels of IL-6 and TNF-α, whereas the percentages of cIAP1$^+$ or cIAP2$^+$ cells in LPMCs were positively correlated with the expression of IL-6 and TNF-α, in our previous studies [14, 16]. As shown in Fig. 7A, specimens exhibiting higher percentages of IRF4$^+$ cells in total CD11c$^+$ DCs contained fewer cells positive for cIAP1 or cIAP2 staining. In contrast, specimens exhibiting lower percentages of IRF4$^+$ cells in total CD11c$^+$ DCs contained higher numbers of cIAP1 or cIAP2 cells. Semiquantitative analyses also showed that the numbers of cells positive for cIAP1 and cIAP2 were significantly lower in samples with high percentages of IRF4$^+$ cells in total CD11c$^+$ DCs as compared with those in samples with low percentages of IRF4$^+$ cells in total CD11c$^+$ DCs (Fig. 7B). These experiments utilizing clinical samples support our findings that IRF4 expression is negatively correlated with cIAP1, cIAP2, and pro-inflammatory cytokine levels in both human CD and murine experimental colitis.

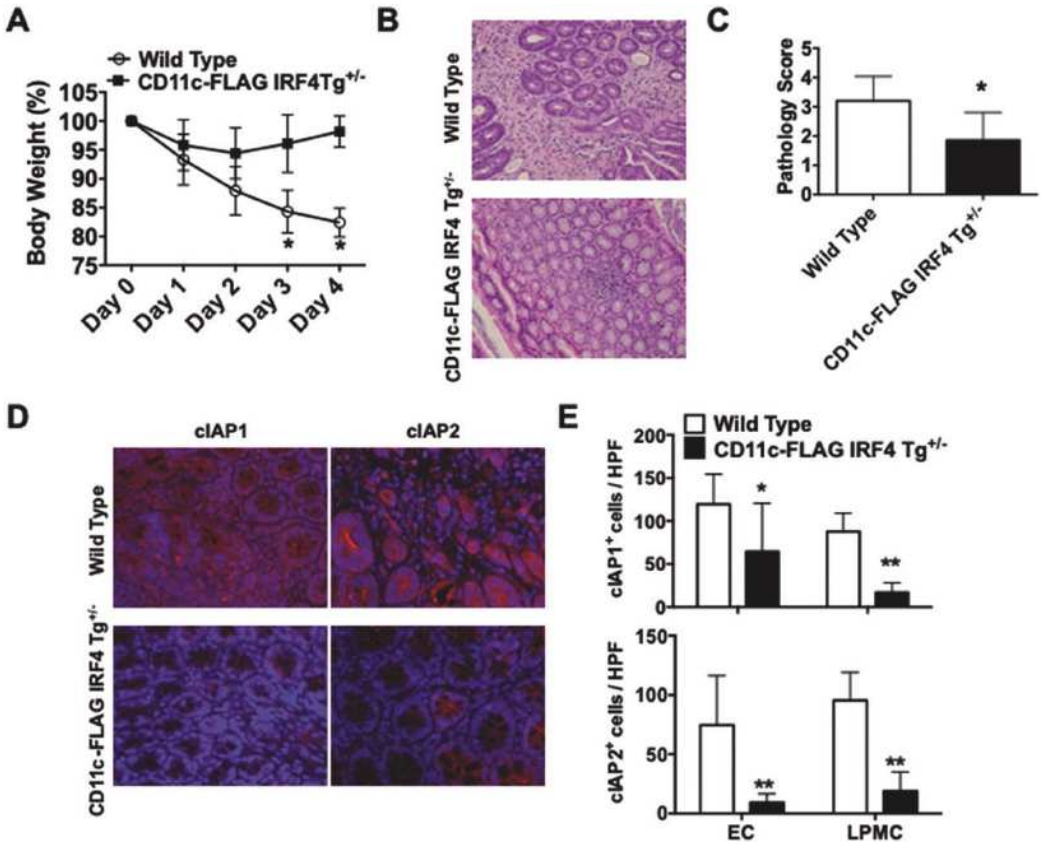

Figure 5: Resistance of mice with DC-specific IRF4 overexpression to colitis induced by trinitrobenzene sulfonic acid (TNBS). Wild-type mice (n = 13) and CD11c-FLAG IRF4 $Tg^{+/-}$ mice (n = 23) were intrarectally administered 3.75 mg TNBS in 50% ethanol on day 0. (A) Body weight curves. Mice were sacrificed on day 4. (B) Hematoxylin and eosin staining of colonic tissues on day 4. Magnification: 200×. (C) Pathological scores of TNBS-induced colitis on day 4. (D) Expression of cIAP1 and cIAP2 in colonic tissues on day 4. Cells positive for cIAP1 or cIAP2 are shown in red. Magnification: 800×. (E) Cells positive for cIAP1 or cIAP2 were semiquantitatively counted in epithelial cells (ECs) and lamina propria mononuclear cells (LPMCs). Results are expressed as means ± standard deviations. $*P < 0.05$, $**P < 0.01$ versus wild-type mice, as assessed by two-tailed Student's t-tests. HPF, high-powered field.

Discussion

In this study, we show that IRF4 maintains intestinal homeostasis and protects against the development of experimental colitis through the downregulation of cIAP1 and cIAP2 expression. The chain of evidence supporting this conclusion was initiated by the observation of markedly reduced cIAP1 and cIAP2 expression in the presence of IRF4 in human DCs. In physiological and unstimulated conditions, human DCs stably expressed both cIAP1 and cIAP2 but lacked IRF4 expression. Stimulation with LPS and MDP in human DCs led to reduced cIAP expression and enhanced IRF4 expression, respectively. Thus, stimulation with LPS or MDP is absolutely required for induction of IRF4 expression. In contrast, such stimulation led to marked reductions in cIAP1 and cIAP2 expression, suggesting that the expression of cIAP1 and cIAP2 at a steady state may be necessary for induction of pro-inflammatory cytokine responses mediated by LPS or MDP and that the expression of IRF4 may be inversely correlated with that of cIAP1 and cIAP2. In addition, transfection of *IRF4* cDNA into IRF4-deficient THP-1 cells resulted in marked reductions in cIAP1 and cIAP2 expression accompanied by decreased production of TNF-α and IL-12p40 upon exposure to TLR2 and TLR4 ligands as compared with that in cells transfected with control cDNA. In overexpression experiments utilizing HEK293 cells, we found that polyubiquitination of cIAP1 and cIAP2 was inhibited in the presence of IRF4. Thus, these *in vitro* experiments suggest that IRF4 downregulates activation of cIAP1 and cIAP2 in DCs. Finally, in experiments utilizing mice with DC-specific IRF4 overexpression (CD11c-FLAG IRF4 $Tg^{+/-}$ mice), we showed that these mice were resistant to the induction of colitis after TNBS treatment. Furthermore, CD11c-FLAG IRF4 $Tg^{+/-}$ mice exhibited marked reductions in cIAP1 and cIAP2 expression as compared with that in wild-type mice. MLN cells isolated from CD11c-FLAG IRF4 $Tg^{+/-}$ mice produced lower amounts of IL-6 and IL-12p40 upon exposure to TLR ligands than MLN cells from wild-type mice. Moreover, the percentages of $IRF4^+$ DCs were inversely correlated with fractions of $cIAP1^+$ or $cIAP2^+$ cells among LPMCs from patients with CD. Collectively, these data reveal a new mechanism of action of IRF4, namely inhibition of colonic inflammation through downregulation of cIAP1 and cIAP2 expression.

In this study, we created a novel mouse model in which FLAG-tagged IRF4 was overexpressed in $CD11c^+$ cells (CD11c-FLAG IRF4 $Tg^{+/-}$ mice). IB studies revealed that FLAG-tagged IRF4 was expressed only in splenic $CD11c^+$ DCs in transgenic mice, but not in wild-type mice. Expression of FLAG-tagged IRF4 was observed in $CD11c^+$ DCs in the

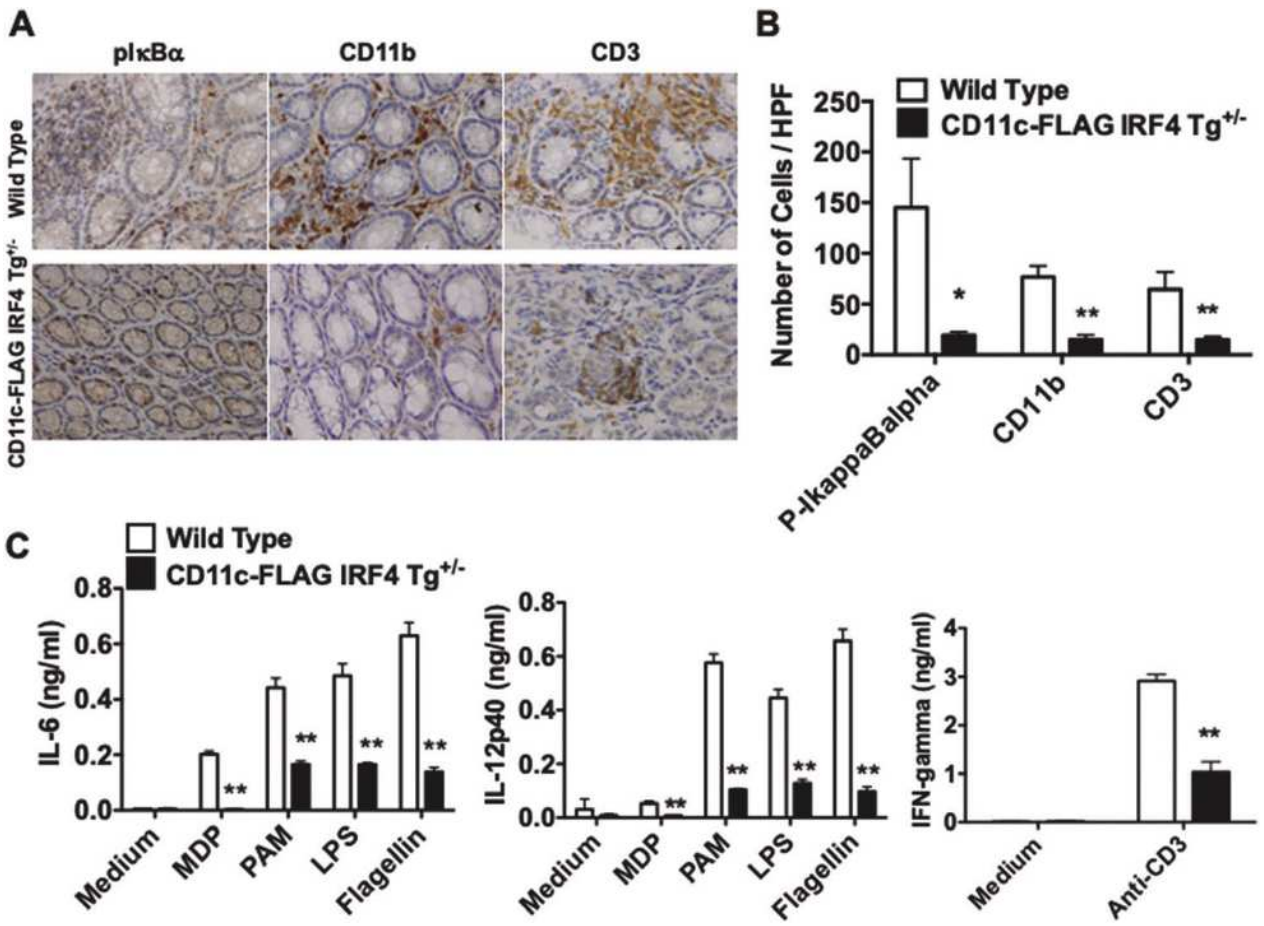

Figure 6: Mice with DC-specific IRF4 overexpression exhibit reduced pro-inflammatory cytokine responses to Toll-like receptor ligands. Wild-type mice (n = 13) and CD11c-FLAG IRF4 $Tg^{+/-}$ mice (n = 23) were intrarectally administered 3.75 mg TNBS in 50% ethanol on day 0. Mice were sacrificed on day 4. (A, B) Colonic tissues harvested on day 4 were subjected to immunohistochemical analyses to visualize cells positive for phospho-IκBα, CD11b, and CD3. Magnification: 800×. Cells positive for phospho-IκBα,CD11b, and CD3 were semiquantitatively counted. HPF, high-powered field. (C) Mesenteric lymph node cells (2×10^6 cells/mL) were stimulated with anti-CD3 antibodies (5 μg/mL), 25 μg/mL MDP, 10 μg/mL PAM, 1 μg/mL LPS, and 5 μg/mL flagellin for 60 h. Culture supernatants were subjected to ELISA to measure IL-6, IL-12p40, and IFN-γ levels. Results are expressed as means ± standard deviations. $*P < 0.05$, $**P < 0.01$ versus wild-type mice, as assessed by two-tailed Student's t-tests.

colonic mucosa of CD11c-FLAG IRF4 $Tg^{+/-}$ mice, but not wild-type mice, whereas $CD3^+$ T cells did not express FLAG-tagged IRF4 in CD11c-FLAG IRF4 $Tg^{+/-}$ mice or wild-type mice. Thus, overexpression of IRF4 was limited to $CD11c^+$ DCs in CD11c-FLAG IRF4 $Tg^{+/-}$ mice. CD11c-FLAG IRF4 $Tg^{+/-}$ mice were resistant to TNBS colitis, accompanied by reduced expression of cIAP1 and cIAP2 not only in immune cells but also in ECs. This reduction of cIAP1 and cIAP2 expression in immune cells of CD11c-FLAG IRF4 $Tg^{+/-}$ mice was consistent with the data obtained in human DCs. However, further studies are needed to elucidate the molecular mechanisms mediating these effects in colonic ECs. We hypothesize that cIAP1 and cIAP2 expression may be regulated by pro-inflammatory cytokine responses [14]. We previously showed that the expression of cIAP1 and cIAP2 is positively correlated with that of pro-inflammatory cytokines, such as IL-6, TNF-α, and IL-12p40 [14]. Therefore, it is likely that the reduced pro-inflammatory cytokine responses in CD11c-FLAG IRF4 $Tg^{+/-}$ mice challenged with TNBS may be involved in the observed decreases in cIAP1 and cIAP2 expression.

IRF4 is a well-established negative regulator of TLR-mediated pro-inflammatory cytokine responses in DCs [18, 19]. We and others have shown that MDP activation of NOD2 induces IRF4 expression in DCs and thereby suppresses the development of experimental colitis through the downregulation of TLR-mediated pro-inflammatory cytokine responses [7–9]. Consistent with these findings, DC-specific IRF4 Tg mice were resistant to TNBS-induced colitis, and their MLN cells produced lower levels of pro-inflammatory cytokines. These previous findings together with the data presented in the current study support the hypothesis that DC-specific IRF4 expression may suppress the development of experimental colitis. This is further supported by our recent findings that the percentages of $IRF4^+$ DCs in total lamina propria DCs were inversely correlated with the expression levels of IL-6 and TNF-α in surgical specimens obtained from patients with CD [16]. In contrast, IRF4 expressed in $CD4^+$ T cells has been shown to play a pathogenic role in the development of CD [22, 23]. Mudter et al. demonstrated that patients with CD exhibit higher IRF4 expression in lamina propria $CD4^+$ T cells as compared with that in control patients. They also showed that adoptive transfer of wild-type but not IRF4-deficient $CD4^+CD45RB^{hi}$ T cells into T cell- and B cell-deficient mice resulted in severe colitis, suggesting that

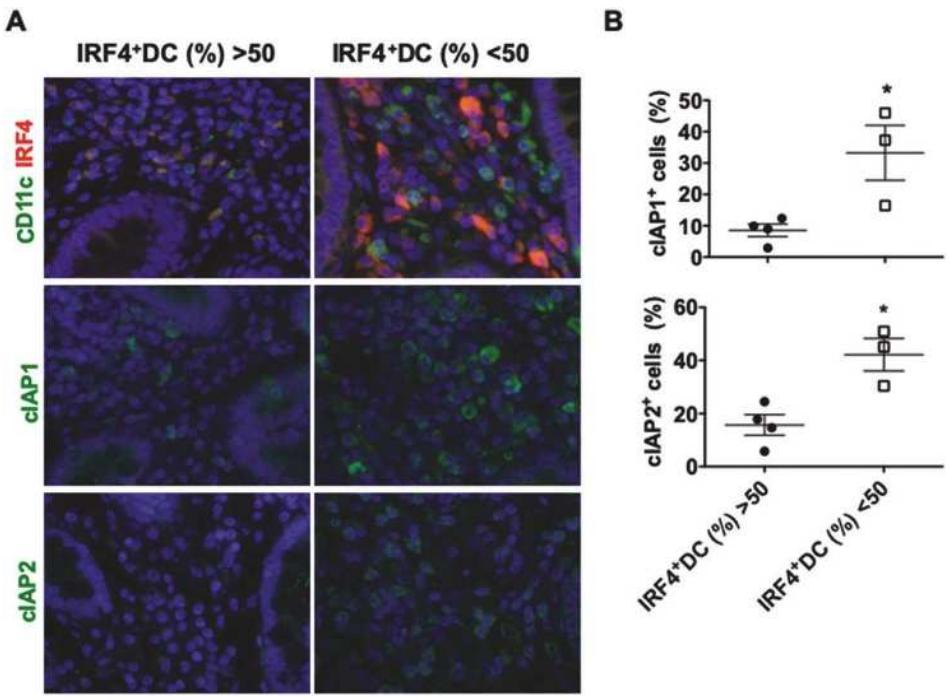

Figure 7: A negative relationship between the percentage of IRF4$^+$ DCs and cIAP1 and cIAP2 expression levels in the colonic mucosa of patients with Crohn's disease. Colonic surgical specimens from patients with Crohn's disease (n = 7) were utilized to determine the expression of IRF4, cIAP1, and cIAP2. (A) IRF4 and CD11c were visualized using red and green colors, respectively. Expression of cIAP1 and cIAP2 was visualized using green color. Nuclei were counterstained with DAPI. Magnification: 1200×. (B) The relationship between the semiquantitatively calculated percentages of IRF4$^+$ cells in total CD11c$^+$ DCs and those of cIAP1$^+$ or cIAP2$^+$ cells in total lamina propria mononuclear cells. $*P < 0.05$ versus samples with high percentages of IRF4$^+$ DCs (> 50%), as assessed by two-tailed Student's t-tests.

the development of experimental colitis required IRF4 in T cells. With regard to the molecular mechanisms mediating the pathogenic roles of IRF4, Mudter et al. proposed that IRF4 is indispensable for IL-6 and IL-17 production by T cells in the colonic lamina propria [22, 23]. Thus, IRF4 expressed in T cells promotes the development of colitis, whereas IRF4 expressed in DCs has protective effects. Therefore, caution must be exercised regarding the clinical application of IRF4 activation for the treatment of patients with CD. DC-specific, but not T cell-specific, activation of IRF4 needs to be achieved for the treatment of patients with CD.

Activation of cIAP1 and cIAP2 is involved in pro-inflammatory cytokine responses mediated by TLRs, NOD2, and TNF-α [11, 12]. Given that pro-inflammatory cytokine responses upon stimulation with TLR ligands and TNF-α underlie the immunopathogenesis of CD and UC [1, 2], it is likely that cIAP1 and cIAP2 may be therapeutic targets. In fact, we previously reported that the expression levels of *BIRC2* and *BIRC3*, which encode cIAP1 and cIAP2, respectively, were markedly higher and positively correlated with those of IL-6, TNF-α, and IL-12p40 in the colonic mucosa of patients with CD and UC [14]. Consistent with these findings, we showed that IL-12p40 production upon exposure to TLR ligands was markedly reduced in human DCs treated with AT406 (a pan-IAP inhibitor) or siRNAs specific to *BIRC2* and *BIRC3*. Moreover, resistance to TNBS-induced colitis in CD11c-FLAG IRF4 Tg$^{+/-}$ mice was accompanied by the downregulation of cIAP1 and cIAP2 expression. Thus, blockade of cIAP1 and cIAP2 function can suppress chronic inflammation through the downregulation of pro-inflammatory cytokine responses mediated by TLRs. In addition to TLR signaling pathways, the activation of cIAP1 and cIAP2 is indispensable for TNFα-mediated pro-inflammatory cytokine responses [11, 12]. In fact, antagonists of cIAP1 and cIAP2 reduce the production of pro-inflammatory cytokines and chemokines induced by TNF-α [24]. Thus, activation of cIAP1 and cIAP2 may increase sensitivity to TNFα-mediated colonic inflammation. Collectively, inhibition of cIAP1 and cIAP2 function may block colonic inflammation by suppressing signaling pathways mediated not only by TLRs but also by TNF-α. However, cIAP1 activation has been shown to suppress TNF-α-induced intestinal epithelial cell death [25], suggesting that the blockade of cIAP1 may promote tissue destruction.

NF-κB activation is tightly regulated by the polyubiquitination of target proteins [20]. cIAP1 and cIAP2 are E3 ligases that polyubiquitinate RIPK2 [7, 8, 10, 14]. In our previous studies, we showed that NOD2-independent and TLR-dependent activation of RIPK2 was negatively regulated by decreased polyubiquitination of RIPK2 [7, 8, 10, 14]. Such inhibition of RIPK2 polyubiquitination ameliorates experimental colitis through downregulation of pro-inflammatory cytokine responses to TLR ligands [7, 8, 10, 14]. Importantly, cIAP1 and cIAP2 function as E3 ligases for RIPK2, and the molecular interaction between RIPK2 and cIAP1 or cIAP2 plays colitogenic roles in both experimental and human inflammatory bowel disease (IBD) [14]. Thus, the cIAP1/cIAP2/RIPK2 axis activated by TLRs underlies the immunopathogenesis of IBD through the activation of NF-κB and subsequent pro-inflammatory cytokine responses. In this study, we found that the expression of cIAP1 and cIAP2 was inversely correlated with that of IRF4 in human DCs. To elucidate the molecular mechanisms of this negative regulation, cIAP1 and cIAP2 were

subjected to K48- and K63-linked polyubiquitination in the presence of IRF4. Our results suggested that the E3 ligases cIAP1 and cIAP2 could become polyubiquitinated in the absence of IRF4, whereas such polyubiquitination on cIAP1 and cIAP2 was markedly suppressed in the presence of IRF4. K48-linked polyubiquitination leads to the degradation of the target protein, whereas K63-linked polyubiquitination induces activation of the targeted protein [20]. However, in contrast to this established notion, overexpression of IRF4 inhibited both K48- and K63-linked polyubiquitination of cIAP1 and cIAP2. This discrepancy can be partially explained by the recent finding that Ub chains branched at K48 and K63 are abundant in mammalian cells and that K48-K63 branched Ub chains are involved in NF-κB signaling [21]. However, we have not addressed whether inhibition of cIAP1 and cIAP2 polyubiquitination is directly mediated by the binding of IRF4 to cIAPs. Therefore, it is possible that the pro-inflammatory functions of cIAP1 and cIAP2 may be regulated by IRF4 in an indirect manner.

In conclusion, IRF4 overexpression protected mice from TNBS-induced colitis partially through downregulation of cIAP1 and cIAP2 expression. Augmentation of IRF4 expression or function may represent a novel therapeutic approach for patients with CD, as shown by the results obtained in CD11c-FLAG IRF4 $Tg^{+/-}$ mice. Therefore, chronic colonic inflammation may be effectively treated by DC-specific upregulation of IRF4 and downregulation of cIAPs.

Supplementary Data

Supplementary data is available at *Clinical and Experimental Immunology* online.

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Disclosures

The authors declare that they have no known competing financial interests or personal relationships that could have influenced the work reported in this paper.

Author contributions

S.M., T.W., Y.A., and K.Y. designed and performed the experiments. S.M., T.W., I.S., A.H., M.Kur., Y.O., Y.M., T.Y., R.T., K.K., and K.M. performed data analysis and interpretation. S.M., T.W., Y.A., K.M., and K.Y. prepared the manuscript draft. M.Kud. supervised the research.

Ethics approval

Ethical permission for this study was granted by the Review Boards of the Kyoto University and Kindai University. Animal experiments were approved by the Review Board of the Kyoto University and adhered to the ARRIVE guidelines.

Data Availability

Data supporting the findings of this study are available from the corresponding author upon reasonable request.

References

1. Strober W, Fuss I, Mannon P. The fundamental basis of inflammatory bowel disease. *J Clin Invest* 2007, 117, 514–21.
2. Strober W, Fuss IJ. Proinflammatory cytokines in the pathogenesis of inflammatory bowel diseases. *Gastroenterology* 2011, 140, 1756–67.
3. Hoshi N, Schenten D, Nish SA, Walther Z, Gagliani N, Flavell RA, et al. MyD88 signalling in colonic mononuclear phagocytes drives colitis in IL-10-deficient mice. *Nat Commun* 2012, 3, 1120.
4. Liew FY, Xu D, Brint EK, O'Neill LA. Negative regulation of toll-like receptor-mediated immune responses. *Nat Rev Immunol* 2005, 5, 446–58.
5. Berglund M, Melgar S, Kobayashi KS, Flavell RA, Hörnquist EH, Hultgren OH. IL-1 receptor-associated kinase M downregulates DSS-induced colitis. *Inflamm Bowel Dis* 2010, 16, 1778–86.
6. Hedl M, Li J, Cho JH, Abraham C. Chronic stimulation of Nod2 mediates tolerance to bacterial products. *Proc Natl Acad Sci USA* 2007, 104, 19440–5.
7. Watanabe T, Asano N, Murray PJ, Ozato K, Tailor P, Fuss IJ, et al. Muramyl dipeptide activation of nucleotide-binding oligomerization domain 2 protects mice from experimental colitis. *J Clin Invest* 2008, 118, 545–59.
8. Watanabe T, Asano N, Meng G, Yamashita K, Arai Y, Sakurai T, et al. NOD2 downregulates colonic inflammation by IRF4-mediated inhibition of K63-linked polyubiquitination of RICK and TRAF6. *Mucosal Immunol* 2014, 7, 1312–25.
9. Udden SMN, Peng L, Gan JL, Shelton JM, Malter JS, Hooper LV, et al. NOD2 Suppresses Colorectal Tumorigenesis via Downregulation of the TLR Pathways. *Cell Rep* 2017, 19, 2756–70.
10. Strober W, Watanabe T. NOD2, an intracellular innate immune sensor involved in host defense and Crohn's disease. *Mucosal Immunol* 2011, 4, 484–95.
11. Estornes Y, Bertrand MJ. IAPs, regulators of innate immunity and inflammation. *Semin Cell Dev Biol* 2015, 39, 106–14.
12. Pedersen J, LaCasse EC, Seidelin JB, Coskun M, Nielsen OH. Inhibitors of apoptosis (IAPs) regulate intestinal immunity and inflammatory bowel disease (IBD) inflammation. *Trends Mol Med* 2014, 20, 652–65.
13. Labbé K, McIntire CR, Doiron K, Leblanc PM, Saleh M. Cellular inhibitors of apoptosis proteins cIAP1 and cIAP2 are required for efficient caspase-1 activation by the inflammasome. *Immunity* 2011, 35, 897–907.
14. Watanabe T, Minaga K, Kamata K, Sakurai T, Komeda Y, Nagai T, et al. RICK/RIP2 is a NOD2-independent nodal point of gut inflammation. *Int Immunol* 2019, 31, 669–83.
15. Qiu W, Liu H, Sebastini A, Sun Q, Wang H, Zhang L, et al. An apoptosis-independent role of SMAC in tumor suppression. *Oncogene* 2013, 32, 2380–9.
16. Honjo H, Watanabe T, Arai Y, Kamata K, Minaga K, Komeda Y, et al. ATG16L1 negatively regulates RICK/RIP2-mediated innate immune responses. *Int Immunol* 2021, 33, 91–105.
17. Watanabe T, Sadakane Y, Yagama N, Sakurai T, Ezoe H, Kudo M, et al. Nucleotide-binding oligomerization domain 1 acts in concert with the cholecystokinin receptor agonist, cerulein, to induce IL-33-dependent chronic pancreatitis. *Mucosal Immunol* 2016, 9, 1234–49.
18. Honma K, Udono H, Kohno T, Yamamoto K, Ogawa A, Takemori T, et al. Interferon regulatory factor 4 negatively regulates the production of proinflammatory cytokines by macrophages in response to LPS. *Proc Natl Acad Sci USA* 2005, 102, 16001–6.

19. Negishi H, Ohba Y, Yanai H, Takaoka A, Honma K, Yui K, et al. Negative regulation of Toll-like-receptor signaling by IRF-4. *Proc Natl Acad Sci USA* 2005, 102, 15989–94.
20. Liu S, Chen ZJ. Expanding role of ubiquitination in NF-κB signaling. *Cell Res* 2011, 21, 6–21.
21. Ohtake F, Saeki Y, Ishido S, Kanno J, Tanaka K. The K48-K63 Branched Ubiquitin Chain Regulates NF-κB Signaling. *Mol Cell* 2016, 64, 251–66.
22. Mudter J, Yu J, Zufferey C, Brüstle A, Wirtz S, Weigmann B, et al. IRF4 regulates IL-17A promoter activity and controls RORγt-dependent Th17 colitis in vivo. *Inflamm Bowel Dis* 2011, 17, 1343–58.
23. Mudter J, Amoussina L, Schenk M, Yu J, Brüstle A, Weigmann B, et al. The transcription factor IFN regulatory factor-4 controls experimental colitis in mice via T cell-derived IL-6. *J Clin Invest* 2008, 118, 2415–26.
24. Kearney CJ, Sheridan C, Cullen SP, Tynan GA, Logue SE, Afonina IS, et al. Inhibitor of apoptosis proteins (IAPs) and their antagonists regulate spontaneous and tumor necrosis factor (TNF)-induced proinflammatory cytokine and chemokine production. *J Biol Chem* 2013, 288, 4878–90.
25. Grabinger T, Bode KJ, Demgenski J, Seitz C, Delgado ME, Kostadinova F, et al. Inhibitor of apoptosis protein-1 regulates tumor necrosis factor-mediated destruction of intestinal epithelial cells. *Gastroenterology* 2017, 152, 867–79.

（謝辞）

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