Induction and rescue of Nod2-dependent Th1-driven granulomatous inflammation of the ileum

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Mutations in the NOD2 gene are strong genetic risk factors for ileal Crohn’s disease. However, the mechanism by which these mutations predispose to intestinal inflammation remains a subject of controversy. We report that Nod2-deficient mice inoculated with Helicobacter hepaticus, an opportunistic pathogenic bacterium, developed granulomatous inflammation of the ileum, characterized by an increased expression of Th1-related genes and inflammatory cytokines. The Peyer’s patches and mesenteric lymph nodes were markedly enlarged with expansion of IFN-γ-producing CD4 and CD8 T cells. Rip2-deficient mice exhibited a similar phenotype, suggesting that Nod2 function likely depends on the Rip2 kinase in this model. Transferring wild-type bone marrow cells into irradiated Nod2-deficient mice did not rescue the phenotype. However, restoring crypt antimicrobial function of Nod2-deficient mice by transgenic expression of α-defensin in Paneth cells rescued the Th1 inflammatory phenotype. Therefore, the regulation of intestinal microbes, Nod2 function in nonhematopoietic cells of the small intestinal crypts is critical for protecting mice from a TH1-driven granulomatous inflammation in the ileum. The model may provide insight into Nod2 function relevant to inflammation of ileal Crohn’s disease.

Crohn’s disease | granuloma | Helicobacter hepaticus | innate immunity | Paneth cells

Under physiological conditions, reciprocal interactions between the intestinal immune system and commensal microbiota elicit a basal level of immune responses that protect the mucosa from both pathogenic and nonpathogenic bacteria. Changes precipitated by either abnormal microbiota and/or dysregulation of immune responses may disrupt this homeostasis, resulting in mucosal inflammation (1–3). For example, the pathogenesis of intestinal inflammation in Crohn’s disease (CD) appears to involve an inappropriate immune response against colonizing microbes (1–3). Mutations in the NOD2 gene were the first defined genetic risk factors identified for CD (4, 5). Nod2 belongs to the NLR family of cytoplasmic proteins and responds to muramyl dipeptide (MDP), a moiety of bacterial peptidoglycan, consisting of N-acetylmuramyl-L-Ala-D-Glu (6, 7). The Rip2 kinase mediates downstream signaling of Nod2, and, upon MDP stimulation, Rip2 activates NF-κB and MAP kinase cascades, resulting in the induction of immune response genes (8–10). Although Rip2-dependent Nod2 function(s) may be fundamentally important, CD penetrance in individuals with either Nod2 homozygous or compound heterozygous mutations is incomplete, indicating that dysregulation of Nod2 signaling alone is insufficient to induce disease (11). Moreover, Nod2-deficient mice do not develop spontaneous intestinal inflammation (10, 12). Therefore, CD pathogenesis is likely to be influenced by additional contributing factors, including the environment, altered immune regulation, and dysbiosis of colonizing microbiota (1–3, 13).

The mechanism by which NOD2 mutations contribute to CD pathogenesis remains a subject of controversy. Three models have been proposed. The first suggests that a NOD2 “gain-of-function” mutation results in heightened sensitivity to MDP, leading to an increased inflammatory response (14). However, several studies using human patient samples have suggested that CD-associated NOD2 mutations are loss-of-function (13, 15, 16). The second model involves altered TLR2 signaling, proposing an inhibitory role of Nod2 in the TLR2-mediated Th1 responses (17). However, other groups have shown that TLR2 responses are normal in different lines of Nod2-deficient mice, and there may be a synergistic rather than a negative effect on TLR2 stimulation in human and mouse cells (10, 18–21). The third model proposes that mutations in NOD2 may result in altered mucosal host–microbe interactions (13). Nod2 is highly expressed in Paneth cells, epithelial cells of the intestinal crypts of Lieberkühn that govern innate immune responses through the secretion of antibacterial proteins and peptides such as α-defensins (22–24). CD-associated NOD2 mutations primarily predispose to the development of small intestinal (ileal) lesions, corresponding to the location of Paneth cells (25). Ileal CD is characterized by a decrease of Paneth cell-produced antimicrobial α-defensins, human α-defensin-5 (HD5) and α-defensin-6 (HD6) (26–28). Although reduced expression of α-defensins was observed regardless of NOD2 genotype, individuals with the common frame-shift mutation NOD2*2003K had a more pronounced decrease compared with the other genotypes (26). This decreased expression of α-defensins reported in ileal CD, whether or not there was an identified mutation in NOD2, was independent of tissue inflammation (26, 28, 29). Moreover, the levels of α-defensin detected in the ileostomy fluid of CD patients are also lowest in patients with either homozygous or compound heterozygous NOD2 mutations (30). In a murine model, the expression of a subgroup of α-defensins is reduced in Nod2-deficient mice (10, 31). Indeed, Nod2-deficient small intestinal crypts are unable to kill bacteria efficiently and there are increases of commensal and pathogenic bacteria in the terminal ileum of Nod2-deficient mice (32).

Although the idea that CD may result from abnormal host–microbe interactions is appealing, there is no direct experimental evidence that links loss of Nod2 function in small intestinal crypts with intestinal inflammation. Here, we show that Nod2-deficient mice inoculated with an opportunistic pathogenic bacterium, Helicobacter hepaticus, develop Th1-driven granulomatous inflammation of the ileum. Moreover, we find that Rip2-deficient
Nod2 function in nonhematopoietic cells of the ileal crypts is required for protection from the inflammation, and we rescue the Nod2-dependent phenotype by the transgenic expression of HD5, a human Paneth cell α-defensin. The data provide insight into Nod2 function in a model that may prove relevant to ileal CD.

Results

**H. hepaticus** Induces Granulomatous Inflammation in Nod2-Deficient Mice. *H. hepaticus*, a Gram-negative microaerophilic bacterium, is a common intestinal commensal bacterium in most animal facilities in the United States. Although *H. hepaticus* may induce colitis in immunodeficient mice such as common gamma-chain (IL-2Rγ)-deficient mice, it does not cause disease in most strains of wild-type mice, and thus is categorized as an opportunistic pathogen (33). We previously reported that Nod2-deficient mice were unable to regulate bacterial load in the ileum after de novo inoculation of *H. hepaticus* (32). This observation prompted us to characterize the mucosal response in this model. Mice were rederived and maintained under specific pathogen free, Helicobacter-free conditions until experimental challenge. In response to *H. hepaticus* inoculation, numbers of macroscopically visible Peyer’s patches increased significantly at 14 d in Nod2-deficient mice in comparison with wild-type mice (Fig. 1A). In addition, the Peyer’s patches and mesenteric lymph nodes were increased in size in Nod2-deficient mice (Fig. 1B). No gross change in the cecum or colon was observed for either strain. For histological analysis, tissue sections were scored for degree of inflammation and granuloma formation in a blind manner by one pathologist. Interestingly, *H. hepaticus*-inoculated Nod2-deficient mice exhibited granulomatous inflammation, which is a hallmark pathological characteristic of human CD (Fig. 1C and D and Fig. S1) (34). In the ileum, there were increased mononuclear cells in lamina propria and the appearance of epithelioid cells in the granulomatous nodules (Fig. 1D). The expression of proinflammatory cytokines IL-1β and IL-6, as well as the Th1 response-related genes IFN-γ and Spp1, was significantly higher in the Nod2-deficient ileum than in that of the wild-type controls (Fig. 1E). In contrast, no differences in the expression of these genes was observed in the descending colon, highlighting a regional specificity for Nod2 function in this model (Fig. 1E). These data indicate that Nod2 is required to elicit an adequate mucosal response against *H. hepaticus* and to avert development of granulomatous inflammation.

**H. hepaticus** Induces Th1 Immune Responses in Peyer’s Patches and Mesenteric Lymph Nodes of Nod2-Deficient Mice. We found that inoculation of *H. hepaticus* significantly increased levels of CD69, an activation marker, on CD4 and CD8 T cells, and B cells in the Peyer’s patches of Nod2-deficient mice but not of wild-type mice (Fig. S2). Because it is known that both CD and intestinal pathology caused by *H. hepaticus* are characterized by Th1 dominant chronic inflammation, we next assessed CD4 and CD8 T cells isolated from Peyer’s patches and mesenteric lymph nodes for their expression of IFN-γ and IL-4 (35, 36). *H. hepaticus* inoculation resulted in significantly increased IFN-γ producing CD4 and CD8 T cells in both Peyer’s patches and mesenteric lymph nodes in Nod2-deficient but not wild-type mice (Fig. 2A). Expression of Th1-related genes including IFN-γ, T-bet (a Th1 specific transcription factor), and IL-12β2 receptor was significantly higher in the Peyer’s patches and mesenteric lymph nodes of Nod2-deficient mice (Fig. 2B). In contrast, we could not detect any changes in IL-4 or IL-17 producing CD4 T cells (Fig. 2A and Fig. S3A). Consistent with this finding, *H. hepaticus* inoculation produced no change in the expression of genes associated with other T-helper subsets, including IL-4, IL-17, Gata-3, and Foxp3 (Fig. S3B). These observations suggest that Nod2-deficiency renders mice susceptible to *H. hepaticus*-induced Th1 inflammatory responses in Peyer’s patches and mesenteric lymph nodes in this model, which is similar to the immune responses found in the intestines of CD patients.

**Rip2 Deficiency Mimics the Nod2-Dependent Phenotype.** We recently showed a deficiency in the antimicrobial capacity of ileal crypts of Rip2-deficient mice similar to that seen in Nod2-deficient mice (32). Therefore, we examined whether the Rip2-deficient mice also developed inflammatory responses when challenged with *H. hepaticus*.
Similar to Nod2-deficient mice, Rip2-deficient mice inoculated with *H. hepaticus* showed (i) significant increases in the number of macroscopically visible Peyer’s patches after 14 d (Fig. 3A), (ii) enlargement of the Peyer’s patches and mesenteric lymph nodes (Fig. 3B), and (iii) development of granulomatous inflammation (Fig. 3C). In addition, the expression of both IFN-γ and T-bet were also significantly higher in the Peyer’s patches and mesenteric lymph nodes, consistent with a Th1 dominated immune response (Fig. 3D). Finally, similar to Nod2-deficient mice, there were no significant changes in the expression of IL-4, Gata-3, IL-17A, or Foxp3 (Fig. S4). Thus, Rip2 deficiency results in susceptibility to Th1 immune responses in the ileum following *H. hepaticus* inoculation, supporting that the protective functions of Nod2 in this model are dependent on Rip2 signaling.

Adaptive Transfer of Wild-Type Bone Marrow Cells Did Not Rescue the Nod2-Deficient Inflammatory Phenotype. Nod2 is expressed not only in Paneth cells but also in hematopoietic cells including monocytes, macrophages, and dendritic cells (1, 23, 37). To address whether the protective function of Nod2 in the ileum is due to its expression in hematopoietic cells, we attempted to rescue the Nod2-dependent inflammatory phenotype by reconstituting Nod2-deficient mice using adoptive transfer of wild-type bone marrow cells from congenic CD45.1 C57BL/6 (B6.SJL-Ptprc) mice. Six weeks after the bone marrow transfer, efficiency of reconstitution was assessed by analyzing blood cells for the expression of CD45.1 (donors) vs. CD45.2 (recipients). Approximately 95% efficiency of reconstitution was evident in both strains (Fig. S5A). Compared with wild-type recipient mice, Nod2-deficient recipient mice again had more IFN-γ producing CD4 and CD8 T cells (Fig. 4A), as well as higher expression of IFN-γ and T-bet (Fig. 4B), in Peyer’s patches and mesenteric lymph nodes following *H. hepaticus* inoculation. The expression of proinflammatory cytokines IL-1β and IL-6, as well as Th1 response-related genes IFN-γ and Spp1, in the ileum (Fig. S6A), was significantly higher in Nod2-deficient mice than in wild-type recipient controls (Fig. 4C). Similar to Nod2-deficient mice without bone marrow reconstitution, there were no significant changes in IL-4–producing CD4 T cells (Fig. S5B). Collectively, these results indicate that reconstitution with wild-type bone marrow cells could not rescue the Nod2-deficient mice from *H. hepaticus*-induced Th1 immune responses. To determine whether Nod2-deficient hematopoietic cells could account for the inflammation, we performed complementary bone marrow cell reconstitution experiments by adoptively transferring CD45.2+ wild-type or Nod2-deficient bone marrow cells into CD45.1+ wild-type mice (Fig. S6B). Wild-type recipient mice given Nod2−/− bone marrow cells showed no signs of inflammation. Nor was there increased expression of IFN-γ and T-bet in Peyer’s patches and mesenteric lymph node cells or increased expression of IL-1β, IL-6, IFN-γ, and Spp1 in the ileum (Fig. S6B and C). Taken together, these data suggest that Nod2 expression in nonhematopoietic cells protects *H. hepaticus*-challenged mice from small intestinal inflammation.

Restoration of Paneth Cell Antimicrobial Function of Nod2-Deficient Mice Rescued the Ileum from Th1 Inflammatory Responses. Because Nod2-deficient mice have impaired clearance of *H. hepaticus* in the ileum (32), defective antimicrobial function of the crypts may underlie the susceptibility of Nod2-deficient mice to inflammation in this model. We thus examined whether transgenic expression of...
human Paneth cell α-defensin 5 (HD5 or DEFA5) in Nod2-deficient mice could rescue crypt function and avert development of Th1-driven inflammation in the ileum. Prior studies have demonstrated that HD5-transgenic mice express physiologically relevant levels of HD5 in a Paneth cell-specific manner and can regulate both pathogenic and commensal bacteria in the small intestine (38, 39). HD5-transgenic mice were intercrossed with Nod2-deficient mice and the expression of Nod2 and HD5 in the ileum of littermates with three genotypes Nod2+/−, Nod2−/−, and Nod2−/−/HD5−/− was confirmed by RT-PCR (Fig. 5A). Crypts were isolated from the ileum of littermates with the three genotypes and stimulated with carbamylcholine (CCH) to induce secretion by qRT-PCR. (B) The expression of IFN-γ and T-bet examined by qRT-PCR. (C) The expression of IL-1β, IL-6, IFN-γ, and Spp1 in the terminal ileal tissue was examined by qRT-PCR. (B and C) Data were normalized by the expression of the β-actin gene. Each bar represents replicate data from a single mouse. The P values were determined by Student t test.

**Discussion**

Since the discovery of a genetic association between NOD2 mutations and CD in 2001 (4, 5), there has been much debate over how NOD2 mutations lead to disease pathogenesis. Unlike apparent gain-of-function NOD2 mutations associated with Blau syndrome or early onset sarcoidosis, CD-associated mutations in the NOD2 gene seem to be loss-of-function, which may likely alter host–microbe interactions through various mechanisms (13). Several studies have attempted to recapitulate CD-like intestinal inflammation using chemical inducers (such as DSS or TNBS) or adoptive transfer of hematopoietic cells in mice with Nod2 null or frame-shift mutations (14, 40–43). Despite many interesting observations, these mouse models were limited due to the fact that inflammation was induced mainly in the colon, whereas in CD, NOD2 mutations are mostly associated with ileal inflammation (25). Unlike these previous studies, our current model shares multiple features with NOD2 mutation-associated CD. First, the inflammation in this model is localized to the ileum. The expression of proinflammatory cytokine genes was increased in the ileum but not in the descending colon in H. hepaticus inoculated Nod2-deficient mice (Fig. 1E). Second, like CD, our model shows a profound Th1-mediated inflammation with elevated expression of Th1 related genes in the ileal mucosa and stimulated CD4 and CD8 T cells in ileal Peyer’s patches and mesenteric lymph nodes (Figs. 1A and 2A). Third, the inflammation in our model is accompanied with granuloma formation, which is one of pathological hallmarks of human CD (Fig. 1C and D). Fourth, an interplay of intestinal microbiota and genetic susceptibility is important in both human CD and this model (2) because H. hepaticus inoculation was required to induce inflammatory responses in Nod2-deficient mice. Lastly, both human CD and our model involve Peyer’s patches (Fig. 1A and B). Clinical observations suggest that Peyer’s patches and M cells are the initial sites of inflammation in ileal CD (44), and significant inflammation of Peyer’s patches was evident in this mouse model. Taken together, these observations indicate that our model shares many salient features with ileal CD. Therefore, although Nod2 is pleiotropic in both expression and function, our model may help discern the physiological function of Nod2 most relevant to ileal inflammation. Consistent with our previous data (32), Nod2−/− crypts failed to induce efficient bacterial killing (Fig. 5B). However, crypts from Nod2−/−/HD5 transgenic mice showed efficient bacterial killing, which was comparable to wild-type crypts, indicating that transgenic expression of HD5 can successfully restore bacterial killing activity of the ileal crypts from Nod2-deficient mice (Fig. 5B). Moreover, the HD5 transgene successfully regulated H. hepaticus in vivo because the increased loads of H. hepaticus in feces and the terminal ileum of Nod2−/− mice were reduced in Nod2−/−/HD5 mice to levels similar to Nod2−/− control mice (Fig. S7A and B). We then examined the outcome of restored crypt function on H. hepaticus-induced Th1-driven inflammation. Consistent with earlier findings (Fig. 1E), the expression of IL-1β, IL-6, IFN-γ, and Spp1 in the ileum, and of IFN-γ and T-bet in the Peyer’s patches and mesenteric lymph nodes, was significantly higher in Nod2-deficient (Nod2−/−) mice than in heterozygous (Nod2+/−) littermate controls after 14 d (Fig. 5C and D). In contrast, the expression of those genes in crypt-rescued Nod2−/−/HD5 mice were comparable to both heterozygous (Nod2+/−) littermate controls (Fig. 5C and D) and wild-type mice (Figs. 1E and 2B). Moreover, blind scoring for granulomatous inflammation of the ileal mucosa showed that Nod2−/− mice, but not Nod2−/−/HD5 transgenic mice, had significantly increased inflammation compared with Nod2+/− controls (Fig. S7C). Together, these data indicate that H. hepaticus-induced Th1 inflammatory responses in the ileum are dependent on crypt antimicrobial function and that restoration of the bactericidal activity of small intestinal crypts is sufficient to rescue the phenotype of Nod2-deficient mice and to protect them from inflammatory responses in the ileum.
observed that reconstitution of Nod2-deficient mice with wild-type bone marrow cells did not reverse the *H. hepaticus*-induced Th1-driven inflammation (Fig. 4). Conversely, wild-type mice reconstituted with Nod2-deficient bone marrows cells were not susceptible to *H. hepaticus*-induced Th1 inflammation (Fig. S6). These observations point to the role of Nod2 in nonhematopoietic cells as most relevant to the observed phenotype of our model. Impaired antimicrobial function of Paneth cells in Nod2-deficient mice was previously reported (10, 31, 32), and here we show restoration of crypt function in bacterial killing activity by introducing defensin transgene into Nod2-deficient mice. Crypts isolated from the terminal ileum of Nod2−/−, Nod2−/−, and Nod2−/−/HD5 tg littermates were stimulated with CCH for 30 min. Secretions were mixed with *E. coli* (1 × 10⁶ cells) and bacterial killing was measured by counting colonies of serial dilutions. (C and D) Nod2−/−, Nod2−/−, and Nod2−/−/HD5 tg littermates were inoculated with *H. hepaticus* (5 × 10⁶/mouse) via gastric gavage. (C) The expression of IL-1β, IL-6, IFN-γ, and Spp1 in the terminal ileum was examined 14 d postinoculation by qRT-PCR. (C and D) Data were normalized by the expression of the β-actin gene. Each bar represents replicate data from a single mouse. The *P* values were determined by Student *t* test. (E and F) Model of ileal CD. Please see Discussion for details. (E) Normal terminal ileum without NOD2 mutations. (F) Terminal ileum of ileum with loss-of-function NOD2 mutations or Nod2 deficiency. Altered Paneth cell function, dysbiosis, and abnormalities of Peyer’s patch (PP) and mesenteric lymph nodes (MLN) are depicted.

We propose the following model of Nod2 function in the small intestinal mucosa. In mice with functional Nod2, Paneth cells in the crypts secrete antimicrobial proteins that regulate mucosal immunity (Fig. 5). Under physiological conditions, only limited immune activation occurs through responses to nonpathogenic bacteria and associated antigens, as detected by epithelial cell receptors, uptake by M cells and/or sampling by transepithelial dendrites of dendritic cells. These responses are host-beneficial and the steady-state level of activation, referred to as “physiological inflammation,” bolsters mucosal protection. In contrast, with Nod2 loss-of-function or null mutations, the host–microbiota balance in the ileum shifts, in part due to impaired antimicrobial functions of Paneth cells (Fig. 5F). The resulting changes in composition, surface-association or concentration of bacteria, or bacterial antigens, can overstimulate the mucosal immune system, particularly if the surface epithelium is compromised. This scenario may invoke a tendency for lymphocytes in Peyer’s patches and mesenteric lymph nodes to initiate a Th1 immune response, which initially may be insufficient to cause overt mucosal inflammation in most cases and remains subclinical. However, if additional risk factors exist, be they genetic, environmental, dietary, or microbiological, the Th1 immune responses may escalate and the ileum may develop chronic pathological inflammation. Although our animal experimental data supports this disease model, future studies in human CD disease are required to further test the validity of this proposed mechanism.
Materials and Methods

Mouse Strains and H. hepaticus Infection. C57BL/6 mice were purchased from Taconic Farms. CD45.1 C57BL/6 (B6.5Jl-Ptpcr) mice were kindly provided by Dr. Shannon Turley (DFCI, Boston, MA). Nod2- and Rip2-deficient mice (kindly provided by Dr. Richard Flavell, Yale University, New Haven, CT) were backcrossed to C57BL/6 for 12 generations and rederived into specific pathogen-free, Helicobacter-free conditions and maintained in isolated barrier units thereafter at Taconic Farms (B, 10). HDS transgenic mice were established as described (38) and backcrossed to C57BL/6 mice for seven generations. HDS transgenic mice were rederived into specific-pathogen-free, Helicobacter-free conditions and crossed with Nod2-deficient mice to generate Nod2−/− HDS transgenic mice. Culture and inoculation of H. hepaticus was performed as described previously (32) and is detailed in SI Materials and Methods.

**Histological Scoring of Granulomatous Inflammation.** H&E-stained paraffin sections of intestinal tissue were randomly coded and scored for degrees of granulomatous inflammation in a blind manner by a pathologist (A.M.) as previously described and detailed in SI Materials and Methods (46).

**Isolation and Activation of Cells from Peyer’s Patch and Mesenteric Lymph Node.** Cells from Peyer’s patches and mesenteric lymph nodes were isolated by crushing them between the rough surface of glass slides and passing them through a 40-μm cell strainer. The cells were cultured in RPMI-1640 containing 10% FBS, 100 U/mL penicillin, and 100 U/mL streptomycin and activated in the presence of PMA (20 ng/mL) and ionomycin (1 μM) or left untreated for 3 h before isolation of RNA from the cells. For the detection of intracellular cytokines, the cells were stimulated in the presence of 2 μM monensin (eBioscience) for 6 h.

**Flow Cytometry.** Antibodies and detailed methods are described in SI Materials and Methods. Cells were analyzed by FACSCalibur (Becton Dickinson) followed by analysis using FlowJo software.

**Cryopreservation and Bacterial Killing Assays.** Cryopreservation was performed as described (32) and is detailed in SI Materials and Methods.

**Quantitative Real-Time PCR Analysis.** Quantitative real-time PCR (qRT-PCR) analysis was performed as described and is detailed in SI Materials and Methods (10).

**Statistical Analysis.** Data were subjected to Student’s t test for analysis of statistical significance, and P < 0.05 was considered to be significant.

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Supporting Information

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SI Materials and Methods

**H. hepaticus Infection of Mice.** *H. hepaticus* (ATCC 51448, kindly provided by David Schauer, MIT, Cambridge, MA) was cultured on TSA 5% sheep blood agar plates at 37 °C in an anaerobic gas chamber filled with a gas mixture of 5% CO2, 5% H2, and 90% N2. After 2 d, a suspension of *H. hepaticus* in *Brucella* broth was made to a concentration of 25 × 106 cells/mL. Each mouse was intragastrically gavaged with 5 × 105 cells/200 μL.

**Histological Scoring of Granulomatous Inflammation.** Segments of intestines from mice were removed and paraffin sections were made and stained with H&E. Histological sections were randomly numbered and scored for degrees of pathology in a blind manner by a pathologist (A.M.). The degree of granulomatous inflammation was assessed by a combined criteria as previously described (1). Granuloma score: 0, no granulomas present; 1, aggregation of cells without definite granuloma formation that is characterized by nodular collections of epithelioid cells; 2, a definite granuloma formation present; 3, multiple granulomas present. Inflammatory score: 0, no obvious inflammatory cell infiltration; 1, mild inflammatory cell infiltration; 2, moderate cell infiltration; 3, severe inflammation with complete loss of crypts.

**Flow Cytometry.** Anti-mouse IFN-γ, IL-4, IL-17A, CD4, CD8, B220, and CD69 monoclonal antibodies used in this study were obtained from ebioscience. Cells were incubated with anti-CD16/ CD32 antibody for 15 min on ice. Following Fc-block, the cells were stained for surface markers. The cells were then fixed and permeabilized using a permeabilization/fixation buffer (eBioscience) for 20 min at RT, and subsequently stained with IFN-γ, IL-4, or IL-17A antibodies diluted in permeabilization buffer for intracellular staining. Stained cells were washed, resuspended in PBS/1% FBS/0.05% NaN3 and counted for degrees of pathology in a blind manner by a pathologist (A.M.). The degree of granulomatous inflammation was assessed by a combined criteria as previously described (1). Contaminating DNA was removed using the Turbo DNA-free kit (Ambion), and cDNA synthesis was performed using the qScript cDNA synthesis kit (Quanta Biosciences) as per manufacturer's instructions. Two hundred nanograms of cDNA was used to perform qRT-PCR analysis using the PerfeCTa TaqSYBER Green SuperMix, ROX (Quanta Biosciences) on an Applied Biosciences ABI 7700 real time machine. Primer pairs used in the qPCR analysis were as follows: β-actin: GCCTGTGCTGCTCCTGATATGCTCT (forward) CTTCCTACGTGGTTGTTGAA (reverse); IFN-γ: ATGAAACGCTACACACTGTCATC (forward), CCACTCTTTTGACGT TTCTCTC (reverse); T-bet: CGAGGGCAAGCAGCC (forward), GAAACCTCCTGACCTGATG (reverse); IL-4: ACAGGAGAGGGCACCGCATG (forward), GAAGGGTATCAGGGTGCAAAGTGA (reverse); Spp1: AGCAAAAGGATCTCCAGACG (forward), GCCCTCTTTCTCCAGAGCCA (forward), GTTGGATTCGTCAGATTCCTC (reverse); IL-12p2: AGAGAATGCTCAGTTGGC (forward), AACTGGGTATAAGGACCCG (reverse); IL-1β: GGCTCTGTCGTTGACC (forward), TGTGCTGCCGTTGTTTCCTGTG (reverse); IL-6: CCAGAAAACTGTAATGGTTCC (forward), TTGTCCACCAGCATGCTC (reverse); Nod2: TTGACTTGTGGCTAATGCTTTG (forward), TTTATCTGCGTCTCTAG (reverse); HD5: CGCCATTTACAGAACTGGT (forward), TTTGCGCATAGGCTTTTCTGG (reverse).

**Reconstitution of Hematopoietic Cells with Bone Marrow (BM) Cells.** Bone marrow cells were eluted from the tibia and femur of donor mice (CD45.1+) grown in PBS. A single-cell suspension devoid of RBC was prepared in PBS. In NSG-deficient (CD45.2-/-) recipient mice. Mice were given drinking water supplemented with Baytril (70 mg/L water) for 3 d. Donor bone marrow cells were injected i.v. into reconstituted mice. Mice were reconstituted through FACS analysis.

**Crypt Isolation and Bacterial Killing Assays.** Mouse intestinal crypts were isolated from the terminal ileum as described previously (2). Briefly, 10 cm of mouse small intestine measure from the ileal terminus were cut longitudinally and washed in cold PBS. Intestines were cut in 2-cm fragments and shaken in a 15-mL falcon tube in 30 mM EDTA in DPBS (without Ca and Mg), pH 7.4, for 5 min. Washes were repeated six times for a total of seven fractions and crypts were spun down and rapidly replaced into DPBS. Fractions were observed under a light microscope to identify crypt-enriched fractions. Bactericidal assays were performed as described previously (2). Crypts from crypt-enriched fractions were counted in a cell-counter and numbers were normalized to 1,000-2,000 crypts/well in 50 μL of iPIPES (10 mM Pipes, 150 mM NaCl). Crypts were stimulated with 5 μM CCH (Sigma) for 30 min at 37 °C. Crypts were spun down and supernatants were incubated with 1 × 106 cfu of *E. coli* DH5α for 1 h at 37 °C and cells were plated in serial dilutions up to 10-5. Plates were incubated overnight at 37 °C and bacterial colonies were counted. Percent killing was calculated by the following formula.

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\text{Percent killing} = \left[ 1 - \left( \frac{\text{CFU in stimulated samples}}{\text{CFU in unstimulated controls}} \right) \right] \times 100.
\]

**Detection of H. hepaticus from Stool and Small Intestine.** DNA from mouse feces was isolated using the Qiagen DNA stool isolation kit according to manufacturer’s instructions. DNA from mouse small intestine was isolated by treating 1-cm sections of terminal ileum in lysing buffer (100 mM NaCl, 20 mM Tris pH 7.6, 10 mM EDTA, 0.5% SDS, 0.4 mg/mL Proteinase K) overnight at 55 °C. DNA was precipitated with a high salt/ethanol precipitation method and washed extensively with 70% ethanol. *H. hepaticus* was detected from DNA isolated from stool samples using primers specific for *H. hepaticus* 16S RNA. Primer pairs used in qPCR analysis are as follows: *H. hepaticus*: (5′-ATGTTGCCAAACTGTTCTGAGG-3′) (5′-CGTTTTTCAAGCTCCGGAG-3′); *Eubacteria*: (5′-ACTCTACGGGAGGCACAG-3′) (5′-ATTACCGCGCTTGTCGCCG-3′).

Fig. S1. Granulomatous inflammation in Nod2-deficient mice. Age- and sex-matched wild-type and Nod2-deficient mice were inoculated with *H. hepaticus* (5 × 10^8/mouse) via gastric gavage. Inflammation and granuloma score of the ileocecal junction of wild-type (*n* = 29) and Nod2-deficient (*n* = 31) mice at d 14 postinoculation. The scoring was performed in a blind manner as described in Materials and Methods. The *P* values were determined by Student *t* test.
Fig. S2. Increased activated lymphocytes in Peyer’s patches in *H. hepaticus*-inoculated *Nod2*-deficient mice. (A–C) Age- and sex-matched wild-type and *Nod2*-deficient mice were inoculated with *H. hepaticus* (5 × 10⁸/mouse) via gastric gavage. (A) Peyer’s patch cells were isolated at d 0 and 14 postinoculation and the expression of CD4 and CD69 was analyzed by flow cytometry. (B) The expression of CD8 and CD69 in Peyer’s patch cells was analyzed as described in A. (C) The expression of B220 and CD69 in Peyer’s patch cells was analyzed as described in A. Data shown are representative of three independent experiments.
**Fig. S3.** *H. hepaticus* inoculation does not induce Th2, Th17, or Treg related genes in Peyer’s patches and mesenteric lymph nodes in Nod2-deficient mice. (A and B) Age- and sex-matched wild-type and Nod2-deficient mice were inoculated with *H. hepaticus* (5 × 10⁸/mouse) via gastric gavage. Peyer’s patch and mesenteric lymph node cells were isolated at d 14 postinoculation and stimulated with PMA and ionomycin for 6 (A) or 3 (B) h. (A) The expression of CD4 and IL-17A was analyzed by flow cytometry. Data shown are representative of 3 independent experiments. (B) The expression of IL-4, IL-17, GATA3, and Foxp3 was examined by qRT-PCR. Data were normalized to the expression of the b-actin gene. Each bar represents replicate data from a single mouse. NS: not significant.
Fig. S4. *H. hepaticus* inoculation does not induce Th2, Th17 or Treg related genes in Peyer’s patches and mesenteric lymph nodes in Rip2-deficient mice. Age- and sex-matched wild-type and Rip2-deficient mice were inoculated with *H. hepaticus* (5 × 10⁸/mouse) via gastric gavage. The Peyer’s patch and mesenteric lymph node cells were isolated 14 d after the inoculation and stimulated with PMA and ionomycin for 3 h. The expression of IL-4, GATA3, IL-17A and Foxp3 was examined by qRT-PCR. Each bar represents replicate data from a single mouse. NS: not significant.
Fig. S5. Reconstitution of wild-type hematopoietic cells into Nod2-deficient mice does not influence IL-4 producing CD4 T cells. (A) Successful reconstitution of lethally irradiated Nod2-deficient mice with bone marrow cells from wild-type mice. Five-week-old wild-type and Nod2-deficient mice were lethally irradiated and 1 × 10^7 bone marrow cells from CD45.1 C57BL/6 mice were i.v. transferred. Six weeks after the transfer, heparinized blood cells were subjected for flow cytometric analysis for the expression of CD45.1 (donor) and CD45.2 (recipient). (B) H. hepaticus (5 × 10^8/mouse) was inoculated into wild-type and Nod2-deficient mice 6 wk after reconstitution with CD45.1 C57BL/6 bone marrow cells. Peyer’s patch and mesenteric lymph node cells were isolated at d 14 post-inoculation and stimulated with PMA and ionomycin for 6 h. The expression of CD4 and IL-4 was analyzed by flow cytometry. Data shown are representative of two independent experiments.
**Fig. S6.** Reconstitution of Nod2-deficient hematopoietic cells into wild-type mice does not induce Th1 type response. (A) Successful reconstitution of lethally irradiated wild-type mice with bone marrow cells from wild-type and Nod2-deficient mice. Five-week-old wild-type (CD45.1 +) mice were lethally irradiated and 1 × 10⁷ bone marrow cells from CD45.2 wild-type and Nod2-deficient mice were i.v. transferred. Six weeks after the transfer, heparinized blood cells were subjected for flow cytometric analysis for the expression of CD45.1 (recipient) and CD45.2 (donor). (B) H. hepaticus (5 × 10⁸/mouse) was inoculated into wild-type mice 6 wk after reconstitution. Peyer’s patch and mesenteric lymph node cells were isolated at d 14 postinoculation and stimulated with PMA and ionomycin for 3 h. The expression of IFN-γ, and T-bet examined by qPCR. (C) The expression of IL-1β, IL-6, IFN-γ, and spp1 in the terminal ileal tissue was examined by qRT-PCR. Each bar represents replicate data from a single mouse.
Fig. S7. Defensin transgene can inhibit the ileal colonization of *H. hepaticus* and rescue the inflammation in Nod2-deficient mice. *Nod2*+/−, *Nod2*−/−, and *Nod2*−/−*HD5* tg littermates were inoculated with *H. hepaticus* (5 × 10⁸ /mouse) via gastric gavage. (A) Feces samples were collected freshly from mice at 0, 7, and 14 d after inoculation and DNA was purified. *H. hepaticus* colonization was quantified by real-time PCR using *H. hepaticus*-specific primers and 100 ng of purified DNA per reaction. Data were normalized by real-time PCR data for *Eubacteria* using the bacteria 16S RNA gene primer sets, which detects all bacterial strains. (B) Terminal ilea were isolated from *Nod2*+/−, *Nod2*−/−, and *Nod2*−/−*HD5* tg littermates and DNA samples were prepared. *H. hepaticus* colonization was quantified by real-time PCR using *H. hepaticus*-specific primers and 100 ng of purified DNA per reaction. Data were normalized by real-time PCR data for β-actin gene in host genomic DNA. Each dot represents replicate data from a single mouse. (C) Granulomatous inflammation in the ileocecal junction of *Nod2*+/− (n = 8), *Nod2*−/− (n = 10), and *Nod2*−/−*HD5* tg (n = 9) littermates at d 14 postinoculation was scored in blind manner as described in Materials and Methods. The *P* values were determined by Student t test.