博士学位論文

クローン病疾患感受性遺伝子 NOD2 の欠損は

T 細胞依存性腸炎の発症を抑制する

近 畿 大 学 大 学 院
医 学 研 究 科 医 学 系 専 攻

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Doctoral Dissertation

NOD2 deficiency protects mice from the development of

adoptive transfer colitis through the induction of

regulatory T cells expressing forkhead box P3

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NOD2 deficiency protects mice from the development of adoptive transfer colitis through the induction of regulatory T cells expressing forkhead box P3

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ABSTRACT

Nucleotide-binding oligomerization domain 2 (NOD2) is an intracellular receptor for muramyl dipeptide derived from the intestinal microbiota. Loss-of-function mutations in *Nod2* are associated with the development of Crohn's disease, suggesting that NOD2 signaling plays critical roles in the maintenance of intestinal immune homeostasis. Although NOD2 activation prevents the development of short-term experimental colitis, it remains unknown whether the sensitivity to long-term experimental colitis is influenced by NOD2. In this study, we explored the roles played by NOD2 in the development of long-term adoptive transfer colitis. Unexpectedly, we found that $Rag1^{-/-}Nod2^{-/-}$ mice were more resistant to adoptive transfer colitis than $Rag1^{-/-}$ mice and had reduced proinflammatory cytokine responses and enhanced accumulation of regulatory T cells (Tregs) expressing forkhead box P3 in the colonic mucosa. Prevention of colitis in $Rag1^{-/-}Nod2^{-/-}$ mice was mediated by TGF-β1 because neutralization of TGF-β1 resulted in the development of more severe colitis due to reduced accumulation of Tregs. Such paradoxical Treg responses in the absence of NOD2 could explain why *Nod2* mutations in humans are not sufficient to cause Crohn's disease.

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1. Introduction

Nucleotide-binding oligomerization domain 2 (NOD2) is a cytosolic receptor involved in innate immunity [1—3]. NOD2 is a peptidoglycan sensor that detects muramyl dipeptide (MDP), a small molecule derived from bacterial cell wall components [1—3]. Sensing of MDP by intracellular NOD2 activates nuclear factor κB (NF-κB) and mitogen-activated protein kinases, leading to pro-inflammatory cytokine responses, which contribute to the host defense against microbial infections [1—3]. In addition, activation of NOD2 by MDP plays a critical role in the maintenance of intestinal immune homeostasis [1—3]. This is fully supported by the fact that polymorphisms in *Nod2* are one of the strongest risk factors for Crohn's disease (CD), a chronic inflammatory disorder of the gastrointestinal (GI) tract driven by excessive immune responses to gut microbiota.

Loss-of-function mutations in *Nod2* are associated with the development of CD [1—3], suggesting that intact NOD2 signaling pathways prevent the development of chronic GI inflammatory responses. In line with this idea, activation of NOD2 by MDP protected mice from experimental colitis induced by dextran sodium sulfate (DSS) and trinitrobenzene sulfonic acid (TNBS) [4—6]. Thus, recognition of intestinal bacteria derived MDP by NOD2 suppresses harmful immune responses towards the intestinal bacteria. As for the molecular mechanisms underlying NOD2-mediated maintenance of intestinal immune homeostasis, three hypotheses have been proposed. First, impaired production of antimicrobial peptides in the presence of CD-associated *Nod2* mutations may promote bacterial overgrowth in the GI tract [1—3]. Second, impaired autophagy in the presence of CD-associated *Nod2* mutations may lead to bacterial overgrowth in the GI tract [1—3]. Thus, loss-of-function *Nod2* mutations contribute to the development of CD through bacterial overgrowth. Third, MDP activation of NOD2 negatively

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Abbreviations

CD	Crohn's disease
cLPMC	colonic lamina propria mononuclear cell
DSS	dextran sodium sulfate
FOXP3	forkhead box P3
GI	gastrointestinal
IHC	immunohistochemical
IRF4	interferon regulatory factor 4
MDP	muramyl dipeptide
NF-κB	nuclear factor κB
NOD2	nucleotide-binding oligomerization domain 2
SEM	standard error of the mean
STAT3	signal transducer and activator of transcription 3
TLR	Toll-like receptors
TNBS	trinitrobenzene sulfonic acid
Treg	regulatory T cell

regulates proinflammatory cytokine responses to Toll-like receptors (TLRs) derived from intestinal bacteria. In the presence of CD-associated *Nod2* mutations, excessive proinflammatory colitogenic cytokine responses are induced in antigen-presenting cells upon exposure to TLR ligands [1—3]. It should be noted, however, that these hypotheses have been established following observations in short-term experimental models of colitis induced by DSS or TNBS. It is unknown whether a complete NOD2 deficiency affects susceptibility to long-term colitis. Adoptive transfer of naïve CD4$^+$ T cells from donor mice into $Rag1^{-/-}$ mice causes long-lasting colonic inflammation [7]. In this study, we explored the role of NOD2 in the development of long-term colitis using $Rag1^{-/-}Nod2^{-/-}$ mice and $Rag1^{-/-}$ mice. Here, we provide evidence that $Rag1^{-/-}Nod2^{-/-}$ mice exhibited decreased susceptibility to adoptive transfer colitis compared to that in $Rag1^{-/-}$ mice. Interestingly, enhanced accumulation of regulatory T cells (Tregs) expressing forkhead box P3 (FOXP3) was also associated with decreased susceptibility to adoptive transfer colitis.

2. Materials and methods

2.1. Mice

Nod2-deficient and *Rag1*-deficient mice were generated as previously reported [4,8]. Mice deficient in both *Rag1* and *Nod2* ($Rag1^{-/-}Nod2^{-/-}$ mice) were created by crossing $Rag1^{-/-}$ and $Nod2^{-/-}$ mice. The mice were reared in the Animal Facility of the Kindai University Faculty of Medicine. Protocols for animal experiments were approved by the review board of the Kindai University Faculty of Medicine.

2.2. Induction of adoptive transfer colitis

Adoptive transfer colitis was induced as previously described [9]. Briefly, naïve CD4$^+$CD45RB^{high} T cells were isolated from the spleen of C57BL/6 mice using a naïve CD4 T cell isolation kit (Miltenyi Biotec). The purity of the CD4$^+$CD45RB^{high} cells was more than 90% as assessed by flow cytometry (data not shown). The severity of colitis was assessed using a pathological scoring system as previously described [10]. Each sample was graded semi-quantitatively from 0 to 3 for four criteria: (1) degree of epithelial hyperplasia and goblet cell depletion; (2) leukocyte infiltration in the lamina propria; (3) area of tissue affected; and (4) the presence of markers of severe inflammation, such as crypt abscesses,

submucosal inflammation, and ulcers. Scores for each criterion were summed to obtain an overall inflammation score of 0—12 for each sample. In some experiments, mice received intraperitoneal injection of a neutralizing antibody against TGF-β1 (0.5 mg, Bio X Cell) or with control mouse IgG (Sigma-Aldrich) once a week. Colonic lamina propria mononuclear cells (cLPMCs) were isolated as previously reported [4,5]. The proportions of Tregs in cLPMC were determined using an Accuri C6 flow cytometer (BD Biosciences) and CFlow Plus software (BD Biosciences) as previously described [8]. A regulatory T cell staining kit (eBioscience) was used to determine the proportion of CD4$^+$FOXP3$^+$ T cells.

2.3. Cytokine assay

cLPMCs (1×10^6/mL) were stimulated with an anti-CD3 antibody (5 μg/mL, BD Biosciences), lipopolysaccharide (LPS; InvivoGen, 1 μg/mL), Pam3CSK4 (PAM; InvivoGen, 5 μg/mL), and MDP (InvivoGen, 10 μg/mL) for 48 h as previously described [4,5]. Culture supernatants were used in the cytokine assay. The concentrations of cytokines (IFN-γ, IL-6, IL-10, and IL-17) and TGF-β1 were measured using enzyme-linked immunosorbent assay kits obtained from eBioscience and R&D Systems, respectively.

2.4. Immunohistochemical analyses

Immunohistochemical (IHC) analyses were performed as previously described [8]. Mouse antibodies against phosphorylated signal transducer and activator of transcription 3 (p-STAT3) and phosphorylated IκBα (p-IκBα) (Cell Signaling Technology) and a rabbit anti-FOXP3 antibody (Abcam) were used as primary antibodies. Deparaffinized sections were incubated with primary antibodies and then visualized with the Dako Envision + system (DAKO).

2.5. Statistical analysis

Student's *t*-test was used to assess the significance of differences. Statistical analyses were performed using Prism (GraphPad Software). Differences were considered statistically significant when *p* values were less than 0.05.

3. Results

3.1. $Rag1^{-/-}Nod2^{-/-}$ mice are resistant to adoptive transfer colitis

Loss-of-function mutations in *Nod2* are a major susceptibility factor for the development of CD [1—3]. Thus, impaired activation of NOD2 predisposes mice to experimental colitis. In fact, MDP activation of NOD2 inhibited the development of experimental colitis induced by DSS and TNBS in *Nod2*-intact mice, but not in *Nod2*-deficient mice, indicating the susceptibility of the latter animals to chemical-induced colitis [4,5]. However, the roles of NOD2 in the development of adoptive transfer colitis are poorly understood. In this study, we investigated whether NOD2 deficiency promotes the development of adoptive transfer colitis by using mice deficient in RAG1 alone ($Rag1^{-/-}$) or in both RAG1 and NOD2 ($Rag1^{-/-}Nod2^{-/-}$) and injecting them with naïve CD4$^+$CD45RB^{high} T cells.

As shown in Fig. 1A, $Rag1^{-/-}$ mice exhibited significantly greater body weight loss than $Rag1^{-/-}Nod2^{-/-}$ mice. Thus, unexpectedly, $Rag1^{-/-}Nod2^{-/-}$ mice were more protected from adoptive transfer colitis than $Rag1^{-/-}$ mice bearing an intact *Nod2* gene. Pathological analysis revealed destruction of the crypt architecture and accumulation of immune cells in the colon of $Rag1^{-/-}$ mice (Fig. 1B). Indeed, the semi-quantitative colitis severity score was greater in the colon of $Rag1^{-/-}$ mice than in that of $Rag1^{-/-}Nod2^{-/-}$ mice
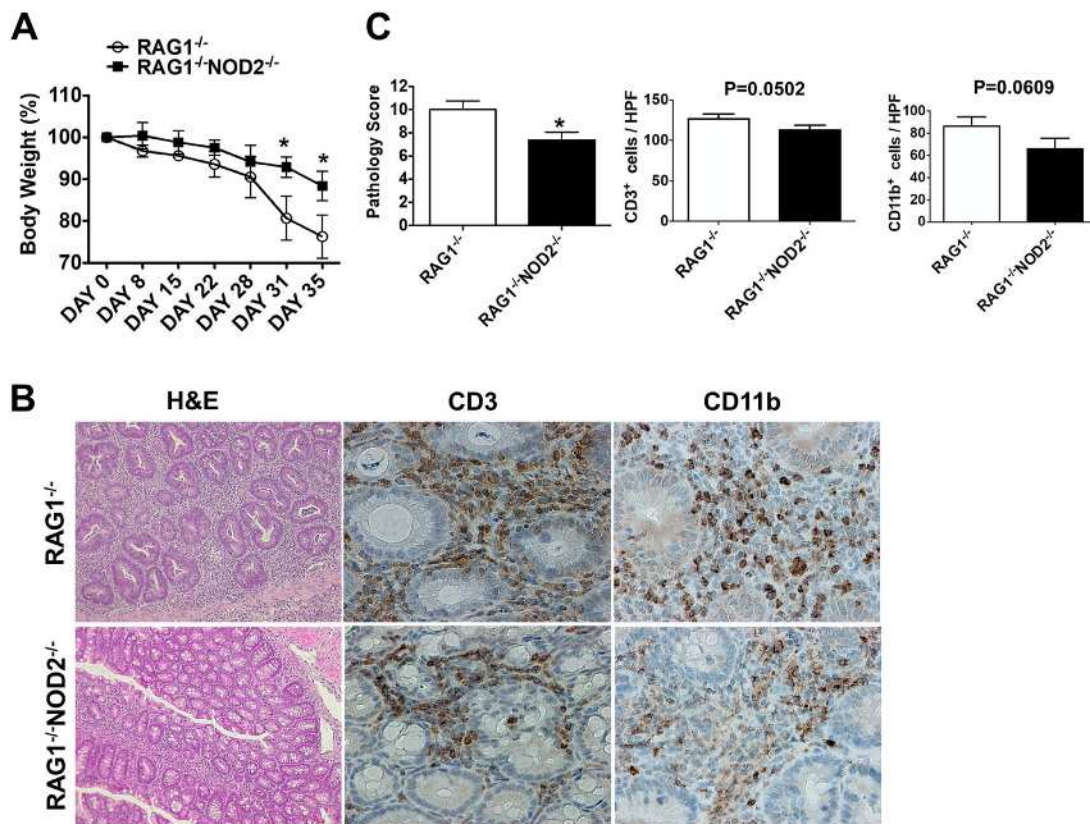
Fig. 1. Resistance of $Rag1^{-/-}Nod2^{-/-}$ mice to adoptive transfer colitis. $Rag1^{-/-}$ and $Rag1^{-/-}Nod2^{-/-}$ mice were adoptively transferred with naïve CD4+ T cells (8×10^5/mouse) isolated from the spleen of C57BL/6 mice. **(A)** Body weight curves. Day 0 was defined as the day of adoptive transfer, and the mice were sacrificed on day 35. **(B, C)** Pathological analyses of the colon of $Rag1^{-/-}$ and $Rag1^{-/-}Nod2^{-/-}$ mice. Representative hematoxylin and eosin (H&E) staining (B, magnification × 200) and pathology scores (C) are shown. Colonic sections were subjected to immunohistochemical analyses to visualize CD3+ T cells and CD11b+ myeloid cells (B, magnification × 800). Cells positive for CD3 or CD11b were counted in high-powered fields (HPFs). The results shown are a summary of two experiments ($Rag1^{-/-}$ mice; n = 9, $Rag1^{-/-}Nod2^{-/-}$ mice; n = 8). Results are shown as the mean ± standard error of the mean (SEM). *$P < 0.05$.

(Fig. 1C). IHC analyses also revealed that the accumulation of CD3+ T cells and CD11b+ myeloid cells in the colonic mucosa were nominally greater in $Rag1^{-/-}$ mice than in $Rag1^{-/-}Nod2^{-/-}$ mice, although the difference did not reach statistical significance (Fig. 1B and C). Thus, these data suggest that $Rag1^{-/-}$ mice were more susceptible to adoptive transfer colitis than $Rag1^{-/-}Nod2^{-/-}$ mice.

3.2. Greater suppression of STAT3 activation in the colonic mucosa of $Rag1^{-/-}Nod2^{-/-}$ mice

It is generally assumed that activation of STAT3 and NF-kB underlies the immunopathogenesis of colitis [11,12]. Having found that $Rag1^{-/-}Nod2^{-/-}$ mice were less susceptible to adoptive transfer colitis than $Rag1^{-/-}$ mice, we next examined the activation status of STAT3 and NF-κB by determining expression levels of p-STAT3 and p-IκBα in colonic tissues using IHC.

As shown in Supplementary Fig. 1A and B, there were more p-STAT3+ cells in the colonic mucosa in $Rag1^{-/-}$ mice than in $Rag1^{-/-}Nod2^{-/-}$ mice. In contrast, the number of p-IκBα+ cells in the colonic mucosa of both mutants was comparable. Both epithelial cells and immune cells in the colonic mucosa expressed significantly higher levels of p-STAT3 in samples from $Rag1^{-/-}$ mice than in samples from $Rag1^{-/-}Nod2^{-/-}$ mice. Thus, the protection from adoptive transfer colitis in $Rag1^{-/-}Nod2^{-/-}$ mice was accompanied by reduced expression of p-STAT3, suggesting that inhibition of the STAT3 signaling pathway was key to the protective effect.

3.3. Accumulation of FOXP3+ Tregs in the colonic mucosa of $Rag1^{-/}$ $^-Nod2^{-/-}$ mice upon induction of adaptive transfer colitis

The development of adoptive transfer colitis is dependent upon colitogenic Th1 and Th17 responses [7]. We next addressed whether the suppression of colitis in $Rag1^{-/-}Nod2^{-/-}$ mice was associated with reduced Th1 and/or Th17 responses. For this purpose, cLPMCs isolated from $Rag1^{-/-}$ and $Rag1^{-/-}Nod2^{-/-}$ mice were stimulated with an anti-CD3 antibody to measure cytokine production.

As shown in Fig. 2A, production of IFN-γ and IL-17 by cLPMCs was markedly lower in $Rag1^{-/-}Nod2^{-/-}$ mice than in $Rag1^{-/-}$ mice. IL-10 and TGF-β1 are prototypical anti-inflammatory cytokines mainly produced by FOXP3+ Tregs [13]. Production of TGF-β1 by cLPMCs was much greater in $Rag1^{-/-}Nod2^{-/-}$ mice than in $Rag1^{-/-}$ mice. Production of IL-10 by cLPMCs was also nominally higher in $Rag1^{-/-}Nod2^{-/-}$ mice than in $Rag1^{-/-}$ mice, although the difference was not statistically significant. Thus, enhanced production of TGF-β1 accompanied by suppression of Th1 and Th17 responses was a prominent feature of the cytokine dynamics in $Rag1^{-/-}Nod2^{-/-}$ mice. We also measured IL-6 production by cLPMCs because
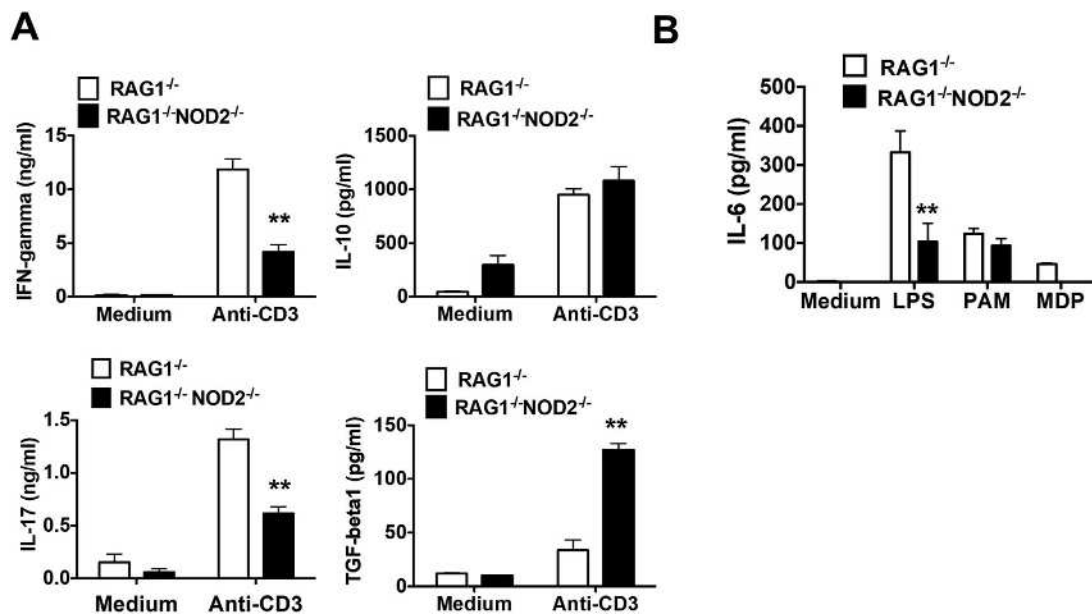
Fig. 2. Cytokine profiles in the colonic mucosa of *Rag1*$^{-/-}$*Nod2*$^{-/-}$ mice. Adoptive transfer colitis was induced as described in Fig. 1. **(A, B)** Colonic lamina propria mononuclear cells (1 × 10^6/mL) isolated from *Rag1*$^{-/-}$*Nod2*$^{-/-}$ or *Rag1*$^{-/-}$ mice were stimulated with an anti-CD3 antibody (5 μg/mL), lipopolysaccharide (LPS, 1 μg/mL), Pam3CSK4 (PAM, 5 μg/mL), and muramyl dipeptide (MDP, 10 μg/mL) for 48 h. Culture supernatants were subjected to cytokine assays. The concentrations of cytokines (IFN-γ, IL-6, IL-10, IL-17, and TGF-β1) were measured using enzyme-linked immunosorbent assay. Results are shown as the mean ± SEM. **P < 0.01.

activation of STAT3, a critical downstream signaling molecule for IL-6 [11], was attenuated in the colonic mucosa of *Rag1*$^{-/-}$*Nod2*$^{-/-}$ mice. As shown in Fig. 2B, IL-6 production by cLPMCs was significantly lower in *Rag1*$^{-/-}$*Nod2*$^{-/-}$ mice than in *Rag1*$^{-/-}$ mice upon stimulation with LPS.

Enhanced production of TGF-β1 led us to explore whether the number of Tregs expressing FOXP3 was higher in *Rag1*$^{-/-}$*Nod2*$^{-/-}$ mice than in *Rag1*$^{-/-}$ mice. Flow cytometry revealed that the percentage and absolute number of FOXP3$^+$ Tregs were indeed significantly greater in the colonic mucosa of *Rag1*$^{-/-}$*Nod2*$^{-/-}$ mice (Fig. 3A). Consistent with these results, the number of FOXP3$^+$ Tregs was also significantly greater in the colonic mucosa of *Rag1*$^{-/}$ $^{-}$*Nod2*$^{-/-}$ mice according to IHC analysis (Fig. 3B). Thus, protection from colitis in *Rag1*$^{-/-}$*Nod2*$^{-/-}$ mice was accompanied by enhanced production of TGF-β1 and accumulation of Tregs. Taken together, these data suggest that NOD2 deficiency suppressed the development of adoptive transfer colitis through the induction of TGF-β1 production and activation of FOXP3$^+$ Tregs.

3.4. TGF-β1 protects against colitis in Rag1$^{-/-}$Nod2$^{-/-}$ mice by inducing FOXP3$^+$ Tregs

TGF-β1 plays critical roles in the maintenance of intestinal immune homeostasis through the induction of Treg differentiation [14,15]. In the final series of experiments, we addressed whether the protection against colitis in *Rag1*$^{-/-}$*Nod2*$^{-/-}$ mice was dependent upon the TGF-β1 response. As shown in Fig. 4A, *Rag1*$^{-/-}$*Nod2*$^{-/-}$ mice treated with an anti-TGF-β1 antibody had lower body weights than mice treated with a control antibody, although the difference was not statistically significant. This body weight loss was accompanied by significantly higher pathological scores in *Rag1*$^{-/-}$*Nod2*$^{-/-}$ mice treated with an anti-TGF-β1 antibody (Fig. 4B and C). We examined the cytokine responses and found that cLPMCs isolated from mice treated with an anti-TGF-β1 antibody

produced higher amounts of IFN-γ and IL-17 upon stimulation with an anti-CD3 antibody than animals treated with a control antibody (Fig. 4D). On the contrary, IL-10 production by cLPMCs from mice treated with an anti-TGF-β1 antibody was lower than those treated with a control antibody. Furthermore, flow cytometry revealed that neutralization of TGF-β1-mediated signaling pathways markedly reduced the percentage and number of CD4$^+$FOXP3$^+$ Tregs in the colonic mucosa of *Rag1*$^{-/-}$*Nod2*$^{-/-}$ mice. These data indicated that the blockade of TGF-β1-mediated signaling pathways increased the severity of adoptive transfer colitis by impairing the activation of Tregs. Taken together, these results suggest that NOD2 deficiency protected mice against adoptive transfer colitis through the induction of TGF-β1 responses and subsequent activation of Tregs.

4. Discussion

In this study, we aimed to determine the roles played by NOD2 in the development of adoptive transfer colitis. Given that *Nod2* mutations are one of the strongest risk factors for the development of CD [1—3], we assumed that NOD2 deficiency would predispose mice to severe colitis. Contrary to this expectation, *Rag1*$^{-/-}$ mice displayed more severe colitis than *Rag1*$^{-/-}$*Nod2*$^{-/-}$ mice, as assessed by the body weight curves and pathological scores. As for the molecular mechanisms accounting for this protection against adoptive transfer colitis under NOD2 deficiency, we observed greater accumulation of FOXP3$^+$ Tregs in the colonic mucosa of *Rag1*$^{-/-}$*Nod2*$^{-/-}$ mice. Enhanced TGF-β1 production is involved in the protection against adoptive transfer colitis in the absence of NOD2 because the neutralization of TGF-β1-mediated signaling pathways exacerbated chronic inflammation of the colon in *Rag1*$^{-/}$ $^{-}$*Nod2*$^{-/-}$ mice. Importantly, this exacerbation of experimental colitis was accompanied by reduced accumulation of FOXP3$^+$ Tregs. This shows that NOD2 deficiency protects mice from the development of adoptive transfer colitis through TGF-β1-mediated
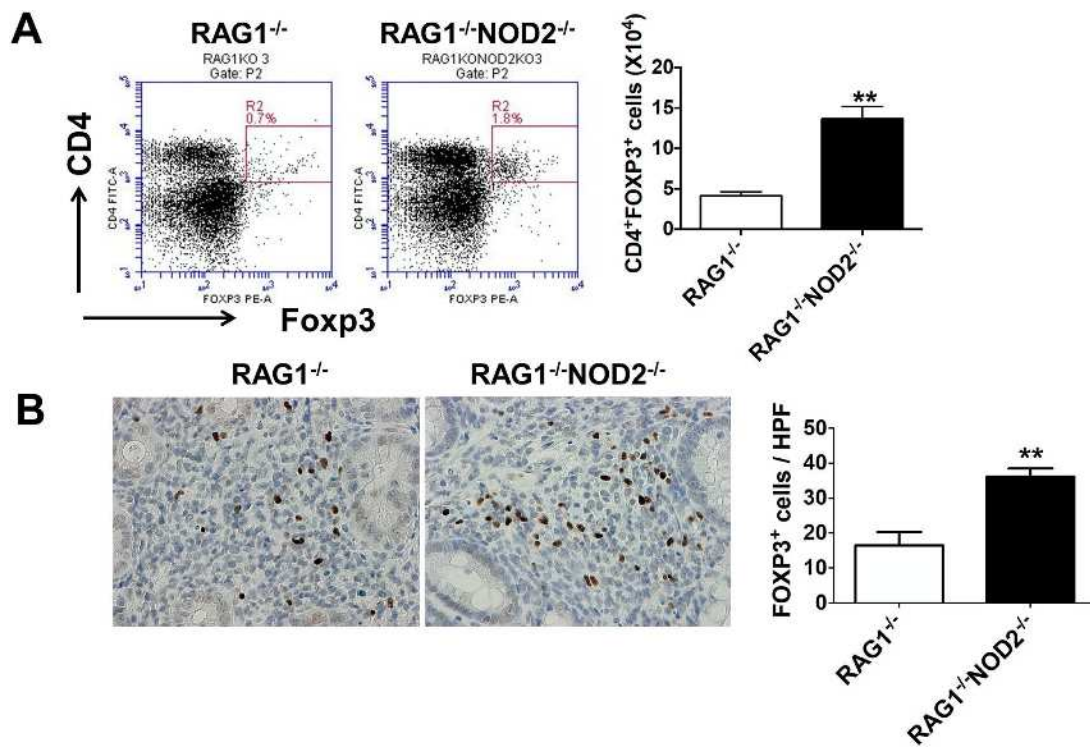
Fig. 3. Accumulation of regulatory T cells in the colonic mucosa of $Rag1^{-/-}Nod2^{-/-}$ mice. Adoptive transfer colitis was induced as described in Fig. 1. Colonic lamina propria mononuclear cells were isolated from $Rag1^{-/-}Nod2^{-/-}$ mice or $Rag1^{-/-}$ mice. **(A)** The number and percentage of CD4+FOXP3+ positive cells were determined by flow cytometry in $Rag1^{-/-}$ (n = 4) and $Rag1^{-/-}Nod2^{-/-}$ (n = 5) mice. **(B)** Colonic sections were subjected to immunohistochemical analysis to visualize FOXP3+ cells, which were counted in high-powered fields (HPFs, magnification × 800). Results are shown as the mean ± SEM. **$P < 0.01$.

induction of Treg differentiation.

MDP activation of NOD2 protected mice from DSS and TNBS colitis [4—6,16]. Regarding the molecular mechanisms of this protection, we and others reported that MDP activation of NOD2 induced the expression of interferon regulatory factor 4 (IRF4) in dendritic cells, which, in turn, downregulated the production of proinflammatory cytokines upon exposure to TLR ligands derived from intestinal microbiota [4,5,16]. Thus, the expression of IRF4, a pivotal negative regulator of TLR-mediated signaling pathways, is associated with the protection against DSS and TNBS-induced colitis. In contrast to the observations in models of short-term, chemically induced colitis, $Rag1^{-/-}Nod2^{-/-}$ mice exhibited greater resistance to the induction of long-term adoptive transfer colitis than $Rag1^{-/-}$ mice. Such unexpected results can be partially explained by the enhanced accumulation and activation of Tregs in the colonic mucosa in the absence of NOD2. In line with these data, other researchers have shown that NOD2 deficiency is associated with increased mucosal regulatory responses mediated by Tregs expressing TGF-β1/latency-associated peptide [17,18]. However, the molecular mechanisms underlying the enhanced Treg responses in the absence of NOD2 remain unknown. In this regard, we assume that the intestinal dysbiosis induced by NOD2 deficiency may be involved in enhanced Treg responses. In fact, Butera et al. reported that NOD2 deficiency was associated with alterations in the intestinal microbiota and caused the expansion of Tregs expressing TGF-β1/latency-associated peptide [18].

Consistent with our results, de Souza et al. showed that the percentage of FOXP3+ Tregs in the mesenteric lymph nodes was greater in $Nod2^{-/-}$ mice than in wild-type mice upon exposure to DSS [19]. However, such enhanced Treg responses did not lead to the suppression of short-term DSS colitis [19]. In contrast, in our experiments, the enhanced Treg responses associated with NOD2 deficiency prevented the development of long-term adoptive transfer colitis. Thus, NOD2 deficiency promoted the accumulation of FOXP3+ Tregs in the GI tract, which led to the suppression of long-term colitis but not short-term colitis. A potential molecular mechanism whereby NOD2 deficiency causes augmented Treg responses could involve impairment of IL-6-mediated signaling pathways. IL-6, which is a cytokine induced during the acute phase of inflammation, has been reported to inhibit the differentiation of FOXP3+ Tregs induced by TGFβ1 [20]. Accordingly, IL-6 production and STAT3 activation in the colonic mucosa were markedly lower in $Rag1^{-/-}Nod2^{-/-}$ mice than in $Rag1^{-/-}$ mice. Therefore, the enhanced Treg responses observed in the colonic mucosa of $Rag1^{-/-}Nod2^{-/-}$ mice can be partially explained by a reduction in IL-6 production.

Three major $Nod2$ loss-of-function mutations (R702W, G908R, and L1007 fs) are associated with CD, suggesting that intact NOD2 signaling pathways protect against the development of CD [1—3]. CD patients with $Nod2$ mutations manifest severe phenotypes, including ileal disease [21,22]. Thus, it is well established that $Nod2$ polymorphisms are one of the strongest risk factors for the development of CD. However, CD does not always occur in individuals with CD-associated $Nod2$ mutations [23]. Although approximately, 11—14% of white Europeans are heterozygous for CD-associated $Nod2$ variants and 0.4—0.9% of them are homozygous for these
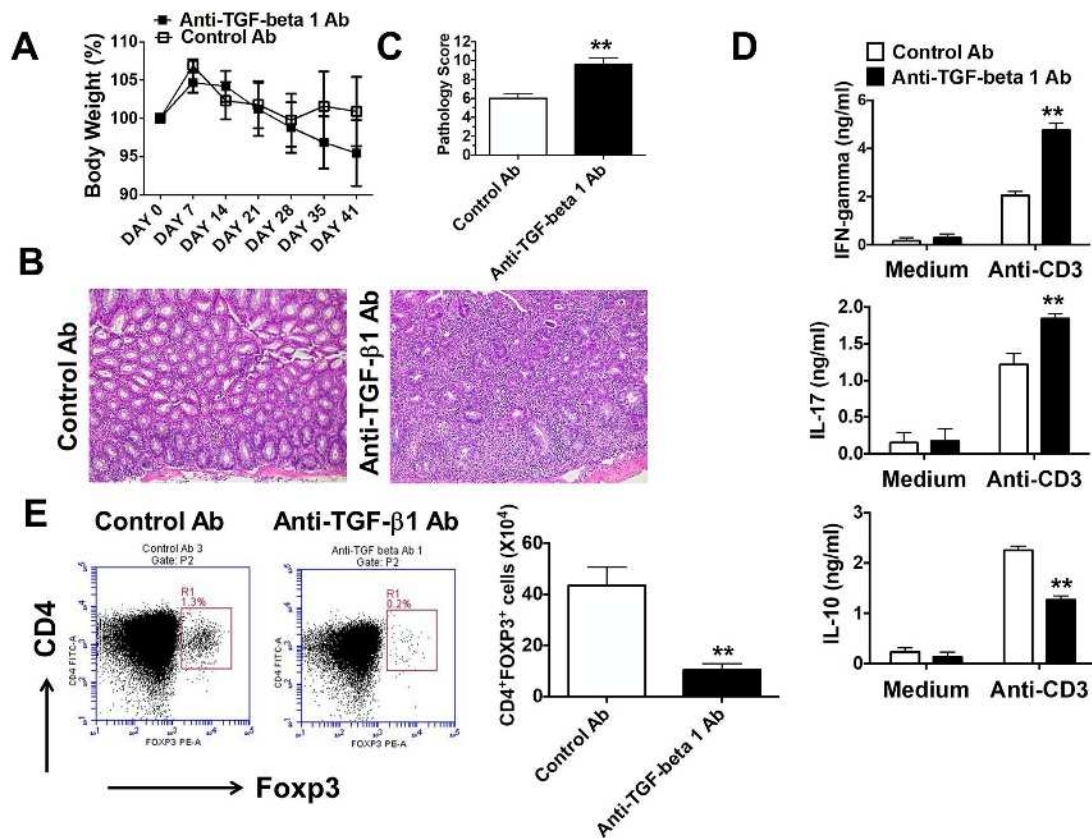
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Fig. 4. Requirement of TGF-β1 for the differentiation of regulatory T cells in the colonic mucosa of $Rag1^{-/-}Nod2^{-/-}$ mice. Adoptive transfer colitis was induced in $Rag1^{-/-}Nod2^{-/-}$ mice as described in Fig. 1. Mice were treated with a neutralizing antibody (Ab) against TGF-β1 (0.5 mg once a week/mouse, n = 5) or with control mouse IgG (0.5 mg/mouse, n = 5). **(A)** Body weight curve. Day 0 was defined as the day of adoptive transfer, and the mice were sacrificed on day 42. **(B, C)** Pathological analyses of the colon in $Rag1^{-/-}Nod2^{-/-}$ mice treated with an anti-TGF-β1 or control Ab. Representative hematoxylin and eosin (H&E) staining (magnification × 200) and pathological scores are shown. **(D)** Colonic lamina propria mononuclear cells were isolated from $Rag1^{-/-}Nod2^{-/-}$ mice (1×10^6/mL) and stimulated with an anti-CD3 antibody (5 μg/mL) for 48 h. Culture supernatants were subjected to cytokine assays. The concentrations of cytokines (IFN-γ, IL-10, and IL-17) were measured using enzyme-linked immunosorbent assay. **(E)** The numbers and percentages of CD4+FOXP3+ cells were determined by flow cytometry. Results are shown as the mean ± SEM. **$P < 0.01$.

variants, the majority of them remain healthy [23]. Genetic and epidemiological studies suggest that *Nod2* mutations alone are not sufficient to cause the disease and that environmental factors are required for the initiation of CD. Our data show that NOD2 deficiency protects mice from adoptive transfer colitis through the induction of Tregs in the colonic mucosa. Such paradoxical protection against colitis in the absence of NOD2 partially explains why some individuals bearing *Nod2* mutations do not always develop CD. Further studies are needed to confirm whether enhanced Treg responses are associated with protection against CD in individuals with *Nod2* mutations.

In conclusion, $Rag1^{-/-}Nod2^{-/-}$ mice were more resistant to adoptive transfer colitis than $Rag1^{-/-}$ mice. Although MDP activation of NOD2 protected wild-type mice against chemically induced colitis [4—6,16], NOD2 deficiency paradoxically contributed to protection against adoptive transfer colitis. The improvement in colitis in the absence of NOD2 can be partially explained by the enhanced accumulation of Tregs in the colonic mucosa. These observations provide an explanation for the insufficiency of NOD2 deficiency alone to cause intestinal inflammation, and confirm that additional factors are necessary for CD manifestations in individuals with *Nod2* mutations.

Declaration of competing interest

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

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrc.2021.06.068.

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