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The NLRP1 Inflammasome Attenuates Colitis and Colitis-Associated Tumorigenesis

Tere M. Williams, Rachel A. Leeth, Daniel E. Rothschild, Sheryl L. Coutermarsh-Ott, Dylan K. McDaniel, Alysha E. Simmons, Bettina Heid, Thomas E. Cecere, and Irving C. Allen

Nucleotide-binding domain and leucine-rich repeat (NLR) proteins are a diverse family of pattern recognition receptors that are essential mediators of inflammation and host defense in the gastrointestinal system. Recent studies have identified a subgroup of inflammasome forming NLRs that modulate the mucosal immune response during inflammatory bowel disease (IBD) and colitis associated tumorigenesis. To better elucidate the contribution of NLR family members in IBD and cancer, we conducted a retrospective analysis of gene expression metadata from human patients. These data revealed that NLRP1, an inflammasome forming NLR, was significantly dysregulated in IBD and colon cancer. To better characterize the function of NLRP1 in disease pathogenesis, we used Nlrp1b−/− mice in colitis and colitis-associated cancer models. In this paper, we report that NLRP1 attenuates gastrointestinal inflammation and tumorigenesis. Nlrp1b−/− mice demonstrated significant increases in morbidity, inflammation, and tumorigenesis compared with wild-type animals. Similar to data previously reported for related inflammasome forming NLRs, the increased inflammation and tumor burden was correlated with attenuated levels of IL-1β and IL-18. Further mechanistic studies using bone marrow reconstitution experiments revealed that the increased disease pathogenesis in the Nlrp1b−/− mice was associated with nonhematopoietic-derived cells and suggests that NLRP1 functions in the colon epithelial cell compartment to attenuate tumorigenesis. Taken together, these data identify NLRP1 as an essential mediator of the host immune response during IBD and cancer. These findings are consistent with a model whereby multiple NLR inflammasomes attenuate disease pathobiology through modulating IL-1β and IL-18 levels in the colon. The Journal of Immunology, 2015, 194: 3369–3380.

Pattern recognition receptors (PRRs) modulate mucosal inflammation in the gut through maintaining a balanced immune response to commensal flora and damage to the epithelial cell barrier (1). Members of the nucleotide-binding domain and leucine-rich repeat (NLR) containing family of PRRs have recently emerged as significant modulators of inflammatory bowel disease (IBD) and cancer pathogenesis (2, 3). In human populations, single nucleotide polymorphisms (SNPs) have been identified in a variety of NLRs, including NOD1, NOD2, and NLRP3, that are associated with a genetic predisposition to IBD. Likewise, recent animal studies have characterized several NLRs that are critical modulators of gastrointestinal inflammation and colitis-associated cancer (4–8). However, of the 23 human NLR and NLR-like proteins, only about half have been adequately characterized and the clinical relevance of the majority of NLR family members in IBD is unknown (9–11).

The bulk of recent studies have focused on a subgroup of NLRs that function in inflammasome formation. Inflammasomes are macromolecular scaffolds that are composed of an NLR, the adaptor protein ASC, and caspase-1, which form in the cytosol following NLR activation in response to specific microbe- and/or damage-associated molecular patterns (MAMPs and DAMPs) (12). NLR inflammasomes are responsible for the activation of caspase-1 and the subsequent cleavage and maturation of pro–IL-1β and pro–IL-18 into their mature, bioactive cytokines. Prior animal studies using Acr−/− and Casp1/11−/− mice have demonstrated that loss of either essential inflammasome-associated protein results in severe experimental colitis and colitis-associated tumorigenesis in common chemical induced models (4, 5, 7, 8, 13–16). Because of the robust effects of ASC and Caspase-1 on IBD pathogenesis, it is critical to identify and characterize the specific inflammasome-forming NLRs associated with mucosal immune system homeostasis in the gut.

Of the inflammasome forming NLRs, NLRP1 is a highly interesting candidate to explore in the context of IBD. In human populations, mutations in NLRP1 have been linked to a variety of diseases associated with dysfunctional immunoregulation, including celiac disease, vitiligo, and type 1 diabetes (17–20). In the context of IBD, genome-wide association studies have identified NLRP1 mutations that are associated with Crohn’s disease and in particular were associated with extraintestinal co-occurring inflammatory manifestations (21). NLRP1 polymorphisms were also found to be associated with IBD steroid responsiveness in a pediatric study (22). The NLRP1 inflammasome was the first inflammasome characterized in vitro but has yet to be sufficiently
characterized in vivo. NLRP1 is activated by muramyl dipeptide in humans and is activated by *Bacillus anthracis* lethal toxin (LeTx) and *Toxoplasma gondii* in rodents (23–28). There are multiple species-specific and structurally diverse orthologs of NLRP1. For example, rodents have multiple paralogs of the Nlrp1 gene, including three in mice that are poorly characterized (24). The NLRP1a paralog in mice has been shown to regulate hemo poetic hematopoiesis, and the NLRP1b paralog is associated with LeTx sensitivity (24, 25, 29, 30).

NLRP1 has not been directly evaluated in mouse models of IBD or cancer. Recently, two independent mouse lines lacking NLRP1 (Nlrp1b<sup>−/−</sup> and Nlrp1b<sup>−/−</sup>) have been described previously (25, 29). Both mouse lines were found to be susceptible to *T. gondii* infection and develop attenuated acute lung injury following LeTx exposure (25, 29, 31). Although exposure to either *T. gondii* or LeTx are unlikely mediators of IBD pathogenesis, further characterization of NLRP1 will likely identify a range of additional MAMPs and DAMPs of greater relevance to gastrointestinal inflammation and cancer. In this study, we evaluate the hypothesis that NLRP1 attenuates the progression of colitis and colitis-associated tumorigenesis. Specifically, we used Nlrp1b<sup>−/−</sup> mice in models of experimental colitis using dextran sulfate sodium (DSS) and azoxymethane (AOM)/DSS-mediat ed-inflammation-driven tumorigenesis. To our knowledge, this study is the first to functionally evaluate the NLRP1 inflammasomes in IBD and demonstrates that NLRP1 is a robust modulator of colitis pathology and colitis associated cancer progression.

**Materials and Methods**

**Experimental animals**

The generation and characterization of Nlrp1b<sup>−/−</sup> and Asc<sup>−/−</sup> mice has been described previously (25, 32). All experiments were conducted with 8- to 10-wk-old male mice, unless otherwise noted, that were backcrossed onto the C57BL/6 background. All studies were conducted with either littermate and/or cohoused wild-type (WT) animals that were maintained under specific pathogen-free conditions and received 5010 chow (LabDiet) and water ad libitum. All experiments were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were conducted under Institutional Animal Care and Use Committee approval.

**Induction and assessment of experimental colitis and colitis-associated tumorigenesis**

Experimental colitis was induced using a single cycle of either 2.5% or 5% DSS (MP Biomedicals) for 4–5 d and the mice were evaluated for up to 10 d (33). To evaluate the effects of antibiotics on colitis progression, mice were given water (pH ~ 3) containing ampicillin (1 mg/ml), streptomycin (5 mg/ml), and vancomycin (0.25 mg/ml) daily for 2 wk prior to DSS exposure and maintained on this regimen throughout the experimental colitis study. For cohousing experiments, age- and gender-matched WT and Nlrp1b<sup>−/−</sup> mice were weaned and housed in a 1:1 ratio for 8 wk prior to DSS exposure and maintained on this regimen throughout the experimental colitis study. For cohousing experiments, age- and gender-matched WT and Nlrp1b<sup>−/−</sup> mice were weaned and housed in a 1:1 ratio for 8 wk prior to DSS exposure. For cytokine reconstitution studies, mice were given daily i.p. injections with either 0.25 μg/g recombinant mouse IL-18 (Life Technologies) or 10 μg/g recombinant mouse IL-1β (Sigma-Aldrich). Relapsing/remitting experimental colitis was induced with three cycles of 2.5% DSS for 5 d, with 14-d intervals between each cycle (33). Mice were evaluated 10–14 d following the last cycle of DSS. To induce inflammation-driven tumorigenesis in the colitis-associated cancer model, mice were given one i.p. injection of 10 mg/kg body weight AOM (Sigma Aldrich) to the relapsing polycolitis model using experimental colitis model with 2.5% DSS (34). Mice were euthanized, and disease pathogenesis was evaluated at specific time points in each model or when moribund. Morbidity and mortality evaluations included assessments of body weight and the presence of blood around the rectum or in the stool and stool consistency. Each of these parameters were scored (scale of 0–4) and averaged to generate a cumulative semiquantitative clinical score, as described previously (4, 35). For all animals, whole blood was collected by cardiac puncture, and serum was isolated under sterile conditions. Endotoxin levels in the serum were quantified using the Limulus amebocyte lysate assay (Pierce) following the manufacturer’s instructions for serum.

**Macroscopic polypl analysis and histopathology evaluation**

Colonos were harvested from the cecum to the rectum and flushed with 1× PBS containing penicillin/streptomycin. Each colon was opened longitudinally, and macroscopic polyps were identified under ×10 magnification. Size was calculated by taking multiple measurements across the maximum diameter of each macroscopic polyp (4, 35). The colon sections were subsequently fixed and embedded in 5% buffered formalin for paraffin embedding. Paraffin-embedded tissues were sectioned at 5 μm and H&E stained. H&E-stained sections were evaluated by an experienced investigator (I.C.A.) and/or a board certified veterinary pathologist (T.E.C. or S.L.C.-O.), who was blinded to genotype and treatment. Each section was scored (scale of 0–4) for inflammation, epithelial defects, crypt atrophy, hyperplasia, dysplasia/ neoplasia, and area affected by disease, as detailed previously (4, 35, 36). The score for each parameter in the distal and mid-colon was summed to generate the histological activity index (HAI) score. Goblet cell hyperplasia and mucus in the colon was evaluated on 5-μm sectioned paraffin embedded colon tissues, which were stained using the Alcian blue/periodic acid–Schiff (AB/PAS) reaction (37). Briefly, 2-μm sections of the colon located ~1 cm proximal from the beginning of the tail region were identified and digitally imaged in an effort to consistently observe similar regions across all colon samples and experiments. The length and area of the AB/PAS-stained regions of the epithelium were assessed using ImageJ software (National Institutes of Health, Springfield, VA), and data are expressed as the mean volume density (Vs) (38). Cell death was evaluated on colon tissues using TUNEL staining (EMD Millipore). Computer-assisted image analysis with ImageJ software was also used to evaluate TUNEL-positive cells in each colon section and reported as pixels per field of view under ×20 magnification. Three sections per mouse were evaluated for both TUNEL and AB/PAS assessments.

**Colon organ culture and cytokine assessments**

Colon cytokine levels were determined using an organ culture system, as described previously (4, 35, 39). Briefly, bisection colon was cut into 1-cm sections and washed with 1× PBS containing penicillin/streptomycin. Each section was weighed and incubated overnight in 500 μl of RPMI 1640 medium at 37°C in an atmosphere containing 5% CO₂. The medium contained penicillin/streptomycin but no additional supplements. Cell-free supernatants were collected following centrifugation, and cytokine levels were evaluated by ELISA.

**Generation of chimeric mice by bone marrow reconstitution**

Chimeric mice were generated by bone marrow transplantation following standard protocols (40). Recipient mice were lethally irradiated with 1100 rad (equivalent) using two equal doses of x-ray irradiation (Rad Source Technologies) with a 6-h interval, and 12 h later, the mice received 5 × 10<sup>6</sup> bone marrow cells from the femurs of WT and Nlrp1b<sup>−/−</sup> mice. Reciprocal bone marrow reconstitution was performed, which resulted in the generation of the following four groups of experimental mice: WT→WT, Nlrp1b<sup>−/−</sup>→Nlrp1b<sup>−/−</sup>, Nlrp1b<sup>−/−</sup>→WT, and WT→Nlrp1b<sup>−/−</sup>. Surviving mice were subjected to the AOM/DSS model, 6 wk after reconstitution.

**Human metadata analysis**

Human NLRP1 and mouse Nlrp1 expression was evaluated using a publicly accessible microarray meta-analysis search engine (http://www.nextbio.com/b/nextbioCorp.nb), as described previously (41). The following array data series were analyzed to generate the human patient data: colon cancer—GSE10972, GSE31279, GSE33126, GSE21815, GSE41328, GSE37364, GSE4107, and GSE35279; and colitis—GSE11223, GSE13367, and GSE6731.

**Statistical analysis**

We used GraphPad Prism 5 Statistical software to conduct ANOVA followed by either Tukey–Kramer honest significant difference or Newman–Keuls posttest to evaluate statistical significance for multiple comparisons. Single data point comparisons were evaluated by the Student two-tailed t test. Growth of the relapse was assessed using the Kaplan–Meier test. All data are presented as the mean ± the SEM, and in all cases, a p value < 0.05 was considered statistically significant.

**Results**

NLRP1 attenuates acute gastrointestinal inflammation during experimentally induced colitis

Previous studies have shown that the NLRP3, NLRP6, and NLRC4 inflammasomes significantly contribute to immune system ho-
meostasis during IBD. The current paradigm suggests that each inflammasome forming NLR functions to attenuate IBD pathogenesis through context-specific and nonredundant mechanisms. Thus, we initially sought to evaluate the gene expression of all of the currently identified inflammasome forming NLRs during IBD using a retrospective metadata analysis of publicly available gene expression data. During this screen, we discovered that NLRP1 expression was significantly altered in several datasets associated with IBD. Data from three independent studies revealed that NLRP1 was significantly increased in colon biopsies from patients with active ulcerative colitis compared with biopsies collected from healthy donors (Fig. 1A). These data suggest that NLRP1 is either directly or indirectly induced by intestinal inflammation.

We next sought to functionally evaluate NLRP1 using Nlrp1b−/− mice (25). These animals were exposed to DSS, which is a common model of ulcerative colitis and previously used to evaluate other NLR inflammasomes in similar studies. Mice were exposed to acute DSS (5%) for 4–5 d, and morbidity and mortality were evaluated for up to 10 d. Nlrp1b−/− mice were found to be hypersensitive to the DSS administration and demonstrated a significant decrease in survival, with over half of the animals requiring euthanasia by day 8 (Fig. 1B). In the DSS model, weight loss is typically considered to be a surrogate measurement of disease progression, including increased weight loss, stool consistency, and bleeding, compared with the WT animals (Fig. 1C). Hallmark clinical parameters associated with disease progression were also evaluated and scored. The clinical scores for mock-treated Nlrp1b−/− mice tended to skew higher than the scores for the mock-treated WT animals (Fig. 1D). In this model, the average daily clinical score typically ranges from 0.0 to 0.75; thus, the animals are still considered to be within normal limits of the assessment but trend higher compared with the WT animals (Fig. 1D). DSS administration induced a significant increase in clinical features associated with disease in WT and Nlrp1b−/− mice following DSS administration; however, disease progression was significantly greater in the Nlrp1b−/− animals (Fig. 1D). Taken together, these data indicate that NLRP1 plays a protective role during experimental colitis in mice.

Previous studies have revealed that components of the host microbiota significantly contribute to disease pathology. For example, the commensal microbiota composition in the Nlrp6−/− mice was found to be significantly altered compared with WT animals, and this distorted microbiota was suggested to be directly associated with disease pathogenesis in IBD (6, 14). NLRP1 is one of the few NLRs functionally shown to detect both prokaryotic and eukaryotic MAMPs (26, 31). Thus, to broadly evaluate the contribution of the bacterial components of the host microbiota to disease pathogenesis in the Nlrp1b−/− mice, animals were treated daily with a broad spectrum antibiotic mixture prior to and throughout exposure to DSS. Antibiotic ablation significantly reversed the sensitivity of both WT and Nlrp1b−/− mice to DSS (Fig. 1E, Supplemental Fig. 1A, 1B). Although the antibiotic ablation studies are broad, these data indicate a robust contribution of the bacterial components of the host microbiota in driving experimental colitis pathogenesis in the Nlrp1b−/− mice.

**FIGURE 1.** NLRP1 dysregulation is associated with ulcerative colitis. (A) Retrospective analysis of metadata from colonic biopsies collected from ulcerative colitis (UC) patients revealed that NLRP1 expression was significantly upregulated. The fold-change values were averaged from three separate datasets and reflect the change in the expression between the affected tissues of UC patients with active disease compared with healthy controls. *p < 0.01. (B) Kaplan–Meier plot of Nlrp1b−/− and WT mouse survival. Nlrp1b−/− animals were euthanized on day 8 because of increased weight loss and clinical parameters associated with disease progression. *p < 0.01, †p < 0.05. (C) Nlrp1b−/− mice exhibited significant weight loss following DSS exposure compared with the WT animals. *p < 0.05, †p < 0.01. (D) Nlrp1b−/− mice exhibit enhanced clinical parameters associated with disease progression, including increased weight loss, stool consistency, and bleeding, compared with the WT animals. *p < 0.05, †p < 0.01. (E) Nlrp1b−/− mice and WT animals were treated with an antibiotic mixture throughout the duration of DSS exposure (2.5% DSS) and disease progression was evaluated. *p < 0.01, †p < 0.05, ‡p < 0.01. WT Mock, n = 3; WT DSS, n = 5; Nlrp1b−/− Mock, n = 5; Nlrp1b−/− DSS, n = 6. (F) Nlrp1b−/− mice and WT animals were treated with an antibiotic mixture plus Ab, n = 15, Nlrp1b−/− DSS, n = 5; Nlrp1b−/− DSS+Ab, n = 15. (G) Nlrp1b−/− mice and WT animals were weaned and cohoused with WT [Nlrp1b−/− (WT)], n = 10; Nlrp1b−/− mice cohoused with WT [Nlrp1b−/− (WT)], n = 10. Data are representative of greater than three independent experiments. *p < 0.01, †p < 0.05, ‡p < 0.01.
Prior studies have shown that DSS sensitivity is transmissible from \( \text{Asc}^{-/-} \) and \( \text{Nlrp6}^{-/-} \) animals to WT mice due to significant alterations in the composition of the fecal microbiota (16, 42). To further evaluate the contribution of the microbiota and transmissibility of DSS hypersensitivity, we weaned and cohoused \( \text{Nlrp1b}^{-/-} \) mice with age-matched WT animals for 8 wk prior to DSS exposure. Similar to the previous findings reported for \( \text{Asc}^{-/-} \) and \( \text{Nlrp6}^{-/-} \) mice, cohousing WT animals with \( \text{Nlrp1b}^{-/-} \) mice resulted in the development of severe experimental colitis pathogenesis in the WT mice. WT animals demonstrated a significant increase in weight loss and morbidity, which was comparable to the levels observed in the \( \text{Nlrp1b}^{-/-} \) mice (Fig. 1F). Taken together, the findings from the antibiotic ablation and cohousing studies suggest that the phenotype associated with the \( \text{Nlrp1b}^{-/-} \) mice is transmissible and further implicates alterations of the host microbiota in facilitating DSS hypersensitivity in these animals.

Acute distal colon inflammation and damage to the epithelial cell barrier are hallmark pathological characteristics associated with the experimental colitis model. All of the DSS treated mice demonstrated increases in both of these parameters (Fig. 2). However, \( \text{Nlrp1b}^{-/-} \) mice developed histological features associated with disease progression that were increased compared with the WT animals (Fig. 2A). Previous studies have shown that mice lacking the inflammasome adaptor protein ASC develop severe colitis, which is significantly increased compared with \( \text{Nlrp3}^{-/-} \), \( \text{Nlrc4}^{-/-} \), and \( \text{Nlrp6}^{-/-} \) mice. Consistent with these previous findings, \( \text{Asc}^{-/-} \) mice used in the current study were more hypersensitive to DSS compared with the \( \text{Nlrp1b}^{-/-} \) mice and presented with augmented histopathological features associated with disease progression (Fig. 2A). In the experimental colitis model, the HAI score provides a semiquantitative assessment of colon histopathology (36). All of the mice exposed to DSS demonstrated a significant increase in HAI compared with the mock-treated animals. However, HAI assessments revealed that the \( \text{Nlrp1b}^{-/-} \) mice developed intermediate disease that was significantly increased compared with the WT animals and significantly attenuated compared with the \( \text{Asc}^{-/-} \) mice (Fig. 2B). Further evaluation of the components of the HAI score revealed that distal colon inflammation and epithelial cell defects were the greatest contributors to the increased histopathology observed in the \( \text{Nlrp1b}^{-/-} \) animals, whereas all of the individual scores in the mid and distal colon were significantly increased in the more severe \( \text{Asc}^{-/-} \) mice (Fig. 2B, Supplemental Fig. 2A–F). In addition to histopathology assessments, we also evaluated the level of serum endotoxin following DSS exposure. Previous studies have correlated the level of serum endotoxin with epithelial barrier dysfunction in the experimental colitis model (43). Following DSS administration, serum endotoxin levels were significantly increased in all mice following DSS administration (Supplemental Fig. 2G). Consistent with the increased epithelial barrier damage in the \( \text{Asc}^{-/-} \) and \( \text{Nlrp1b}^{-/-} \) animals, we routinely observed increased levels of serum endotoxin in these mice compared with WT animals; however, these data did not reach statistical significance due to high variability in the inflammasome-deficient mice (Supplemental Fig. 2G). Taken together, these data identify the NLRP1 inflammasome as an essential mediator of inflammation in the gut and significantly contributes to mucosal immune system homeostasis during colitis.

Colitis sensitivity in the \( \text{Nlrp1b}^{-/-} \) mice is correlated with IL-1\( \beta \) and IL-18 attenuation

NLR inflammasome formation results in the cleavage of pro–IL-1\( \beta \) and pro–IL-18, which results in the maturation of these cytokines into their bioactive states. Prior studies evaluating ASC and Caspase-1 in similar models of ulcerative colitis have revealed that the levels of these cytokines are commonly found to be ablated in \( \text{Asc}^{-/-} \) and \( \text{Casp1}^{-/-}/\text{Il1}^{-/-} \) mice (4–6, 8). Likewise, IL-1\( \beta \) and IL-18 levels are also attenuated in the absence of NLRP3, NLRC4, and NLRP6 in the DSS model (4, 8–9). To evaluate local cytokine levels in the colon, we generated organ cultures following necropsy (4, 35). Following overnight incubation, the colon supernatants were collected, and protein levels were assessed by ELISA. DSS treatment increased IL-1\( \beta \), IL-18, and IL-6 in all of the animals compared with levels observed in the mock-treated mice (Fig. 3A–C). However, IL-1\( \beta \) and IL-18 levels in the supernatants collected from the \( \text{Nlrp1b}^{-/-} \) animals were significantly reduced compared with the WT mice (Fig. 3A, 3B). The levels of IL-6 between the DSS-treated WT and \( \text{Nlrp1b}^{-/-} \) animals were not significantly altered (Fig. 3C).

The contribution of IL-1\( \beta \) and IL-18 during IBD is currently unclear and in many cases contradictory. Previous studies have shown that attenuated levels of both of these cytokines are typically observed in the absence of NLR inflammasomes and are correlated with enhanced disease pathogenesis in the experimental colitis and colitis-associated tumorigenesis models (4–6, 8, 16). To directly evaluate the contribution of each of these cytokines, \( \text{Nlrp1b}^{-/-} \) mice that received IL-18 demonstrated significantly reduced weight loss and attenuated disease pathogenesis compared with the saline-treated animals (Fig. 3D, 3E). Likewise, we also observed a significant attenuation in disease pathogenesis in the \( \text{Nlrp1b}^{-/-} \) mice that were treated with IL-1\( \beta \) (Fig. 3D, 3E). Previous studies have shown that attenuated levels of IL-18 are associated with diminished epithelial barrier function, which increases colitis severity in other NLR inflammasome-deficient mouse strains (13). Although these data are consistent with these prior studies, our findings also suggest that IL-1\( \beta \) plays an important role in attenuating disease pathogenesis in the \( \text{Nlrp1b}^{-/-} \) mice.

NLRP1 contributes to attenuation of tumorigenesis during colorectal carcinoma and colitis-associated tumorigenesis

We next sought to evaluate the contribution of NLRP1 in the development of intestinal malignancies and colitis associated colorectal carcinoma. To evaluate the possibility that NLRP1 may contribute to colorectal cancer pathogenesis, we conducted a retrospective evaluation of publically available gene expression metadata compiled from eight independent studies that evaluated colon biopsies from areas of cancer versus adjacent tissue or biopsies/tissue from colon cancer patients compared with healthy controls (Fig. 4A). NLR gene expression from each study was averaged and revealed that NLRP1 was significantly downregulated in all of the evaluated datasets (Fig. 4A). In addition to NLRP1, we also evaluated the other three NLRs previously reported to modulate tumorigenesis in the colon. Although none of the NLRs were dysregulated in studies comparing colon cancer to adjacent tissue, NLRP3 and NLRC4 were found to be significantly upregulated in cancer biopsies compared with healthy or normal patients, whereas NLRP6 expression was unchanged in all studies (Fig. 4A). Taken together, these data indicate that NLRP1 is downregulated in the context of tumorigenesis in human patient populations, whereas NLRP3 and NLRC4 are directly induced or upregulated, likely as part of a feedback response during disease progression. Likewise, these data underscore the value of additional mechanistic studies to better determine the function of these NLRs in patient populations.

To gain additional insight into the role of the NLRP1 inflammasome in the development and progression of colitis-associated colon cancer, we used the AOM/DSS model of inflammation-driven colon tumorigenesis. AOM is a mutagen that
exerts mild colonotrophic carcinogenicity when administered by itself; however, this process is greatly enhanced by combining with DSS in a model of relapsing remitting colitis. The Nlrp1b−/− mice have been thoroughly evaluated by our laboratory and others, and no overt spontaneous inflammation or cancer phenotypes have been observed. Thus, to induce tumorigenesis in these animals, the Nlrp1b−/− mice were treated with a single dose of AOM, followed by three rounds of 2.5% DSS over the course of 2 mo. By day 60, 16% of the Nlrp1b−/− mice had progressed to the point of re- quiring euthanasia, which was statistically significant compared with 3% of the WT animals (Fig. 4B). Further assessments of weight change throughout the course of the colitis-driven tumo- rigenesis model revealed that the Nlrp1b−/− mice are hypersensi- tive to the lower dosages of DSS and show significant weight loss during the early stages of the model compared with the WT animals (Fig. 4C, Supplemental Fig. 3A). Following each DSS ad- ministration, the mice recover; however, the recovery never fully reaches the levels observed for the WT animals until the final DSS administration (Fig. 4C). The Nlrp1b−/− mice were also found to be hypersensitive to DSS administration alone; however, the weight changes were not as robust compared with the AOM/DSS- treated animals (Supplemental Fig. 3A). No significant differences in disease progression were noted between the WT and Nlrp1b−/− AOM-only or mock-treated animals (Supplemental Fig. 3A, 3B). These clinical findings suggest that NLRP1 is necessary for allevi- ating mouse morbidity and mortality during inflammation- driven colon tumorigenesis. Furthermore, when combined with the findings that NLRP1 is downregulated in human patients during colon cancer, these data suggest that NLRP1 functions as a critical modulator of colon homeostasis during tumorigenesis.

Prior studies have revealed NLR inflammasome activity attenuates colitis-associated cancer. Specifically, Asc−/− and Casp1−/−/Ili−/− mice develop severe gastrointestinal inflammation and colon tumorigenesis in AOM/DSS models (4, 5, 8). Because ASC and Caspase-1 are shared components of all NLR inflammasomes, we sought to specifically assess the contribution of NLRP1 in the CAC model. Upon completion of the AOM/DSS challenge (day 64), colons were removed and macroscopic polyps were evaluated. Polyps were detected in all of the AOM/DSS- treated mice (Fig. 5A). However, we observed a significant increase in the average number of polyps in the Nlrp1b−/− (2.57 ± 0.75) and Asc−/− animals (4.25 ± 1.33) compared with the WT mice (0.75 ± 0.23) (Fig. 5A). In addition to being more numerous, the polyps in the Nlrp1b−/− (10.60 ± 1.78 mm²) and Asc−/− mice (7.97 ± 1.46 mm²) were also significantly larger than those observed in the WT animals (2.34 ± 0.44 mm²) (Fig. 5B). Histopathology assessments revealed increased inflammation, hyperplasia, and dysplasia in the Nlrp1b−/− and Asc−/− mice (Fig. 5C). HAI assessments revealed a significant increase in colon histopathology in all of the DSS- and AOM/DSS-treated animals, compared with the mock- and AOM-only treated mice (Fig. 5D). However, consistent with the increase in macroscopic polyps, AOM/DSS-treated Nlrp1b−/− and Asc−/− mice had significantly greater HAI scores compared with WT animals (Fig. 5D). Further assessments of the parameters that compose the HAI score revealed significant increases in colon hyperplasia, and dysplasia was a major contrib- utor to the higher composite scores observed in these mice (Fig. 5E, 5F, Supplemental Fig. 3C–J). Typically, AOM/DSS administration results in disease pathogenesis that is restrained to the distal colon. However, in both Nlrp1b−/− and Asc−/− animals, extensive histopathology was observed to extend into the mid and proximal colon, which was also a contributing factor associated with the higher composite scores (Supplemental Fig. 3C–J). In addition to histopa- thological assessments of inflammation and tumorigenesis, we
also conducted basic assessments of epithelial cell death in the 
Nlrp1b−/− mice. In this study, we used TUNEL staining of colon sections following relapsing remitting experimental colitis and colitis-associated tumorigenesis. TUNEL-stained colon sections were imaged and digitally analyzed to generate a semiquantitative assessment of epithelial cell death. Following DSS exposure, we observed significantly increased TUNEL-positive cells in the Nlrp1b−/− mice compared with WT animals (Fig. 5G). Likewise, Nlrp1b−/− mice also demonstrated increased cell death in the context of colitis-associated tumorigenesis compared with the WT animals (Fig. 5G). Consistent with the increased cell death and epithelial barrier dysfunction, we also observed increased levels of endotoxin in serum collected from Nlrp1b−/− animals compared with WT mice (Supplemental Fig. 3K). Serum endotoxin levels were routinely higher in the Nlrp1b−/− and Asc−/− mice compared with WT but did not reach statistical significance because of high but variable levels detected in the Nlrp1b−/− and Asc−/− animals (Supplemental Fig. 3K).

Enhanced tumorigenesis in the Nlrp1b−/− mice is correlated with attenuated levels of IL-1β and IL-18

Previous studies evaluating NLR inflammasome signaling during colitis-associated cancer revealed that both relapsing remitting colitis progression and colitis-associated tumorigenesis were correlated with attenuation of IL-1β and IL-18. Indeed, similar to the findings reported in the experimental colitis models, Asc−/− and Casp1−/−/Il-11−/− mice typically demonstrate ablation of these cytokines (4, 5, 8, 14). To assess this mechanism in the Nlrp1b−/− mice, we evaluated these and other inflammatory mediators in the organ culture supernatants following completion of the CAC model. We observed a significant increase in all of the cytokines evaluated in colons collected from the WT mice compared with mock-treated animals (Fig. 6A–D). However, IL-1β and IL-18 levels were significantly attenuated in the Nlrp1b−/− mice compared with the WT animals (Fig. 6A, 6B). In addition to IL-1β and IL-18, IL-6 levels also have been routinely reported to be increased in NLR inflammasome–deficient mice (4). Consistent with these previous observations, IL-6 was significantly increased in the Nlrp1b−/− and Asc−/− colons compared with WT (Fig. 6C). The increased IL-6 is presumably associated with the heightened disease state in these animals, rather than directly associated with NLR inflammasome function. The immunomodulatory cytokine IL-10 is an essential regulator of gastrointestinal inflammation in the gut, and IL-10−/− mice develop spontaneous colitis and are prone to developing adenocarcinoma. Thus, we also evaluated IL-10 levels and found that this cytokine was significantly increased in all of the animal groups,
with no differences between genotypes (Fig. 6D). Taken together, these data support our hypothesis that the increased colitis-associated tumorigenesis in the \textit{Nlrp1b}^{2/-} mice is associated with attenuated levels of IL-1\(\beta\) and IL-18 in the colon.

In addition to modulating IL-1\(\beta\) and IL-18, recent studies evaluating the mechanism associated with NLRP6 inflammasome attenuation of colitis-associated tumorigenesis have shown an increased correlation between disease severity and attenuated mucus production in the \textit{Nlrp6}^{2/-} and \textit{Asc}^{2/-} mice (14, 42). To assess this mechanism in the context of NLRP1 inflammasome deficiency, we evaluated goblet cell hyperplasia in the colon using AB/PAS staining (Fig. 6E). All of the mice exposed to AOM/DSS demonstrated increased goblet cell hyperplasia compared with the mock-treated animals, with no differences observed among WT, \textit{Asc}^{2/-}, and \textit{Nlrp1b}^{2/-} mice (Fig. 6E). To better characterize these observations, we used a semiquantitative scoring system, previously proven to be effective in evaluating goblet cell hyperplasia and mucus production in the lungs, to quantify mucus production in the colon (37, 38). Consistent with the histopathology assessments, all of the AOM/DSS-treated mice demonstrated significantly increased goblet cell hyperplasia compared with the mock-treated animals, with no significant differences observed between genotypes (Fig. 6F). Thus, NLRP1 does not appear to affect mucus production in the gut during the inflammation-driven tumorigenesis model and appears to function through a mechanism with characteristics that are distinct from those described for NLRP6.

**FIGURE 4.** NLRP1 attenuates the progression of colitis associated tumorigenesis. (A) Retrospective analysis of metadata from colonic biopsies collected from colorectal carcinoma patients. Expression of NLRP1 and other NLRs previously associated with cancer were evaluated. Expression of each NLR was assessed from biopsies collected from polyps or characterized adenocarcinomas and compared with either adjacent polyp free areas of the colon or from biopsies collected from healthy control subjects as indicated. The fold-change values were averaged from five separate datasets. *\(p<0.001\), †\(p<0.01\). (B and C) \textit{Nlrp1b}^{2/-} mice were hypersensitive to the AOM/DSS inflammation–driven colon tumorigenesis model. (B) Kaplan–Meier plot of \textit{Nlrp1b}^{2/-} and WT mouse survival. *\(p<0.05\), †\(p<0.05\). (C) \textit{Nlrp1b}^{2/-} mice demonstrated significant weight loss throughout the majority of the AOM/DSS model compared with WT animals. WT Mock, \(n=3\); WT AOM, \(n=3\); WT DSS, \(n=5\); WT AOM/DSS, \(n=12\); \textit{Nlrp1b}^{2/-} Mock, \(n=3\); \textit{Nlrp1b}^{2/-} AOM, \(n=3\); \textit{Nlrp1b}^{2/-} DSS, \(n=6\); \textit{Nlrp1b}^{2/-} AOM/DSS, \(n=9\). Data are representative of three independent experiments. *\(p<0.05\).
FIGURE 5. The NLRP1 inflammasome attenuates tumorigenesis during colitis associated cancer. (A) The number of macroscopic polyps were determined in Nlrp1b−/−, Asc−/− and WT colons upon necropsy following the completion of the CAC model. *p < 0.05, †p < 0.05. (B) The maximal cross-sectional area of macroscopic polyps was determined for each genotype. The percentage of polyps per size range is shown in the table *p < 0.01, †p < 0.01. (C) Representative histopathology illustrating increased inflammation, hyperplasia, and dysplasia in the Nlrp1b−/− and Asc−/− mice compared with the WT animals. Areas of neoplasia in the Nlrp1b−/− andAsc−/− colons are denoted with an asterisk. Scale bar, 250 μm. (D) The composite HAI score was calculated for each set of experimental groups evaluated upon completion of the CAC model. *p < 0.05, †p < 0.05, ‡p < 0.05. (E and F) Histopathology analysis revealed increased colon hyperplasia and dysplasia in all of the animals treated with AOM/DSS. However, a significant increase was observed in both parameters in colons evaluated from the Nlrp1b−/− and Asc−/− mice. *p < 0.05, †p < 0.05. (G) TUNEL staining of histopathology sections revealed a significant increase in TUNEL-positive cells following either DSS or AOM/DSS exposure. Computer-assisted image analysis revealed a significant increase in TUNEL-positive cells in Nlrp1b−/− mice in both models compared with WT animals. WT Mock, n = 3; WT AOM, n = 3; WT DSS, n = 5; WT AOM/DSS, n = 12; Nlrp1b−/− Mock, n = 3; Nlrp1b−/− AOM, n = 3; Nlrp1b−/− DSS, n = 6; Nlrp1b−/− AOM/DSS, n = 9; Asc−/− mock, n = 3; Asc−/− AOM/DSS, n = 8. All studies were evaluated on day 64. Data are representative of three independent experiments. *p < 0.05, †p < 0.05, ‡p < 0.01, †p < 0.01, †p < 0.01.

Following 6 wk of reconstitution, near-complete chimerism was achieved, and the animals were evaluated in the AOM/DSS model. It should be noted that all of the chimera studies were conducted on female mice. Because of the long-term nature of these studies, female mice tend to be less aggressive; however, in our hands, they also have attenuated responses in the AOM/DSS model. All of the animals experienced weight loss following the first DSS administration. However, all of the Nlrp1b−/− chimeric mice failed to gain significant weight compared with the 16.63 ± 2.04% gain observed in the WT→WT mice (Fig. 7B). As the model progressed, we observed increased clinical features associated with disease pathogenesis in the WT→Nlrp1b−/− and Nlrp1b−/−→Nlrp1b−/− animals. These two groups of mice demonstrated significant increases in their clinical scores compared with the WT→WT and Nlrp1b−/−→WT mice (Fig. 7C). Mice were necropsied on day 61, and the colon length was evaluated. Colon length is a surrogate evaluation for disease pathogenesis in the AOM/DSS model. Consistent with the clinical score findings, we observed a significant decrease in colon length in the Nlrp1b−/−→Nlrp1b−/− animals and an intermediate difference in colon length for the WT→Nlrp1b−/− mice (Fig. 7D). Evaluation of macroscopic polyps revealed a significant increase in the number of polyps observed in the Nlrp1b−/−→Nlrp1b−/− and WT→Nlrp1b−/− mice, with no polyps detected in the WT→WT or Nlrp1b−/−→WT animals (Fig. 7E). Similar to our previous observations in the nonchimeric animals, many of these polyps were detected in the proximal colon as opposed to the distal regions. Further evaluation of histopathology revealed that WT→Nlrp1b−/− and Nlrp1b−/−→Nlrp1b−/− mice had significantly increased HAI scores compared with the WT→WT mice (Fig. 7F). Although, no significant difference was observed between the WT→WT and Nlrp1b−/−→WT animals (Fig. 7F). Further evaluation of the components of the HAI score revealed that increased distal and proximal inflammation and hyperplasia were the most significant contributors to the higher HAI scores observed in these chimeric mice (Fig. 7G, 7H). Taken together, these data suggest that
NLRP1 functions through the nonhematopoietic compartment, likely intestinal epithelial cells, to attenuate inflammation driven tumorigenesis.

**Discussion**

NLRs function as sentinel PRRs that sense components of the microbiota and maintain immune system homeostasis in the gut. In human populations, several SNPs in NLR genes, including NOD2 and NLRP3, have been associated with genetic predispositions to IBD. However, the spatial and temporal specificities and mechanisms associated with immunoregulation by the NLRs are not adequately characterized in either human or rodent models of disease. In the retrospective metadata analysis shown in this paper, it is clear that NLRP1 is significantly dysregulated in the context of IBD and colon cancer (Figs. 1A, 4A). In this paper, we expand on these data and show that NLRP1 plays a protective role during experimental colitis and colitis-associated tumorigenesis in the mouse.

The adaptor protein ASC and Caspase-1 exert robust protective effects on IBD and cancer pathogenesis. In the context of inflammasome formation, these proteins interact with a wide range of NLRs. There are eight NLR and NLR-like proteins that have been strongly characterized as being capable of forming an inflammasome following activation, including NLRP3, NLRC4, and NLRP6. All three of these inflammasome forming NLRs have been previously shown to attenuate the progression of IBD and colitis associated cancer (4, 6, 8, 44). In general, the phenotypes observed in the $Nlrp1b^{−/−}$ mice are similar to those previously reported for the other NLR-deficient mouse lines, including those observed for $Nlrp6^{−/−}$ animals in these models (4, 6, 8, 16). However, the polyps and tumors in the $Nlrp1b^{−/−}$ mice occur much more proximal than those observed in the $Nlrp3^{−/−}$ animals and are much larger in size (Fig. 5) (4). Likewise, the phenotypes observed in the $Nlrp1b^{−/−}$ mice are also associated with the nonhematopoietic compartment, whereas the phenotype in the $Nlrp3^{−/−}$ animals

**FIGURE 6.** The NLRP1 inflammasome modulates IL-1β and IL-18 levels during colitis-associated tumorigenesis. (A and B) Colon IL-1β and IL-18 levels were significantly reduced in the $Nlrp1b^{−/−}$ and $Asc^{−/−}$ mice compared with the WT animals. $p < 0.05$, $p < 0.05$, $p < 0.01$. (C) IL-6 levels were increased in the colons harvested from all animals treated with AOM/DSS and were significantly increased in the colons collected from the $Nlrp1b^{−/−}$ and $Asc^{−/−}$ animals compared with the WT tissues. $p < 0.05$, $p < 0.05$, $p < 0.05$. (D) IL-10 was significantly upregulated in the colon tissue following induction of colitis-associated tumorigenesis; however, no significant differences were observed between genotypes. $p < 0.01$, $p < 0.01$, $p < 0.05$. (E) Mucus production in the colon was evaluated by AB/PAS staining. Representative histopathology illustrating increased goblet cell hyperplasia (AB/PAS staining) and mucus production in all of the AOM/DSS-treated animals. Scale bar, 250 μm. (F) AB/PAS staining was quantified using imaging analysis software (ImageJ) along a 2-mm section of the colon, located ~1 cm proximal from the termination of the rectum. Data are represented as Vs. WT Mock, $n = 3$; WT AOM/DSS, $n = 12$; $Nlrp1b^{−/−}$ Mock, $n = 3$ (data not shown); $Nlrp1b^{−/−}$ AOM/DSS, $n = 9$; $Asc^{−/−}$ Mock, $n = 3$ (data not shown); $Asc^{−/−}$ AOM/DSS, $n = 8$. Data shown were generated from colon sections collected from mice on day 64 and are representative of five independent experiments. $p < 0.05$, $p < 0.05$, $p < 0.05$. The Journal of Immunology 3377

NLRP1 functions through the nonhematopoietic compartment, likely intestinal epithelial cells, to attenuate inflammation driven tumorigenesis.
have been previously associated with the hematopoietic compartment when assessed under similar conditions (Fig. 7) (4). Although several studies have suggested that the epithelial cell compartment may be a source of IL-1β and IL-18, our data may also reflect other potential biological functions of NLRP1 in nonhematopoietic-derived cells that may include modulation of reactive oxygen species production, cell death, or autophagy. Taken together, these data are consistent with the hypothesis that multiple NLRs function to initiate inflammasome formation and maintain mucosal immune system homeostasis though cell type, temporal, and stimuli specific mechanisms that are similar but nonredundant.

Similar to the findings from other NLR inflammasome studies, IL-1β and IL-18 levels in the Nlpr1b−/− colons were significantly decreased. Likewise, our reconstitution studies indicated that both of these cytokines play a protective role in the absence of NLRP1. IL-1β and IL-18 are highly related proinflammatory cytokines that are strongly associated with IBD and cancer pathogenesis. Indeed, high levels of IL-1β are often observed in patients with active IBD, where this cytokine functions as a potent stimulator of macrophages, dendritic cells, neutrophils, and epithelial cells (45–47). Also, in the context of IBD, IL-1β regulates leukocyte migration into the lamina propria of the intestine and strongly promotes T cell activation, survival, and differentiation (48–52). However, although the role of IL-1β is well characterized in IBD and tumorigenesis, IL-18 has only recently emerged as a primary modulator of mucosal immune system homeostasis. Similar to IL-1β, IL-18 has strong proinflammatory activities and is essential for the proper regulation of CD4+ T cell function and Th1 cell differentiation (53). Likewise, IL-18 levels are commonly dysregulated in human IBD patients and mutations in the IL-18 gene are associated with ulcerative colitis (54, 55). In mice, both IL-18

FIGURE 7. NLRP1 attenuates colitis-associated tumorigenesis through nonhematopoietic-derived cells. (A) Schematic of bone marrow chimera mice. (B) Weight change of each chimeric mouse group revealed that all of the chimeric mice demonstrated a significant reduction in weight loss compared with the WT→WT mice. Nlpr1b−/−→Nlpr1b−/− (P1→P1), *p < 0.05, Nlpr1b−/−→WT (P1→WT), †p < 0.05, WT→Nlpr1b−/− (WT→P1), ‡p < 0.05. (C) Scoring associated with clinical disease parameters revealed that WT→Nlpr1b−/− mice phenocopied the Nlpr1b−/−→Nlpr1b−/− animals, whereas no difference was observed between the Nlpr1b−/−→WT and WT→WT animals. *p < 0.01, †p < 0.01, ‡p < 0.01. (D) Colons from the Nlpr1b−/−→Nlpr1b−/− animals were significantly truncated compared with the Nlpr1b−/−→WT and WT→WT animals, whereas the colons from the WT→Nlpr1b−/− mice were an intermediate length. *p < 0.01, †p < 0.01. (E) The number of macroscopic polyposis was determined in colons from the chimeric mice upon necropsy following the completion of the CAC model. *p < 0.01, †p < 0.01, ‡p < 0.01. (F) The composite HAI score was calculated for each set of experimental groups evaluated upon completion of the CAC model. *p < 0.05, †p < 0.05. (G and H) Histopathology analysis revealed increased inflammation (G) and hyperplasia (H) in both the proximal and distal colon in the WT→Nlpr1b−/− and Nlpr1b−/−→Nlpr1b−/− animals compared with the WT→WT mice. WT→WT, n = 4; WT→Nlpr1b−/−, n = 9; Nlpr1b−/−→WT, n = 4; Nlpr1b−/−→Nlpr1b−/−, n = 6. Female mice were used in the chimera studies. Data shown are representative of two independent experiments.
overexpression and attenuation have been demonstrated to increase pathogenesis in IBD models, which suggest that this cytokine has multiple functions, both positive and negative, during disease progression (6, 56–58). This dichotomy between IL-1β and IL-18 in IBD and cancer models can be reconciled by recent findings, which revealed that both IL-1β and IL-18 play essential roles in maintaining the integrity of the epithelial barrier in the colon (6, 59). Thus, the current paradigm indicates that NLR inflammasomes exert protective effects in the gut during IBD and inflammation driven tumorigenesis by either directly or indirectly maintaining the function of the epithelial barrier. The findings presented in this paper for IL-1β and IL-18 levels in the Nlrp1b−/− mice in both models of colitis and colitis-associated tumorigenesis are consistent with this proposed model.

Taken together, our data strengthen the hypothesis that each NLR functions to attenuate IBD pathogenesis and cancer through context-specific and nonredundant mechanisms. For example, previous studies have indicated that NLRP3 and NLRP6 strongly influence colitis and cancer progression; however, the proposed mechanisms significantly differ. NLRP3 is responsible for sensing a wide range of MAMPs and DAMPs that are associated with damage to the colonic epithelial layer and bacteria translocation following the wound healing response that is initiated by DSS administration. Because of the wide range of stimuli and biological function associated with NLRP3 activation, it is highly probable that this NLR functions through an indirect mechanism to attenuate disease pathogenesis. For example, our previous studies have suggested that IBD and cancer sensitivity in the Nlrp3−/− mice are associated with a failure of the hematopoietic cells to properly sense and respond to epithelial cell damage in the DSS and AOM/DSS models (4). However, NLRP6 appears to function more directly through the epithelial cell compartment to modulate IBD and tumor progression (6, 14). Recent studies using Nlrp6−/− mice have revealed that these animals have a significant expansion of Bacteroidetes (Prevotellaceae) that is highly correlated with the severity of IBD pathogenesis (14). The bacteria expansion has been associated with defective goblet cell autophagy and subsequent reductions in mucin granule exocytosis in the Nlrp6−/− and Asc−/− mice. This appears to allow Prevotellaceae to colonize in the crypts of these animals and establish a persistent infection (42). It is clear that NLRP1 senses a less diverse range of signals compared with NLRP3. NLRP1 has only been shown to sense B. anthracis LeTx and T. gondii in rodents (23–28). These pathogens are not common constituents of the mouse microbiota and are unlikely to be associated with the increased sensitivity of the Nlrp1b−/− mice to the induction of colitis and tumorigenesis. Likewise, we also evaluated goblet cell hyperplasia in the Asc−/− and Nlrp1b−/− mice and did not observe significantly attenuated levels of mucus production in either mouse line. Thus, NLRP1 appears to function through a mechanism that is independent of that recently suggested for NLRP6. With the recent generation of multiple NLRP1-deficient mice, we anticipate that future studies will reveal and better characterize the mechanism associated with IBD and cancer.

During the past decade, significant progress has been made in the identification and characterization of NLR family members. It is becoming increasingly clear that NLRs are essential mediators of the balance that exists in mucosal tissues between the microbiota and the host immune response. However, the clinical relevance and mechanistic details associated with NLR modulation of immunity and therapeutic potential in IBD and cancer is still inadequately understood. Likewise, their contributions to biological functions beyond microbe recognition are relatively unknown in the context of gastrointestinal health and disease. We anticipate that additional studies will reveal and better characterize the microbial elements sensed by NLRP1 in the gut and will provide additional mechanistic insight associated with its role in attenuating disease pathobiology.

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Disclosures
The authors have no financial conflicts of interest.

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