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Peptidoglycan Recognition Protein 3 and Nod2 Synergistically Protect Mice from Dextran Sodium Sulfate–Induced Colitis

Xuefang Jing,* Fareeha Zulfiqar,* Shin Yong Park,* Gabriel Núñez,† Roman Dziarski,*, and Dipika Gupta*

Aberrant immune response and changes in the gut microflora are the main causes of inflammatory bowel disease (IBD). Peptidoglycan recognition proteins (Pglyrp1, Pglyrp2, Pglyrp3, and Pglyrp4) are bactericidal innate immunity proteins that maintain normal gut microbiome, protect against experimental colitis, and are associated with IBD in humans. Nucleotide-binding oligomerization domain 2 (Nod2) is an intracellular bacterial sensor and may be required for maintaining normal gut microbiome. Mutations in Nod2 are strongly associated with Crohn’s disease, but the causative mechanism is not understood, and the role of Nod2 in ulcerative colitis is not known. Because IBD is likely caused by variable multiple mutations in different individuals, in this study, we examined the combined role of Pglyrp3 and Nod2 in the development of experimental colitis in mice. We demonstrate that a combined deficiency of Pglyrp3 and Nod2 results in higher sensitivity to dextran sodium sulfate–induced colitis compared with a single deficiency. Pglyrp3–/–Nod2–/– mice had decreased survival and higher loss of body weight, increased intestinal bleeding, higher apoptosis of colonic mucosa, elevated expression of cytokines and chemokines, altered gut microbiome, and increased levels of ATP in the colon. Increased sensitivity to dextran sodium sulfate–induced colitis in Pglyrp3–/–Nod2–/– mice depended on increased apoptosis of intestinal epithelium, changed gut microflora, and elevated ATP. Pglyrp3 deficiency contributed colitis-predisposing intestinal microflora and increased intestinal ATP, whereas Nod2 deficiency contributed higher apoptosis and responsiveness to increased level of ATP. In summary, Pglyrp3 and Nod2 are both required for maintaining gut homeostasis and protection against colitis, but their protective mechanisms differ.

Pglyrp3 and Nod2 Protect Against Colitis

Materials and Methods

Mice

Pglyrp3+/− (22) and Nod2+/− (24, 41) mice on BALB/c background were described previously. Pglyrp3+/− mice were crossed with Nod2−/− mice to generate the homozygous double-KO Pglyrp3+/−Nod2−/− mice. Deletion of the Pglyrp3 and Nod2 genes was confirmed by PCR analysis of genomic DNA (22, 24). Pglyrp3+/−Nod2−/− mice were viable, bred normally, and produced the expected male:female ratios and similar litter sizes as the WT mice. They had similar weight as WT mice and developed normally with no obvious defects. Their major internal organs had normal macroscopic appearance and normal histological appearance on H&E-stained sections. The original colony founder WT BALB/c mice were obtained from Harlan Sprague Dawley. WT germ-free mice (Swiss Webster female) were obtained from Taconic Farms (Hudson, NY).

We crossed and KO mice were on BALB/c background, female, 8–9 wk old, bred and kept under conventional pathogen-free conditions in the same room in our facility to minimize the influence of differences in the environment. For each experiment, mice from several different cages and breeder pairs were used. We did not use WT and homozygous KO littermates from heterozygous breeding pairs for three reasons: first, this strategy cannot be used for double-KO mice; second, this strategy may skew the results to the particular microflora present only in this breeding pair; and third, the effect of Pglyrp3 on the composition of the microbiome is not instantaneous, but takes time, and stabilization of microbiome characteristic of a given mutant strain takes more than one generation. To avoid changes in microbiome that could accumulate over an extended period, we backcross our mutant mice to WT females once every other year and redrive our homozygous KO breeding pairs. The latter strategy also minimized genetic drift in the population. The BALB/c background of KO mice and their negative status for all common viral and bacterial pathogens and parasites (including negative PCR stool tests for mouse norovirus) were confirmed as previously described (24). The Indiana University School of Medicine–Northwestern Institutional Animal Care and Use Committee approved all experiments with mice.

Colitis model in conventional and germ-free mice

Experimental colitis was induced in WT, Nod2−/−, Pglyrp3+/−, and Pglyrp3+/−Nod2−/− mice with 5% DSS (MP Biomedical) in drinking water (47). DSS-induced intestinal inflammation is a well-established animal model for colitis and its manifestations include bloody diarrhea, weight loss, shortening of the colon, mucosal ulceration, and epithelial dysplasia. Manifestations such as predominant left-sided colitis, epithelial dysplasia, and lack of granulomas are similar with UC; however, the complexity of the human disease is not completely reproduced in the DSS model (47). The development and severity of colitis were evaluated as previously described (22), using: 1) mortality, 2) weight loss, and 3) score of rectal bleeding (0–3). For evaluation of histopathology, untreated control mice and mice treated with 5% DSS were sacrificed on day 7, and proximal and distal sections of the colon were fixed in 10% buffered formalin, embedded in paraffin, and sectioned. Sections were stained with H&E and scored for hyperplasia, loss of crypts, infiltration of immune cells, loss of epithelium, loss of goblet cells, and extent of ulceration, to evaluate severity of colitis. The scoring scale was from 0 to 5, with 0 being no change and 5 being the greatest change.

To determine the role of gut microflora in the development of colitis, we treated female WT germ-free mice, 4–5 wk old, maintained under sterile conditions, with sterile 4% DSS in drinking water and gavaged daily into the stomach with 12-mg stools from WT, Nod2−/−, Pglyrp3−/−, or Pglyrp3−/−Nod2−/− mice, prepared as previously described (22). In brief, fresh stools were collected from 12 mice/strain, which were obtained from different breeding parents (2 mice from 6 different parents for each strain), and kept in separate cages after weaning. This strategy minimizes the variability observed between different litters because of parent-to-parent and cage-to-cage differences. Stools were immediately resuspended in 0.2 ml reduced anaerobic medium and frozen at −80°C until use.

To determine whether gut microflora from Pglyrp3−/− mice was also predisposing to colitis in Nod2−/− mice, we used an established non-germ-free model (48, 49), in which Nod2−/− mice were depleted of their intestinal microflora with a 3-wk treatment with antibiotics in drinking water (containing 0.33 mg/ml ciprofloxacin, 1.25 mg/ml metronidazole, and 20 mg/ml Kool-Aid mix), followed by 2 d of sterile drinking water, followed by treatment with 4% DSS in drinking water and daily gavages into the stomach with 12-mg stools from Pglyrp3−/− or WT mice. Development of colitis was monitored as described earlier.
Intestinal permeability

WT, Nod2−/−, Pglyrp3−/−, and Pglyrp3−/−Nod2−/− mice were given 5% DSS in drinking water, and on days 0, 3, 6, and 7, mice were gavaged into the stomach with FITC-dextran solution (MW 4000; Sigma) at 0.6 g/kg body weight and sacrificed 4 h later. FITC-dextran in the serum was measured in duplicate samples with a fluorescence spectrophotometer. The concentration of FITC-dextran (in μg/ml) was determined using a standard curve generated with serial dilutions of FITC-dextran.

Cell proliferation

To determine the numbers of proliferating cells, we gave WT, Nod2−/−, Pglyrp3−/−, and Pglyrp3−/−Nod2−/− mice were given 5% DSS in drinking water, and on days 0 and 4, mice were injected i.p. with bromodeoxyuridine (BrdU; Sigma) at 2 mg/mouse, twice at 12-h intervals. Mice were sacrificed 12 h after the second injection. Proximal sections of the colon were fixed in 10% buffered formalin, embedded in paraffin, and sectioned. TUNEL⁺ cells were detected by DeadEnd Colorimetric TUNEL System (Promega). Cleaved caspase-3⁺ cells were detected by using rabbit mAb (clone 5A1E; Cell Signalling) and VECTASTAIN ABC Kit with DAB substrate Kit. TUNEL⁺ or cleaved caspase-3⁺ cells and total epithelial cells were counted on the entire section, and percent of TUNEL⁺ or cleaved caspase-3⁺ cells was calculated.

Pglyrp3−/−Nod2−/− mice were given 5% DSS in drinking water and gavaged daily into the stomach with caspase inhibitor, Q-VD-Oph (Oph001; R&D Systems), at 80 μg/mouse, twice at 12-h intervals. Mice were sacrificed 12 h after the second injection. Proximal sections of the colon were fixed in 10% buffered formalin, embedded in paraffin, and sectioned. TUNEL⁺ cells were detected by DeadEnd Colorimetric TUNEL System (Promega). Cleaved caspase-3⁺ cells were detected by using rabbit mAb (clone 5A1E; Cell Signalling) and VECTASTAIN ABC Kit with DAB substrate Kit. TUNEL⁺ or cleaved caspase-3⁺ cells and total epithelial cells were counted on the entire section, and percent of TUNEL⁺ or cleaved caspase-3⁺ cells was calculated.

Pglyrp3−/−Nod2−/− mice were given 5% DSS in drinking water and gavaged daily into the stomach with the caspase inhibitor, Q-VD-Oph (OPH01; R&D Systems), at 80 μg/mouse/day or with 10% DMSO in PBS, to confirm the role of cleaved caspase-3 in the development of DSS-colitis. The development of colitis was monitored as described earlier in the Colitis Model in Conventional and Germ-Free Mice section. Pglyrp3−/−Nod2−/− mice were treated with 5% DSS in drinking water and gavaged daily with caspase inhibitor (80 μg/mouse/day) or vehicle control (10% DMSO in PBS), to determine the effect of the caspase inhibitor, Q-VD-Oph, on apoptosis. On day 4, proximal and distal regions of the colon were fixed in 10% buffered formalin, embedded in paraffin, and sectioned. TUNEL⁺ cells were detected by DeadEnd Colorimetric TUNEL System (Promega). TUNEL⁺ cells and total epithelial cells were counted on the entire section, and percent of TUNEL⁺ was calculated.

DSS-treated gavaged mice show a delay in the onset of colitis and mortality compared with DSS-treated nongavaged mice, because gavaged mice drink less DSS-containing water and thus consume less DSS per day. Mice are reluctant to drink DSS-containing water, and frequent gavaging provides mice with some fluid, and thus allows them to avoid drinking as much DSS-containing water as they would drink without gavagging. This shift in the onset of colitis and mortality did not affect our conclusions, because in each experiment we compared identically treated mice; that is, all mice were not gavaged or all mice were on the same gavage regimen.

Inflammatory arrays

RNA was isolated from individual colons of WT, Nod2−/−, Pglyrp3−/−, and Pglyrp3−/−Nod2−/− mice, untreated or treated with 5% DSS for 48, 72, and 96 h, using the TRIZol method (Invitrogen), followed by digestion with RNase-free DNase (Qiagen) and purification with RNeasy spin columns using RNeasy Mini Kit (Qiagen) (22, 23, 26). Quantitative real-time RT-PCR (qRT-PCR) was used to quantify the amounts of mRNA in the colon using Mouse Inflammatory Arrays (Qiagen/SA Biosciences) or individual primer sets for some genes, with pooled cDNA from three mice/group, in three separate experiments (total nine mice/group). For each gene, a cycle threshold (Ct) was calculated followed by normalization to five housekeeping genes (Hsp90a1, Gash, Hprt1, Gapdh, and Actb) included in each array, followed by calculation of ∆∆Ct for each gene: ∆∆Ct = ∆Ct1 − ∆Ct2, where ∆Ct1 is for DSS-treated mice and ∆Ct2 is for untreated mice, using the program provided by Qiagen/SA Biosciences. This calculation gives the fold increase in expression of each gene in the treated mice versus untreated mice. The genomic DNA contamination controls, reverse transcription controls, and positive PCR controls were included in each array and were all passed. Additional controls to assure amplification from RNA, but not from possible contaminating DNA, included parallel reaction sets from which reverse transcriptase was omitted, and that showed no amplification. The results were reported as mean fold increases after DSS treatment (treated/untreated) for all groups of mice, and the entire data sets were deposited in National Center for Biotechnology Information Gene Expression Omnibus (accession number GSE47588). In some experiments, the expression of additional genes was measured by the same procedure using sets of qRT-PCR primers from SA Biosciences (IL-6, Nod2, Pglyrp1, Pglyrp3, Pglyrp4) or designed by us (24).

Stool flora analysis by quantitative PCR

The abundance of specific bacterial groups in mouse stools was measured by quantitative PCR (qPCR) using group-specific primers for 16S rRNA genes as previously described (22). In brief, 200 mg fresh stools (freshly defe- cated feces) was collected from female mice of each strain (WT, Nod2−/−, Pglyrp3−/−, and Pglyrp3−/−Nod2−/−) and immediately processed for DNA isolation or snap-frozen at −80°C. Stools were collected from all strains and from a total of 18 mice/strain at 3 time points throughout the entire period of this study (6 mice/strain in 2008, 2010, and 2013). Each time for each strain mice originated from three different litters from different parents, two mice per litter, weaned into separate cages, but all were kept in the same room in our animal facility. DNA was isolated from stools from each mouse using Qiagen QIAamp DNA Stool Mini Kit. Abundance of all bacteria and specific bacterial groups was determined by qPCR using 20 μg DNA and common primers for all Eubacteria or primers specific for the following bacterial groups: mouse intestinal Bacteroides, Bacteroides sp., Eubacterium rectale/Clostridium cocoides, Clostridium leptum, Lactobacillus/Lactococcus, segmented filamentous bacteria, Enterobacteriaceae, and Clostridium perfringens, with primer sequences described previously (22, 50, 51). The amounts of DNA for each bacterial group for each mouse were calculated using comparative cycle threshold method with common Eubacteria primers as a control. We combined the data from the three collection time points, because there were no statistically significant differences in the abundance of the bacterial groups tested for each strain between these three time points. The results for WT and Pglyrp3−/− mice with DNA collected in 2008 and 2010 were reported previously (22).

Bone marrow–derived macrophage isolation, colon and macrophage culture, activation, and cytokine assay

Untreated WT and Nod2−/− mice were sacrificed and bone marrow was flushed from femurs with cold RPMI 1640. The cells were cultured overnight in RPMI 1640 supplemented with 10% FBS and antibiotic/antimycotic mix (Sigma) at 37°C, 5% CO₂. The next day, nonadherent hematopoietic cells and nonadherent bacteria. The resuspended feces were centrifuged at 5000 rpm, 4°C, for 15 min, and the amount of ATP in the supernatant was measured in triplicate samples with a fluorescence spectrophotometer. The resuspended feces were centrifuged at 5000 rpm, 4°C, for 15 min, and the amount of ATP in the supernatant was measured in triplicate samples from each mouse using the ATPlite System (Perkin Elmer). To assess the effect of ATP on the sensitivity to colitis, we treated WT and Nod2−/− mice with 5% DSS to induce colitis and gavaged them daily into the stomach with 50 μg ATP (Sigma-Aldrich) per mouse or with PBS. The development of colitis was monitored as described earlier in the Colitis Model in Conventional and Germ-Free Mice section.

Generation of chimeric mice with bone marrow transplantation

Pglyrp3−/− and Pglyrp3−/−Nod2−/− mice, six to eight mice/group from different cages, six pellets per mouse, and resuspended in PBS by gently vortexing to avoid lysis of bacteria. The resuspended feces were centrifuged at 5000 rpm, 4°C, for 15 min, and the amount of ATP in the supernatant was measured in triplicate samples from each mouse using the ATPlite System (Perkin Elmer). To assess the effect of ATP on the sensitivity to colitis, we treated WT and Nod2−/− mice with 5% DSS to induce colitis and gavaged them daily into the stomach with 50 μg ATP (Sigma-Aldrich) per mouse or with PBS. The development of colitis was monitored as described earlier in the Colitis Model in Conventional and Germ-Free Mice section.
ablation, administered in 2 doses, 4 h apart. Bone marrow cells were obtained from the femurs and tibias of donor Pglyrp3+/+ and Pglyrp3−/− Nod2+/− mice. Four hours after irradiation, 5 × 10⁶ bone marrow cells were injected into the retro orbital vein of recipient mice. Four transplanted groups were generated: Pglyrp3+/− > Pglyrp3+/+; Pglyrp3+/− > Pglyrp3−/−; Pglyrp3+/− > Nod2+/−; and Pglyrp3−/− > Pglyrp3−/−. Transplanted mice were kept in a HEPA-filtered flow hood and given sterilized food and water (52). For the first 4 wk, mice were given filtered water with 15 g/l sucrose, 220 μg/ml neomycin, and 25 μg/ml polymyxin B, and then switched to filtered water. To re-establish the recipient gut microflora, 24 h after the end of antibiotic treatment Pglyrp3−/− and Pglyrp3−/− Nod2+/− mice were colonized with microflora from Pglyrp3+/+ or Pglyrp3+/− Nod2+/−, respectively, by gavageing with 0.2 ml suspension containing 12 mg preserved stools (22), three times, were measured then allowed to recover for 1 wk before treatment with DSS to induce colitis. We used 4% DSS, rather than 5%, to avoid early mortality, which would obscure differences in sensitivity to colitis in these highly sensitive strains. The stool groove procedure and measurement of severity of colitis were done as described earlier in the Colitis Model in Conventional and Germ-Free Mice section. Bone marrow reconstitution was verified at 6 wk after irradiation and before the start of DSS. Blood was collected from individual mice, genomic DNA was isolated using the Qiagen Puregene Blood Core Kit, and DNA was isolated using the Qiagen Puregene Blood Core Kit, and DNA was iso-

colitis in these highly sensitive strains. The stool gavage procedure and
measurement of severity of colitis was characterized by diarrhea, increased weight loss, and significantly higher diar-

pseudonyms and data indicate that the double-KO Pglyrp3−/− Nod2+/− mice are more sensitive to DSS-induced colitis than single KOs Pglyrp3+/− and Nod2−/− and WT mice.

**Results**

Pglyrp3−/− Nod2−/− mice are highly susceptible to DSS-induced colitis

To test whether Pglyrp3 and Nod2 deficiencies synergize in increasing sensitivity to colitis, we constructed Pglyrp3−/− Nod2−/− double-deficient mice and tested their sensitivity to colitis. Colitis was induced by oral administration of 5% DSS in drinking water in WT, Nod2+/−, Pglyrp3−/−, and Pglyrp3−/− Nod2−/− mice. Pglyrp3+/− Nod2−/− mice developed more severe colitis than Pglyrp3−/− and Nod2−/− mice (Fig. 1). Pglyrp3−/− Nod2−/− mice had accelerated and significantly higher mortality and significantly greater weight loss and significantly higher diarrhoea and intestinal bleeding than Pglyrp3+/+ Nod2−/− mice. Pglyrp3+/− Nod2−/− mice, as shwon previously, also developed severe DSS-induced colitis (22); however, they were less sensitive than Pglyrp3−/− Nod2−/− mice, as demonstrated by significantly higher survival, significantly lower weight loss, and significantly less severe intestinal bleeding than Pglyrp3−/− Nod2−/− mice (Fig. 1). By contrast, Nod2−/− mice were far less sensitive to colitis and manifested symptoms similar to WT mice (Fig. 1). We also evaluated histopathologic changes as a measure of the severity of colitis by scoring for hyperplasia, loss of crypts, infiltration with inflammatory cells, loss of epithelium, and loss of goblet cells, and extent of ulceration on H&E-stained sections of the colon. These histopathologic changes are characteristic for colitis. Pglyrp3−/− and Pglyrp3−/− Nod2+/− mice had significantly higher scores for hyperplasia, loss of crypts, infiltration with inflammatory cells, loss of epithelium, and loss of goblet cells, and ulceration than WT and Nod2−/− mice (Fig. 1B, 1C). Furthermore, Pglyrp3−/− Nod2−/− mice had significantly higher scores for loss of crypts, loss of epithelial and goblet cells, and ulceration than WT, Nod2−/−, and Pglyrp3−/− mice (Fig. 1B, 1C). Thus, our phenotypic and histopathologic data indicate that the double-KO Pglyrp3−/− Nod2+/− mice are more sensitive to DSS-induced colitis than single KOs Pglyrp3+/− and Nod2−/− and WT mice.

DSS-colitis is an epithelial injury model of IBD, and we next determined whether the higher sensitivity of Pglyrp3−/− Nod2+/− mice to DSS-induced colitis is accompanied by changes in the permeability of the intestine and in the rates of proliferation and apoptosis of colon epithelial cells. To measure permeability changes, we induced DSS-colitis in WT, Nod2−/−, Pglyrp3−/−, and Pglyrp3−/− Nod2+/− mice, and FITC-dextran was administered by gavage before sacrifice. Mice were sacrificed at different time points, and the amount of FITC-dextran in the serum was measured. The concentration of FITC-dextran in the serum was significantly higher in Pglyrp3−/− Nod2+/− mice on days 6 and 7 of DSS treatment compared with WT, Nod2−/−, and Pglyrp3−/− mice (Fig. 2A). Pglyrp3−/− mice had significantly higher levels of FITC-dextran on day 7 than Nod2−/− mice and on days 6 and 7 than WT mice (Fig. 2A). These results indicate that both Pglyrp3 and Nod2 contribute to the integrity of the intestinal mucosa, and a deficiency of both genes significantly increases damage to the intestine in DSS-treated mice. However, Pglyrp3−/− mice have more prolonged and severe damage than Nod2−/− mice.

To identify the changes in the intestinal mucosa that result in increased permeability, we determined the rate of colonic cell proliferation and apoptosis in DSS-treated and untreated mice. To measure the numbers of proliferating cells, we gavaged DSS-treated or untreated WT, Nod2−/−, Pglyrp3−/−, and Pglyrp3−/− Nod2+/− mice with BrdU, and proliferating cells were identified by immunohistochemistry. Pglyrp3−/− Nod2+/− mice all had significantly fewer BrdU+ cells than WT mice (Fig. 2B). Also, Pglyrp3−/− mice had significantly fewer BrdU+ cells than Nod2−/− and Pglyrp3−/− Nod2+/− mice (Fig. 2B). These results indicate that Pglyrp3 deficiency results in a severe inhibition of proliferation, which most likely contributes to the sensitivity to DSS-induced colitis in Pglyrp3−/− and Pglyrp3−/− Nod2−/− mice. By contrast, Nod2 deficiency caused less severe decrease in proliferation (Fig. 2B), which is reflected in less severe colitis in Nod2−/− than in Pglyrp3−/− mice (Fig. 1).

We next measured apoptosis in the colons of DSS-treated and untreated WT, Nod2−/−, Pglyrp3−/−, and Pglyrp3−/− Nod2+/− mice using TUNEL immunohistochemistry. Pglyrp3−/− Nod2+/− mice had significantly more TUNEL+ cells than Pglyrp3−/−, Nod2−/−, and WT mice, and Nod2−/− mice had significantly...
FIGURE 1. Pglyrp3−/−Nod2−/− mice are more susceptible to DSS-induced colitis than Pglyrp3 and Nod2 single-deficient mice. WT, Nod2−/−, Pglyrp3−/−, and Pglyrp3−/−Nod2−/− mice were treated with 5% DSS in drinking water, and development of colitis was evaluated. (A) DSS-treated mice were monitored over time for survival, change in body weight, and stool and rectal bleeding. (B and C) DSS-treated mice were sacrificed on day 7 of treatment, and H&E-stained sections of the colon were (B) qualitatively and (C) quantitatively evaluated for severity of colitis. Epithelial cells (E), LP, goblet cells (G), muscularis mucosa (MM), submucosa (SM), muscularis externa (ME), and inflammatory cell infiltrations (IN) are indicated. Scale bar, 100 μm. Data are (A) mean ± SEM of 19–20 mice/group; (B) representative images or (C) mean ± SEM from 4 mice/group. Significance of differences between Pglyrp3−/−Nod2−/− and WT mice is indicated by number signs: *p ≤ 0.05, **,##p ≤ 0.005.

more TUNEL+ cells than Pglyrp3−/− and WT mice (Fig. 2C). To further evaluate apoptosis in the intestinal epithelium of DSS-treated Pglyrp3−/−Nod2−/− mice, we assayed for cleaved caspase-3, which is the active form of caspase-3, an enzyme essential for apoptosis. We detected cleaved caspase-3 in the intestinal mucosa of untreated and DSS-treated WT, Nod2−/−, Pglyrp3−/−, and Pglyrp3−/−Nod2−/− mice by immunohistochemistry. DSS-treated Pglyrp3−/−Nod2−/− mice had the highest increase of cleaved caspase-3 cells, significantly higher than Pglyrp3−/−, Nod2−/−, and WT mice (Fig. 2D). DSS-treated Nod2−/− mice had significantly higher numbers of cleaved caspase-3 cells than Pglyrp3−/− and WT mice (Fig. 2D). Our TUNEL and cleaved caspase-3 data indicate that a combined deficiency in both Pglyrp3 and Nod2 results in the highest apoptosis of colonic epithelial cells, and that Nod2 deficiency has a greater contribution to this high apoptosis than Pglyrp3 deficiency.

Because Pglyrp3−/−Nod2−/− mice were highly sensitive to DSS-colitis and had significantly higher numbers of apoptotic epithelial cells in the colon, we next tested the effect of a caspase inhibitor on the development of colitis in these mice. Pglyrp3−/−Nod2−/− mice were treated with DSS and gavaged daily with the caspase inhibitor Q-VD-OPh or PBS as a control. The caspase inhibitor significantly decreased all clinical manifestations of colitis. Caspase inhibitor-treated Pglyrp3−/−Nod2−/− mice showed significantly higher survival and lower weight loss and stool scores than PBS-treated control mice in response to DSS (Fig. 2E). We next determined the effect of the caspase inhibitor Q-VD-OPh on apoptosis in the colon. The numbers of TUNEL+ colon epithelial cells were significantly lower in caspase inhibitor-treated than control Pglyrp3−/−Nod2−/− mice (Fig. 2E). These results demonstrate that the caspase inhibitor Q-VD-OPh is effective in decreasing apoptosis of intestinal epithelial cells, and that apoptosis is required for the full severity of DSS-induced colitis in Pglyrp3−/−Nod2−/− mice. However, the caspase inhibitor did not completely abrogate the symptoms of colitis, which indicates that other factors also contribute to the disease severity in DSS-treated Pglyrp3−/−Nod2−/− mice.

In summary, these results demonstrate that both Pglyrp3 and Nod2 are required for maintaining a functional and intact intestinal mucosa. A single deficiency of Pglyrp3, but not Nod2, is sufficient to make mice more sensitive to DSS-induced colitis. However, a combined deficiency of Pglyrp3 and Nod2 synergistically increases sensitivity to DSS-induced colitis, and increased apoptosis of intestinal epithelial cells is one of the factors responsible for this increased sensitivity.

Pglyrp3−/−Nod2−/− mice have increased expression of inflammatory molecules

We next measured the expression of inflammatory molecules in the colon using qRT-PCR microarrays. DSS-treated Pglyrp3−/− and Pglyrp3−/−Nod2−/− mice had significantly higher, but not identical, expression of mRNA for several cytokines and chemokines (Fig. 3 and Supplemental Table I with entire array data). Both Pglyrp3−/− and Pglyrp3−/−Nod2−/− mice had higher expression of Th1 and NK cell markers, IFN-γ, CXCL-9, CXCL-10, and CXCL-11; Th2 markers, CCL-7 and CCL-8; and Th17 markers, CCL-2, CXCL-1, and CXCL-5, than WT and Nod2−/− mice (Fig. 3). DSS-treated Pglyrp3−/− and Pglyrp3−/−Nod2−/− mice also had elevated expression of the cytokines IL-1β, IL-6, and IL-11 (which are produced by many different cell types) compared with
WT and Nod2−/− mice (Fig. 3). In addition, Pglyrp3−/−Nod2−/− mice had significantly higher expression of TNF-α, IL-16, and CCR-7 than Pglyrp3−/−, WT, and Nod2−/− mice (Fig. 3). These results indicate that colitis in Pglyrp3−/−Nod2−/− mice is accompanied by higher induction of several inflammatory mediators, many of which are shared with the Pglyrp3−/− inflammatory profile and some that are unique. Because expression of TNF-α, IL-16, and CCR-7 was higher in Pglyrp3−/−Nod2−/− than in Pglyrp3−/− mice, these molecules may contribute to the higher sensitivity of Pglyrp3−/−Nod2−/− mice to DSS-colitis. Several of these inflammatory gene responses in DSS-treated Pglyrp3−/−Nod2−/− mice were Nod2 independent, because they were similar or higher than in Pglyrp3−/− (Nod2+/+) mice (Fig. 3 and Supplemental Table I).

We determined that expression of Pglyrp1, Pglyrp2, Pglyrp3, and Pglyrp4 genes in tongue, esophagus, and colon was similar in Nod2−/− and WT mice (data not shown). We have previously shown that treatment with DSS increases the expression of Pglyrp3 in the colon (22). We further determined that expression of Pglyrp3 in the colon in DSS-treated WT and Nod2−/− mice was similar, and also that the expression of Nod2 in the colon was similar in untreated or DSS-treated Pglyrp3−/− WT mice (data not shown). These results indicate that the colitis phenotype in Pglyrp3−/−Nod2−/− double-KO mice is not due to changes in the expression of Pglyrp3 in a Nod2-deficient background or changes in the expression of Nod2 in a Pglyrp3-deficient background.

**Pglyrp3-dependent, but not Nod2-dependent, intestinal microflora predisposes to DSS-colitis**

Pglyrp3 is a secreted antibacterial protein, and we have previously shown that Pglyrp3−/− mice have changes in the gut bacterial flora that are responsible for the higher sensitivity of Pglyrp3−/− mice than WT mice to DSS-induced colitis (22). Nod2 is also involved in innate immune responses to bacteria, and Nod2−/− mice also have an altered microbial population in the gut (43-46). Therefore, we next tested whether Pglyrp3−/−Nod2−/− mice have additional changes in the gut microflora and whether these cumulative changes are responsible for the higher sensitivity to colitis in the double-KO mice. We compared the composition of bacterial flora in the stools of WT, Pglyrp3−/−, Nod2−/−, and Pglyrp3−/−Nod2−/− mice by measuring the amounts of DNA for all Eubacteria and for eight major groups of mouse intestinal bacteria using qPCR. Single- and double-KO mice had significant, but not identical changes in the composition of their bacterial flora in their stools. The amounts of bacteria in the following bacterial groups showed significant changes in KO compared with WT mice: Lactobacillus and C. perfringens groups in Nod2−/−, Pglyrp3−/−, and Pglyrp3−/−Nod2−/− mice; E. rectale, C. leptum, and Enterobacteriaceae groups in Pglyrp3−/− and Pglyrp3−/−Nod2−/− mice; and segmented filamentous bacteria in Pglyrp3−/−Nod2−/− mice (Fig. 4A).
We next tested whether these changes in the microflora in Pglyrp3\(^{-/-}\)Nod2\(^{-/-}\) mice are responsible for their increased sensitivity to DSS-colitis. We gavaged WT germ-free mice daily with stools from WT, Nod2\(^{-/-}\), Pglyrp3\(^{-/-}\), or Pglyrp3\(^{-/-}\)Nod2\(^{-/-}\) mice, and we induced colitis with 4% DSS. Germ-free mice receiving stools from Pglyrp3\(^{-/-}\) or Pglyrp3\(^{-/-}\)Nod2\(^{-/-}\) mice were more sensitive to DSS-colitis than mice receiving Nod2\(^{-/-}\) or WT stools, but the sensitivity of mice receiving stools from Pglyrp3\(^{-/-}\) or Pglyrp3\(^{-/-}\)Nod2\(^{-/-}\) mice was similar. Germ-free mice gavaged with stools from Pglyrp3\(^{-/-}\)Nod2\(^{-/-}\) or Pglyrp3\(^{-/-}\)Nod2\(^{-/-}\) mice had significantly higher mortality, higher weight loss, and higher stool scores than mice gavaged with Nod2\(^{-/-}\) or WT stools, and all these indicators were similar in germ-free mice gavaged with stools from Pglyrp3\(^{-/-}\)Nod2\(^{-/-}\) and Pglyrp3\(^{-/-}\) mice (Fig. 4B). The sensitivity to colitis of germ-free mice gavaged with stools from WT or Nod2\(^{-/-}\) mice was similar. These results confirm our previous results that the increased sensitivity to DSS colitis in Pglyrp3\(^{-/-}\) mice is due to an altered gut microflora (22), and they further demonstrate that this altered microflora contributes to a similar extent to the increased sensitivity to DSS colitis in Pglyrp3\(^{-/-}\) mice and in Pglyrp3\(^{-/-}\)Nod2\(^{-/-}\) mice. These results also demonstrate that microflora from Nod2\(^{-/-}\) mice does not predispose germ-free mice to colitis compared with microflora from WT mice. Thus, these results demonstrate that Pglyrp3\(^{-/-}\) mice contribute colitis-predisposing flora to Pglyrp3\(^{-/-}\)Nod2\(^{-/-}\) mice, and that the additional changes in the microflora in Pglyrp3\(^{-/-}\)Nod2\(^{-/-}\) mice, compared with Pglyrp3\(^{-/-}\) mice, do not further contribute to the increased sensitivity of Pglyrp3\(^{-/-}\)Nod2\(^{-/-}\) mice to colitis.

We further tested the effect of microflora from Pglyrp3\(^{-/-}\) mice on the development of DSS-colitis in a Nod2-deficient background. We treated Nod2\(^{-/-}\) mice with antibiotics for 3 wk to deplete their intestinal microflora. At the end of 3 wk, we gavaged the antibiotic-treated mice with stools from WT or Pglyrp3\(^{-/-}\) mice and induced colitis with oral DSS. Nod2\(^{-/-}\) mice receiving stools from Pglyrp3\(^{-/-}\) mice were more sensitive to DSS-colitis than mice receiving WT stools (Fig. 4C). The more severe colitis in Nod2\(^{-/-}\) mice gavaged with stools from Pglyrp3\(^{-/-}\) mice manifested as significantly higher loss in body weight and stool scores, and significantly lower survival (Fig. 4C). These results indicate that Pglyrp3\(^{-/-}\) microflora is stable, Pglyrp3 dependent, and has colitis-predisposing characteristics on both Nod2\(^{-/-}\) and Nod2\(^{+/+}\) backgrounds.

Our previous results demonstrated that the gut microflora from Pglyrp3\(^{-/-}\) mice induces higher production of inflammatory molecules, IL-6 and CXCL-1, than WT microflora (22). Thus, we next tested whether the response to gut bacteria from Pglyrp3\(^{-/-}\) mice is variable in Nod2\(^{-/-}\) and Nod2\(^{+/+}\) backgrounds, because Nod2 is one of the bacterial recognition cell-activating pattern recognition receptors, and because Pglyrp3\(^{-/-}\)Nod2\(^{-/-}\) mice are more sensitive to colitis than Pglyrp3\(^{-/-}\) mice. Colon cells and fragments or bone marrow macrophages from WT or Nod2\(^{-/-}\) mice were stimulated with diluted stools from WT or Pglyrp3\(^{-/-}\) mice, to determine whether cells from WT or Nod2\(^{-/-}\) mice have differential responsiveness to colitis-promoting (Pglyrp3\(^{-/-}\)) and nonpromoting (WT) stools (Fig. 4) (22). Culture supernatants were then assayed for different cytokines and chemokines. Stools from Pglyrp3\(^{-/-}\) mice induced higher production of CXCL-2, CXCL-9, and IL-6 in cells from WT, but not from Nod2\(^{-/-}\) colon cells and fragments (Fig. 5A), and higher production of CXCL-2, CXCL-1, CXCL-10, and IL-6 in WT, but not Nod2\(^{-/-}\) bone marrow macrophages (Fig. 5B) compared with stools from WT mice. These results confirm our previous data that stools from...
*Pglyrp3*<sup>−/−</sup> mice are more proinflammatory than stools from WT mice (22), and show that induction of these proinflammatory chemokines and cytokines is significantly higher on *Nod2*<sup>−/−</sup> than on *Nod2*<sup>−/−</sup> background. The results in Fig. 5 also show that although the responses of cells from *Nod2*<sup>−/−</sup> mice are generally lower than from WT mice (as expected), the cells from *Nod2*<sup>−/−</sup> mice are still responsive to bacteria (presumably through other bacterial sensors), and also that all responses are not equally affected, showing that some responses are *Nod2* dependent and some *Nod2* independent, and that cells from *Nod2*<sup>−/−</sup> mice are differentially responsive to stools from WT and *Pglyrp3*<sup>−/−</sup> mice. For example, CXCL-10 production was not reduced in colon from *Nod2*<sup>−/−</sup> mice, compared with WT mice (Fig. 5A), and bone marrow macrophages from both WT and *Nod2*<sup>−/−</sup> mice produced IL-6 in response to stools only from *Pglyrp3*<sup>−/−</sup> mice, but not WT mice (Fig. 5B). Moreover, several inflammatory gene responses to DSS in the entire colon in vivo involved additional *Nod2*-independent signals, because many of these responses were similar or higher in *Pglyrp3*<sup>−/−</sup>*Nod2*<sup>−/−</sup> compared with *Pglyrp3*<sup>−/−</sup> mice (Fig. 3 and Supplemental Table I).
Pglyrp3−/− and Pglyrp3−/−Nod2−/− mice have increased ATP in the colon, which increases inflammatory T cells and predisposes to colitis

Pglyrp3−/− and Pglyrp3−/−Nod2−/− mice have altered gut microflora, which contributes to the increased sensitivity to DSS-colitis. In order to identify bacterial metabolites that may facilitate damage to the intestine, we assayed for ATP in mouse stools, because: 1) ATP is produced by gut microflora; 2) ATP is known to increase sensitivity to T-cell–mediated colitis by increasing the numbers of inflammatory Th17 cells in the intestine (54); and 3) to increase sensitivity to T-cell–mediated colitis by increasing the stable ATP analog, α,β-ATP. Again, we used Nod2−/− mice for these experiments to re-create the Nod2−/− genetic background of Pglyrp3−/−Nod2−/− mice and one aspect of Pglyrp3 deficiency (higher ATP). The numbers of CD3+, CD4+, CD4+ TGFβ+, CD4+ IL-4− (Th2), and CD4+IL-17+ (Th17) cells were significantly higher in DSS-treated Nod2−/− mice gavaged with α,β-ATP than in the control mice gavaged with PBS (Fig. 6C, 6D). There was no significant difference in CD4+ cells expressing Foxp3 in the presence and absence of the stable ATP analog, α,β-ATP. These data also correlate with our mRNA expression microarray results, which demonstrate higher expression of Th2 and Th17 markers in Pglyrp3−/− and Pglyrp3−/−Nod2−/− mice (Fig. 3 and Supplemental Table 1).

In conclusion, a deficiency in Pglyrp3 results in high levels of ATP in the colon, and high ATP results in increased numbers of inflammatory Th17 and Th2 cells in the colon and causes higher sensitivity to DSS-colitis. These results suggest that in Pglyrp3−/−Nod2−/− mice, Pglyrp3 deficiency contributes to the elevated ATP, and that Nod2−/− mice respond to a higher concentration of ATP with increased numbers of inflammatory Th17 and Th2 cells and higher sensitivity to DSS-colitis. These results offer one possible mechanism of microbiota-induced sensitivity to colitis in Nod2−/− mice, which are generally less responsive to bacteria than WT mice (Fig. 5), yet are more sensitive to colitis induced by microflora from Pglyrp3−/− mice (Fig. 4C).

Lack of Nod2 in bone marrow–derived cells combined with presence of Nod2 in structural cells predisposes Pglyrp3−/− mice to severe colitis

Pglyrp3 is expressed in epithelial cells, whereas Nod2 is expressed in both bone marrow–derived and structural (epithelial and stromal) cells.
mal) cells. We next determined whether the lack of Nod2 expression in bone marrow–derived cells or structural cells was important for enhanced sensitivity to DSS-induced colitis in Pglyrp3−/−Nod2−/− mice. We generated Pglyrp3−/−Nod2−/− and Pglyrp3−/−Nod2−/− mice. Chimerism was confirmed by PCR of genomic DNA and showed that blood cells from Pglyrp3−/−Nod2−/− mice contained only Nod2 KO allele, whereas blood cells from Pglyrp3−/−Nod2−/− mice contained only Nod2 WT allele (Fig. 7B). All groups of mice were treated with DSS to induce colitis, and development of colitis was monitored. Pglyrp3−/−Nod2−/−
Pglyrp3\(^{-/-}\) chimeras developed the most severe colitis compared with Pglyrp3\(^{-/-}\) > Pglyrp3\(^{-/-}\) > Nod2\(^{-/-}\) chimeras and the control Pglyrp3\(^{-/-}\) > Pglyrp3\(^{-/-}\) and Pglyrp3\(^{-/-}\) > Nod2\(^{-/-}\) > Pglyrp3\(^{-/-}\) > Nod2\(^{-/-}\) mice, with significantly higher mortality, significantly higher stool scores, and significantly greater loss of body weight (Fig. 7A). Pglyrp3\(^{-/-}\) > Pglyrp3\(^{-/-}\) > Nod2\(^{-/-}\) and Pglyrp3\(^{-/-}\) > Nod2\(^{-/-}\) > Pglyrp3\(^{-/-}\) > Nod2\(^{-/-}\) mice had intermediate sensitivity to colitis, and Pglyrp3\(^{-/-}\) > Pglyrp3\(^{-/-}\) > Nod2\(^{-/-}\) mice developed the least severe colitis.

These results show that the lack of Nod2 expression in bone marrow–derived cells combined with expression of Nod2 in structural (radio-resistant) cells (Pglyrp3\(^{-/-}\) > Pglyrp3\(^{-/-}\) > Pglyrp3\(^{-/-}\)) predisposes Pglyrp3\(^{-/-}\) mice to very severe colitis, more severe than the lack of Nod2 in both bone marrow–derived and structural cells (Pglyrp3\(^{-/-}\) > Pglyrp3\(^{-/-}\) > Nod2\(^{-/-}\) > Pglyrp3\(^{-/-}\) > Nod2\(^{-/-}\)) or the lack of Nod2 only in structural cells (Pglyrp3\(^{-/-}\) > Pglyrp3\(^{-/-}\) > Nod2\(^{-/-}\) > Nod2\(^{-/-}\)). Also, expression of Nod2 in all cells protects from colitis, because Pglyrp3\(^{-/-}\) > Pglyrp3\(^{-/-}\) mice were less sensitive to colitis than Nod2-chimeric or fully Nod2-deficient mice, which confirms our other results in this study. Altogether, these results further indicate that expression of Nod2 is protective against colitis, and suggest that expression of Nod2 in bone marrow–derived cells is more important for protection against colitis than Nod2 expression in structural cells. These results also suggest that expression of Nod2 in structural cells has a colitis-promoting effect, but only when Nod2 is not expressed in marrow-derived cells.

**Discussion**

IBD is caused by aberrant host immune responses, altered gut microbiome, and environmental factors. Thus, there is no single cause for IBD, and the exact genetic and environmental factors that result in IBD most likely differ in different individuals. In this study, we report that a combined deficiency of two innate immunity genes, Pglyrp3 and Nod2, results in increased sensitivity to an experimental model of colitis. We show that Pglyrp3\(^{-/-}\) > Nod2\(^{-/-}\) double-deficient mice are more sensitive to DSS-induced colitis than Pglyrp3\(^{-/-}\), Nod2\(^{-/-}\), and WT mice. We also confirmed our previous results showing increased sensitivity to DSS-colitis in Pglyrp3\(^{-/-}\) mice (22). Nod2\(^{-/-}\) mice were significantly less sensitive to DSS-colitis than Pglyrp3\(^{-/-}\) and Pglyrp3\(^{-/-}\) Nod2\(^{-/-}\) mice, and had similar sensitivity to WT mice, which confirms the findings by Kobayashi et al. (41), but differs from two other studies (42, 43). These conflicting results with Nod2\(^{-/-}\) mice are difficult to explain and may result from differences in genetic backgrounds, gut bacteria, environment, and/or other unknown factors.

Pglyrp3\(^{-/-}\) > Nod2\(^{-/-}\) mice treated with DSS had high mortality, severe loss in body weight, and high stool scores. These phenotypic manifestations were accompanied by several histopathologic changes including severe hyperplasia, loss of crypts, infiltration with inflammatory cells, loss of epithelium, loss of goblet cells, and ulcers, which are characteristic for colitis. Additional changes associated with colitis in Pglyrp3\(^{-/-}\) > Nod2\(^{-/-}\) mice were in-
creased gut permeability, decreased colonic epithelial cell proliferation, increased colonic epithelial cell apoptosis, increased expression of chemokines and cytokines, changes in gut bacteria, and increased ATP in the colon. Our results demonstrate that Nod2 deficiency contributes significantly to higher apoptosis and that Pglyrp3 deficiency contributes to higher ATP in the colon, which causes increased numbers of inflammatory T cells and increased sensitivity to DSS-colitis. These results suggest that the enhanced sensitivity of Pglyrp3−/−Nod2−/− mice to DSS-colitis is due to multiple factors, and that Pglyrp3 and Nod2 have both overlapping and unique contributions toward sensitivity to colitis in the double-KO mice.

Pglyrp3−/−Nod2−/− mice have significantly higher apoptosis in the colon, which is required for the increased sensitivity to colitis of Pglyrp3−/−Nod2−/− mice. Deficiency in both Pglyrp3 and Nod2 contributed to the enhanced apoptosis in the double-KOs; however, Nod2 deficiency had a stronger effect than Pglyrp3 deficiency. The increased sensitivity of Pglyrp3−/−Nod2−/− mice to DSS-colitis required the protease caspase-3, an important regulatory enzyme that activates downstream execution caspsases during apoptosis. Apoptosis is crucial for the rapid turnover of intestinal mucosal cells and for homeostasis of the gut, and the role of caspase-3 in this process is well documented (55). The mechanism by which Nod2 regulates apoptosis is not known. However, our results also suggest that Nod2 deficiency in bone marrow–derived cells highly increases sensitivity to colitis, which suggests that higher sensitivity to apoptosis of epithelial cells in Nod2−/− mice may result from the lack of protective effects of cytokines or other factors from bone marrow–derived cells. A recent report demonstrated that Nod2 is involved in cross talk with the apoptosis protein, Bid, a member of the Bcl2 family, and that this interaction is required for the proinflammatory function of Nod2, which involves activation of NF-κB and increased expression of chemokines and cytokines (56). However, data showing Nod2–Bid interaction have not been replicated, and it is not known whether this interaction regulates apoptosis. The role of mammalian Pglyrp3 in apoptosis is novel for these proteins, and increased apoptotic cells may be an indirect consequence of mucosal damage by changed microflora in Pglyrp-deficient background.

Pglyrp3−/−Nod2−/− and Pglyrp3−/− mice treated with DSS had higher expression of Th1, Th2, and Th17 markers and higher levels of IL-1, IL6, and IL-11, which are produced by many major groups of gut bacteria compared with WT mice, and some of these changes were also common for Pglyrp3−/− and Nod2−/− mice. Our results using germ-free mice gavaged with stools from WT, Nod2−/−, Pglyrp3−/−, or Pglyrp3−/−Nod2−/− mice demonstrate that Pglyrp3 deficiency, but not Nod2 deficiency, contributes colitis-promoting microflora to the double-KO mice. These data suggest that a deficiency in Pglyrp3, but not in Nod2, is responsible for the colitis-predisposing dysbiosis in Pglyrp3−/−Nod2−/− mice, and that Nod2 deficiency contributes other factors further predisposing these mice to colitis. Pglyrp3-dependent microflora also increases sensitivity to colitis in a Nod2-deficient background, which further indicates that Pglyrp3-dependent microflora is required for the increased sensitivity to DSS-colitis in Pglyrp3−/−Nod2−/− mice.

Our results demonstrating differences in gut microbial populations between Nod2−/− and WT mice are in agreement with other studies (43–46). However, our results showing that microflora from Nod2−/− mice does not increase the sensitivity to colitis in WT germ-free mice differ from the results of Couturier-Maillard et al. (43), who used cohoused WT mice. Moreover, differences in gut microbial populations between F2 WT and Nod2−/− littermates were recently shown to be dependent on maternal lineage and not on the Nod2−/− deficiency (61). It was not determined, however, whether Nod2−/− genotype influences microflora over a longer period. We did not use F2 littermates in our experiments because a change to colitis-predisposing microflora because of a Pglyrp deficiency does not happen immediately or over a few weeks, but is established over more than one generation (R. Dziarski, unpublished observations). Therefore, all mice used in our experiments were derived from KO strains that were established after backcrossing to the same WT stock at least 6 months earlier and bred in the same facility. Furthermore, in our experiments with gut microflora, we used 18 mice/strain, obtained from 9 different breeding pairs for each strain (2 mice/breeding pair), and kept in separate cages after weaning. This strategy allows stabilization of microflora and minimizes the variability observed between different litters because of different parents and different cages. Stools for microflora testing were collected at three time points over a 5-y period, and there were no statistically significant differences in the abundance of the bacterial groups tested for each strain between these three time points, which indicates stable and genotype-dependent stool microflora over the entire 5-y period of the study. Moreover, our results with the transfer of the predisposition to colitis to germ-free mice with stool microflora from either Pglyrp3−/− or Pglyrp3−/−Nod2−/− mice show that Pglyrp3−/− microflora is stable, Pglyrp3 dependent, and has colitis-predisposing characteristics on both Nod2−/− and Nod2−/+ backgrounds.

Pglyrp3−/− and Pglyrp3−/−Nod2−/− mice had higher levels of ATP in the colon than Nod2−/− and WT mice. Administration of a stable ATP analog to Nod2−/− and WT mice made them more sensitive to DSS-colitis. However, Nod2 deficiency further increased sensitivity to DSS-induced colitis in the presence of elevated ATP. Increased colitis in ATP-treated Nod2−/− mice was accompanied by higher numbers of CD3, CD4, Th17, and Th2 treated Pglyrp3−/−Nod2−/− mice may be in response to the altered gut microflora caused by Pglyrp3 deficiency. IL-116 is a new member of the IL-1 family of cytokines, and its elevated expression is associated with the inflammatory disease psoriasis and with psoriasis-like symptoms (60). Our results indicate that IL-116 expression is increased in a Nod2−/−, but not a Nod2−/+ background, and may have a role in sensitivity to DSS-colitis in Pglyrp3−/−Nod2−/− mice.

Pglyrp3−/−Nod2−/− mice had significant differences in the major groups of gut bacteria compared with WT mice, and some of these changes were also common for Pglyrp3−/− and Nod2−/− mice. Our results using germ-free mice gavaged with stools from WT, Nod2−/−, Pglyrp3−/−, or Pglyrp3−/−Nod2−/− mice demonstrate that Pglyrp3 deficiency, but not Nod2 deficiency, contributes colitis-promoting microflora to the double-KO mice. These results also suggest that Pglyrp3 deficiency in bone marrow–derived cells highly increases sensitivity to colitis, which suggests that higher sensitivity to apoptosis of epithelial cells in Nod2−/− mice may result from the lack of protective effects of cytokines or other factors from bone marrow–derived cells. A recent report demonstrated that Nod2 is involved in cross talk with the apoptosis protein, Bid, a member of the Bcl2 family, and that this interaction is required for the proinflammatory function of Nod2, which involves activation of NF-κB and increased expression of chemokines and cytokines (56). However, data showing Nod2–Bid interaction have not been replicated, and it is not known whether this interaction regulates apoptosis. The role of mammalian Pglyrp3 in apoptosis is novel for these proteins, and increased apoptotic cells may be an indirect consequence of mucosal damage by changed microflora in Pglyrp-deficient background.

Pglyrp3−/−Nod2−/− and Pglyrp3−/− mice treated with DSS had higher expression of Th1, Th2, and Th17 markers and higher expression of IL-1β, IL6, and IL-11, which are produced by many cell types. These changes are most likely due to a deficiency of Pglyrp3 and altered microflora, because they are observed in both Pglyrp3−/− and Pglyrp3−/−Nod2−/− mice. These results also confirm our previous discovery of a predominant role of IFN-γ and IFN-γ-regulated chemokines, CXCL9, CXCL10, CXCL11, and CCL-2, in DSS-colitis in Pglyrp-deficient mice (22). CCR7, TNF-α, IL-1β, and IL-116 were significantly higher in the colon of Pglyrp3−/−Nod2−/− than Pglyrp3−/− mice and may indicate a role for Nod2 in their regulation. CCR7 is the receptor for CCL19 and CCL21, and influences both immune responses and tolerance in the gut by regulating migration of T cells to lymphoid organs (57). The role of CCR7 in Pglyrp3−/−Nod2−/− mice during DSS-colitis is not understood but may indicate greater trafficking of T cells in the damaged intestine. TNF-α is a common pathogenic factor in many models of colitis and is also known to trigger apoptosis of epithelial cells (58). Higher expression of TNF-α in Pglyrp3−/−Nod2−/− mice correlates with higher apoptosis and may be one of the contributing factors to the increased mucosal cell death in the double-KO mice. Synthesis of IL-1β is induced by TLRs in response to bacteria and bacterial fragments (59), and the increased expression of IL-1β in DSS-
cells in the colon LP. Extracellular levels of ATP are tightly controlled and increased amounts serve as a danger signal that modulates immune functions (54, 62, 63). High extracellular ATP induces differentiation of LP T cells into Th17 cells, which causes colitis (54) and drives inflammation in asthma (62, 63). High concentrations of extracellular ATP also induce activation of caspase-3 and apoptosis in macrophages (64), gingival epithelial cells (65), and anti-inflammatory Tregs (66). ATP in the colon may be released from injured epithelial cells or from gut bacteria, which are known to secrete large amounts of ATP (67). The most likely source of increased ATP in Pglyrp3−/− and Pglyrp3+/− Nod2−/− mice is the altered microbiota and not injured epithelial cells, because increased ATP is present in untreated mice, which have a healthy gut mucosa (22). Accordingly, changes in the gut bacterial populations are known to modify the amount of ATP produced in the colon (54). Collectively, these studies suggest a possible mechanism for the role of ATP in increased sensitivity to DSS-colitis in Pglyrp3−/− and Pglyrp3+/− Nod2−/− mice, which includes ATP-induced apoptosis of intestinal epithelial cells and LP Tregs, and increased differentiation of LP Th17 cells. Our data demonstrate that Nod2−/− mice treated with DSS and a stable analog of ATP are more sensitive to colitis and have increased numbers of Th17, but unchanged numbers of Tregs in the colon LP compared with PBS-treated mice. Thus, in our model of UC, ATP may regulate sensitivity to DSS-colitis through increased apoptosis of epithelial cells and increased differentiation of Th17 cells, but not through increased apoptosis of Tregs. Thus, our data suggest that Pglyrp3 deficiency results in increased ATP in the colon, which drives differentiation of inflammatory Th17 cells and increased sensitivity to DSS-induced colitis in Nod2−/− mice.

Because both immune and colon structural (epithelial and stromal) cells express Nod2, we performed bone marrow transplant experiments and generated Pglyrp3−/− > Pglyrp3−/− Nod2−/− and Pglyrp3+/− Nod2−/− > Pglyrp3+/− chimeric mice to identify the source of Nod2-deficient cells that mediate increased sensitivity to DSS-induced colitis. Our data indicate that an unbalanced expression of Nod2 because of the lack of Nod2 expression in bone marrow–derived cells combined with the expression of Nod2 in structural (radio-resistant) cells in a Pglyrp3−/− background results in the highest sensitivity to colitis. On the contrary, the lack of Nod2 expression in structural cells combined with the expression of Nod2 in bone marrow–derived cells results in intermediate sensitivity to colitis, and similar to the sensitivity of mice lacking Nod2 in all cells. Finally, expression of Nod2 in all cells results in the lowest sensitivity to colitis. These results suggest that expression of Nod2 in bone marrow–derived cells is more important for protection against colitis than Nod2 expression in structural cells, and also that expression of Nod2 in structural cells has a colitis-promoting effect, but only when Nod2 is not expressed in bone marrow–derived cells. The reasons for the protection from colitis from bone marrow–derived Nod2 and the susceptibility to colitis from structural cell–derived Nod2 (but only when bone marrow–derived Nod2 is missing) are not clear and may include protective effects of cytokines from bone marrow–derived cells or other cross talk between these two cell types.

The role of Nod2 in intestinal disease is complex, often contradictory, and not clearly understood. Different models have been proposed to explain the role of Nod2 in CD and in animal models of colitis. One possible explanation is an impaired response of intestinal mucosa and immune cells in Nod2-deficient individuals to intestinal bacteria, resulting in an inability to control microbiota-induced intestinal damage. A second possible explanation is dysbiosis in Nod2-deficient mice (43–46) and humans with Nod2 mutations (45), resulting in more damaging bacteria in the terminal ileum. A third possible explanation is that Nod2 is a negative regulator of TLR signaling and that Nod2 deficiency results in increased TLR-mediated responses to bacteria, which leads to damaging inflammation (42, 68, 69). However, an inhibitory role of Nod2 in TLR signaling is controversial and has been shown by only one research group, whereas several investigators have determined that during acute responses, Nod2 activation synergizes with TLRs to increase production of proinflammatory cytokines (41, 70–72).

Our data support the first explanation and show that Pglyrp3-controlled changes in intestinal microflora and Pglyrp3-controlled increases in colonic ATP promote colitis on Nod2−/− background. Thus, Nod2 deficiency results in less efficient control of microflora-induced damage, and one contributing factor is the higher sensitivity to apoptosis and ATP in the colon on Nod2−/− than on Nod2+/+ background. Our data do not support the second and third explanations, because Nod2 deficiency did not contribute to colitis-predisposing intestinal microflora (Fig. 4), and because cytokine and chemokine responses of macrophages or colonic cells to gut microflora were lower on Nod2−/− than on Nod2+/+ background (Fig. 5).

In summary, the two innate immunity genes, Pglyrp3 and Nod2, synergistically protect mice in an experimental model of colitis. A combined deficiency of Pglyrp3 and Nod2 results in higher sensitivity to DSS-colitis because of a combination of factors, including higher apoptosis, altered gut microbiota, and increased ATP in the colon. Pglyrp3 deficiency contributes colitis-predisposing intestinal microflora and increased intestinal ATP, whereas Nod2 deficiency contributes higher apoptosis and higher sensitivity to increased ATP and to microflora.

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Disclosures

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