Published in final edited form as:
J Immunol. 2014 September 15; 193(6): 3055–3069. doi:10.4049/jimmunol.1301548.

Peptidoglycan recognition protein 3 and Nod2 synergistically protect mice from dextran sodium sulfate-induced colitis

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Abstract

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 inflammatory bowel disease in humans. 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 *Nod2* role 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 (DSS)-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 DSS-induced colitis in *Pglyrp3*$^{-/-}$*Nod2*$^{-/-}$ mice depended on increased apoptosis of intestinal epithelium, changed gut microflora, and elevated ATP. *Pglyrp3* deficiency contributed colitispredisposing 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.

INTRODUCTION

Mammalian gastrointestinal tract is inhabited by thousands of microbial species that exist in a mutually beneficial relationship with the host. The gut microflora influences normal development of the host immune system, prevents colonization and damage induced by opportunistic bacteria, and regulates development and repair of the intestinal mucosa (1-4).

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qRT-PCR array data were deposited in NCBI GEO (accession number GSE47588), confidential reviewer link: http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=fjghxccmwqucupy&acc=GS

The host immune system along with environmental factors, in turn, shapes the composition of the microflora. Crohn's Disease (CD) and ulcerative colitis (UC) are common inflammatory bowel diseases (IBD) and are associated with deficiencies in the host immune system and with changes in gut microflora (5, 6). Host genetic factors involved in microbial recognition and immune responses are associated with IBD; however, the specific genes and their precise role in the development of both CD and UC remain mostly unknown (7-10). Similarly, the precise changes in the microflora and their role in the pathogenesis of IBD are not clearly understood (4-6).

Peptidoglycan Recognition Proteins (PGRP or Pglyrp) are innate immunity proteins that are conserved from insects to mammals, recognize the bacterial cell wall component peptidoglycan, and are antibacterial (11-13). Mammals have four *Pglyrps*, *Pglyrp1*, *Pglyrp2*, *Pglyrp3*, and *Pglyrp4* (14, 15); Pglyrp1 is expressed in polymorphonuclear leukocytes, whereas Pglyrp2, Pglyrp3, and Pglyrp4 are expressed in epithelial cells of many organs, including salivary glands, throat, tongue, esophagus, stomach, and intestine (15-17). All mammalian Pglyrps are secreted proteins and are bactericidal for both Gram-negative and Gram-positive bacteria (12, 17-19). Pglyrp2 is also an amidase that hydrolyzes bacterial cell wall peptidoglycan (20, 21).

Pglyrps are important for maintaining a normal gut microbiome and for protection against experimental colitis (22). *Pglyrp1*$^{-/-}$, *Pglyrp2*$^{-/-}$, *Pglyrp3*$^{-/-}$, and *Pglyrp4*$^{-/-}$ mice are all more sensitive to experimental (DSS-induced) colitis than WT mice (22). DSS-treated *Pglyrp*-deficient mice show a greater loss of body weight, more severe intestinal bleeding, more severe colon pathology, hyperplasia of the lamina propria, and loss of colonic epithelial cells (22). These changes are accompanied by higher production of IFN-γ and increased numbers of NK cells in the colon (22). In the absence of *Pglyrps* the gut flora changes to a more damaging, proinflammatory microbiome and these changes are responsible for the increased sensitivity to colitis in *Pglyrp*-deficient mice (22).

Pglyrps also modulate sensitivity to other inflammatory diseases. *Pglyrp2* protects mice against psoriasis-like skin inflammation (23) and is required for the development of experimental arthritis (24), whereas *Pglyrp3* and *Pglyrp4* protect mice against atopic dermatitis (25). By contrast, *Pglyrp1* has a pro-inflammatory effect in three mouse models of inflammatory skin diseases (psoriasis, atopic dermatitis, and contact dermatitis) (23, 25) and in experimentally induced asthma (26), but has anti-inflammatory effect in experimentally induced arthritis (24). Thus, each *Pglyrp* has a unique role in the development of different inflammatory diseases, and for this reason *Pglyrps* do not compensate for each other in mice deficient in a single *Pglyrp*. These unique effects of each *Pglyrp* are most likely based on their different effects on the microbiome (22). Moreover, genetic variants in human *Pglyrp1*, *Pglyrp2*, *Pglyrp3*, and *Pglyrp4* genes are associated with CD and UC (27). Several of these *Pglyrp* polymorphisms result in missense amino acid changes, which may indicate a role of the Pglyrp proteins in modulating sensitivity to colitis in humans (27).

Nod2 is a member of the NLR family of cytosolic receptors, is expressed in antigen-presenting cells, such as monocytes (28) and dendritic cells (29), and also in structural cells

such as intestinal epithelial cells (30), and is involved in host immune responses (31-33). Nod2 is an intracellular sensor for muramyl dipeptide, a bacterial peptidoglycan fragment (34, 35), and activation of Nod2 results in the activation of NF-κB and MAP kinase signaling cascades, followed by the synthesis and release of proinflammatory cytokines, chemokines, and antimicrobial peptides (33, 36). *Nod2* was the first susceptibility gene identified for CD and common homozygous or compound heterozygous mutations in *Nod2* increase susceptibility to CD by 20-fold (37-39). However, the functional role of *Nod2* in CD remains unexplained, primarily because CD is characterized by increased inflammation in the intestine, whereas, *Nod2* mutations associated with CD result in reduced activation of NF-κB and decreased production of inflammatory mediators after stimulation with muramyl dipeptide (35, 40). Studies in both mice and humans also present variable and contradictory roles of *Nod2* in innate immune responses and in the development of intestinal inflammation. In few studies that examined the role of *Nod2* in ulcerative colitis the data are also contradictory. Earlier results show no difference between WT and $Nod2^{-/-}$ mice in the sensitivity to DSS-induced colitis (41), whereas later studies show higher sensitivity of $Nod2^{-/-}$ mice to similar treatment (42, 43). Genome-wide association studies have demonstrated that CD and UC share many susceptibility loci, however a *Nod2* loss-of-function variant, which is a risk allele for CD, may have a protective effect for UC (10). These results indicate similarities and difference in the mechanisms of disease development between CD and UC.

Here we tested the hypothesis that deficiencies in both *Pglyrp3* and *Nod2* synergize in increasing the sensitivity to experimental colitis, because both Pglyrp3 and Nod2 recognize the bacterial component peptidoglycan and play a role in maintaining normal intestinal microflora (22, 43-46). We demonstrate that $Pglyrp3^{-/-}Nod2^{-/-}$ double knockout mice are more sensitive to DSS-colitis than $Pglyrp3^{-/-}$ and $Nod2^{-/-}$ single knockouts. The increased susceptibility to colitis in $Pglyrp3^{-/-}Nod2^{-/-}$ mice is accompanied by increased apoptosis of epithelial cells in the colon, elevated expression of chemokines and cytokines, changes in gut bacteria, and higher levels of ATP in the colon. Our data indicate that *Pglyrp3* deficiency is mainly responsible for the change to colitis-predisposing gut bacteria and higher ATP, whereas *Nod2* deficiency is mainly responsible for the higher apoptosis in the colon and responsiveness to higher levels of ATP. Our data also show that the lack of *Nod2* expression in bone marrow-derived cells combined with expression of *Nod2* in structural (radio-resistant) cells predisposes $Pglyrp3^{-/-}$ mice to very severe colitis. We conclude that *Pglyrp3* and *Nod2* deficiencies enhance sensitivity to experimental colitis through different mechanisms, which are synergistic.

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 knockout $Pglyrp3^{-/-}Nod2^{-/-}$ 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 to female ratios and similar litter size 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 hematoxylin/eosin stained sections. The original colony founder WT BALB/c mice were obtained from Harlan-Sprague-Dawly. WT germ-free mice (Swiss Webster female) were obtained from Taconic Farms (Hudson, NY).

All WT and knockout mice were on BALB/c background, female, 8-9 week-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 knockout littermates from heterozygous breeding pairs for three reasons: first, this strategy cannot be used for double knockout mice; second, this strategy may skew the results to the particular microflora present only in this breeding pair; and third, the effect of *Pglyrps* 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 extended period of time, we backcross our mutant mice to WT females once every other year and re-derive our homozygous knockout breeding pairs. The latter strategy also minimized genetic drift in the population. The BALB/c background of knockout 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–Northwest 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 (dextran sulfate sodium, 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 ulcerative colitis; however, the complexity of the human disease is not completely reproduced in the DSS model (47). The development and severity of colitis was evaluated as previously described (22), using: (a) mortality; (b) weight loss; and (c) stool and rectal bleeding scores of 0-16. For evaluation of histopathology, untreated control mice and mice treated with 5% DSS were sacrificed on day 7, proximal and distal sections of the colon were fixed in 10% buffered formalin, embedded in paraffin and sectioned. Sections were stained with hematoxylin and eosin 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, female WT germ-free mice, 4 to 5 week-old, maintained under sterile conditions, were treated 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). Briefly, fresh

stools were collected form 12 mice/strain, which were obtained from six 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 due to parent-to-parent and cage-to-cage differences. Stools were immediately resuspended in 0.2 ml of reduced anaerobic medium and frozen at −80°C until use. Development of colitis was monitored as described above.

To determine whether gut microflora from *Pglyrp3*$^{-/-}$ mice was also predisposing to colitis in *Nod2*$^{-/-}$ mice, an established non-germ-free model (48, 49) was used, in which *Nod2*$^{-/-}$mice were depleted of their intestinal microflora with a 3-week 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 days of sterile drinking water, followed by treatment with sterile 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 above.

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, WT, *Nod2*$^{-/-}$, *Pglyrp3*$^{-/-}$, and *Pglyrp3*$^{-/-}$ *Nod2*$^{-/-}$ were given 5% DSS in drinking water and on days 0 and 4 mice were injected intraperitoneally with bromodexoyuridine (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 and embedded in paraffin. Sections were stained with BrdU In-Situ Detection Kit (BD Pharmingen) and numbers of BrdU-positive cells per 500 epithelial cells were counted for each section.

TUNEL and cleaved caspase-3 staining and inhibition of cleaved caspase-3

The numbers of apoptotic cells in the colon were determined by TUNEL-labeling and by staining for cleaved caspase-3. WT, *Nod2*$^{-/-}$, *Pglyrp3*$^{-/-}$, and *Pglyrp3*$^{-/-}$*Nod2*$^{-/-}$ mice were given 5% DSS in drinking water and on days 0 and 4 mice were sacrificed. Proximal sections of the colon were fixed in 10% buffered formalin, embedded in paraffin, and sectioned. TUNEL-positive cells were detected by DeadEnd Colorimetric TUNEL System (Promega). Cleaved caspase-3 positive cells were detected using rabbit monoclonal antibody (clone 5A1E, Cell Signaling) and VECTASTAIN ABC Kit with DAB substrate Kit. TUNEL-positive or cleaved caspase-3-positive cells and total epithelial cells were counted on the entire section and percent of TUNEL-positive or cleaved caspase-3-positive cells were calculated.

To confirm the role of cleaved caspase 3 in the development of DSS-colitis, *Pglyrp3*$^{-/-}$ *Nod2*$^{-/-}$ mice were given 5% DSS in drinking water and gavaged daily into the stomach with the caspase inhibitor, Q-VD-Oph (R&D Systems, OPH001), at 80 μg/mouse/day or with 10% DMSO in PBS. The development of colitis was monitored as described in the Colitis Model section. To determine the effect of the caspase inhibitor, Q-VD-Oph, on apoptosis, *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). On day 4, proximal and distal regions of the colon were fixed in 10% buffered formalin, embedded in paraffin, and sectioned. TUNEL-positive cells were detected by DeadEnd Colorimetric TUNEL System (Promega). TUNEL-positive cells and total epithelial cells were counted on the entire section and percent of TUNEL-positive were calculated.

DSS-treated gavaged mice show a delay in the onset of colitis and mortality compared with DSS-treated non-gavaged 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 gavaging. This shift in the onset of colitis and mortality did not affect our conclusions, because in each experiment we compared identically treated mice, i.e., all non-gavaged, or all 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 on RNeasy spin columns using RNeasy Mini Kit (Qiagen) (22, 23, 26). Quantitative real-time reverse transcription (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 3 mice/group, in 3 separate experiments (total 9 mice per group). For each gene, ΔCt was calculated followed by normalization to 5 housekeeping genes (Hsp90ab1, Gusb, Hprt1, Gapdh, and Actb) included in each array, followed by calculation of ΔΔCt for each gene: ΔΔCt = ΔCt1 – ΔCt2, where ΔCt1 is for DSS-treated mice and ΔCt2 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 which 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 NCBI GEO (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 qPCR

The abundance of specific bacterial groups in mouse stools was measured by qPCR using group-specific primers for 16S rRNA genes as previously described (22). Briefly, 200 mg of fresh stools (freshly defecated feces) were collected from female mice of each strain (WT, *Nod2*$^{-/-}$, *Pglyrp3*$^{-/-}$, and *Pglyrp3*$^{-/-}$*Nod2*$^{-/-}$) and immediately processed for DNA isolation or snap-frozen at -80ºC. Stools were collected from all strains and from total of 18 mice/strain at three time points throughout the entire period of this study (6 mice/strain each time in 2008, 2010, and 2013). Each time for each strain mice originated from 3 different litters from different parents, 2 mice per litter, weaned into separate cages, but all 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 ng DNA and common primers for all *Eubacteria* or primers specific for the following bacterial groups: Mouse Intestinal *Bacteroides*, *Bacteroides sp*., *Eubacterium rectale*/*Clostridium coccoides*, *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. The cells were cultured overnight in RPMI-1640 supplemented with 10% FBS and antibiotic/antimycotic mix (Sigma) at 37°C, 5% CO_2. The next day non-adherent cells were collected and cultured in complete medium with 10 ng/ml of murine colony stimulating factor (PeproTech Inc.), in 48-well plates at 0.5×10^6 cells per well and incubated for an additional 5 to 6 days or until they became confluent. For colon cultures, colons were excised from untreated WT and *Nod2*$^{-/-}$ mice, washed to remove fecal material and cut into 0.5 cm pieces. Cells and colon fragments were stimulated with diluted stools prepared from WT or *Pglyrp3*$^{-/-}$ mice and supernatants were collected and assayed for cytokines and chemokines, CCL-2, CXCL-9, CXCL-10, IL-6, and CXCL-1 by ELISA using paired capture and detection antibodies and standards from R&D Systems. For stimulation, 30 mg of fresh stools were collected from 6 WT or *Pglyrp3*$^{-/-}$ mice and immediately placed on ice and processed. For each strain, mice originated from 3 different litters from different parents (2 mice per litter), weaned into separate cages, but all kept in the same room in our animal facility. Stools were suspended in 5 ml of DPBS, sonicated on ice for 2 min, and centrifuged at 30g for 2 min to remove debris. The OD_{660} of the supernatants was adjusted to 1.4 and diluted superantants were used for cell activation experiments.

ATP measurement and colitis

Fresh stools were collected from WT, *Nod2*$^{-/-}$, *Pglyrp3*$^{-/-}$, and *Pglyrp3*$^{-/-}$*Nod2*$^{-/-}$ mice, 6 to 8 mice/group from different cages, 6 pellets per mouse, and resuspended in PBS by gently vortexing to avoid lysis of bacteria. The resuspended feces were centrifuged at 5,000 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, WT and *Nod2*$^{-/-}$ mice were treated with 5% DSS to induce colitis and gavaged daily into the stomach with 50 μg of α,β-ATP (Sigma-Aldrich) per mouse or with PBS. The development of colitis was monitored as described in the Colitis Model section.

Generation of chimeric mice with bone marrow transplantation

Pglyrp3$^{-/-}$ and *Pglyrp3*$^{-/-}$*Nod2*$^{-/-}$ mice, 6 to 8 mice/recipient group, were subjected to total body irradiation of 850 rads for marrow 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. 4 h after irradiation, 5×10^6 bone marrow cells were injected into the retro orbital vein of recipient mice. Four transplanted groups were generated: *Pglyrp3*$^{-/-}$ > *Pglyrp3*$^{-/-}$, *Pglyrp3*$^{-/-}$ > *Pglyrp3*$^{-/-}$*Nod2*$^{-/-}$, *Pglyrp3*$^{-/-}$*Nod2*$^{-/-}$ > *Pglyrp3*$^{-/-}$*Nod2*$^{-/-}$, *Pglyrp3*$^{-/-}$*Nod2*$^{-/-}$ > *Pglyrp3*$^{-/-}$. Transplanted mice were kept in a HEPA-filtered flow hood and given sterilized food and water (52). For the first four weeks 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 gavaging with 0.2 ml of suspension containing 12 mg of preserved stools (22), three times over 6 days. Mice were then allowed to recover for one week 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 gavage procedure and measurement of severity of colitis were done as described in the Colitis Model section. Bone marrow reconstitution was verified at 6 weeks 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 *Nod2* WT and KO alleles were determined by PCR analysis (24, 41).

Isolation of lamina propria cells and flow cytometry

Nod2$^{-/-}$ mice were gavaged once daily into the stomach with 50 μg of α,β-ATP per mouse or with PBS and given 5% DSS in drinking water to induce colitis. Mice were sacrificed on day 4 and colon lamina propria (LP) cells were isolated as described (53). Briefly, colons were excised, washed to remove fecal material and cut into 0.5 cm pieces. Colon pieces were shaken in HBSS with 5% FBS and 1 mM EDTA at 37°C, 2 times for 20 min. Epithelial cells were removed by straining through 100 μm filter and the colon tissue was digested with 2 mg/ml collagenase D (Worthington T3), 40 μg/ml DNAse I (Sigma DN25-1G) in RPMI-1640 supplemented with 10% FCS for 80 min. The colon LP cell suspensions were obtained by passing the digested tissue through 100 μm and then 40 μm

filters, centrifuging, and resuspending in RPMI-1640 with 5% FBS. Colon LP cells were stained with the following fluorochrome-conjugated antibodies in different combinations: CD45-Vioblue, CD3-PE, CD8-APC from Miltenyi; CD4-APC, CD4-Pacific Blue, IFN-γ-PE, IL4-PE, IL10-PE, IL17-PE, and TGFβ-APC from Biolegend, and Foxp3-PE from eBioscience. All extracellular and intracellular staining was performed as described previously (25, 26). CD4-stained cells were fixed, permeabilized, and stained for Foxp3. Prior to staining for cytokines, CD4-stained cells were stimulated with TPA (25 ng/ml) and ionomycin (250 ng/ml) in the presence of the Golgi inhibitor, monensin, for 4 h at 37°C, 5% CO_2, fixed, permeabilized, and stained for cytokines (25, 26). Cells were analyzed by flow cytometry using MACSQuant (Miltenyi) cytometer. CD3, CD4, and CD8 positive cells were measured within the CD45 gate. IFN-γ, IL-4, IL-10, IL-17, TGFβ, and Foxp3 positive cells were measured within the CD4 gate.

Statistical analysis

All quantitative results are presented as means ± SEM, with statistical significance of the differences between groups determined by the two-sample one-tailed Student's *t*-test, except for survival, which was analyzed using Chi-square test; $p \leq 0.05$ was considered significant.

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, accelerated and significantly greater weight loss, and significantly higher diarrhea and intestinal bleeding than *Pglyrp3*$^{-/-}$, *Nod2*$^{-/-}$, and WT mice. *Pglyrp3*$^{-/-}$ mice, as shown 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, loss of goblet cells, and extent of ulceration on hematoxylin and eosin 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, loss of goblet cells, and ulceration than WT and *Nod2*$^{-/-}$ mice (Fig. 1B and 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 and 1C). Thus, our phenotypic and histopathologic data indicate that the double knockout *Pglyrp3*$^{-/-}$*Nod2*$^{-/-}$ mice are more sensitive to DSS-induced colitis than single knockouts *Pglyrp3*$^{-/-}$ and *Nod2*$^{-/-}$ and WT mice.

Pglyrp3$^{-/-}$*Nod2*$^{-/-}$ mice are deficient in cell proliferation and have higher apoptosis in the intestine

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. In order to measure permeability changes, DSS-colitis was induced 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.

In order 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, DSS-treated or untreated WT, *Nod2*$^{-/-}$, *Pglyrp3*$^{-/-}$, and *Pglyrp3*$^{-/-}$*Nod2*$^{-/-}$ mice were gavaged with BrdU and proliferating cells were identified by immunohistochemistry. *Pglyrp3*$^{-/-}$, *Nod2*$^{-/-}$, and *Pglyrp3*$^{-/-}$*Nod2*$^{-/-}$ mice all had significantly fewer BrdU-positive cells than WT mice (Fig. 2B). Also, *Pglyrp3*$^{-/-}$ mice had significantly fewer BrdU-positive 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-positive cells than *Pglyrp3*$^{-/-}$, *Nod2*$^{-/-}$, and WT mice and *Nod2*$^{-/-}$ mice had significantly more TUNEL-positive 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 positive cells, significantly higher than *Pglyrp3*$^{-/-}$, *Nod2*$^{-/-}$, and WT mice (Fig. 2D). DSS-treated *Nod2*$^{-/-}$ mice had significantly higher numbers of cleaved caspase-3 positive 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-positive 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 Table S1 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; 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-1f6, 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-1f6, 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 Table S1).

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 the 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*$^{-/-}$ and WT mice (data not shown). These results indicate that the colitis phenotype in *Pglyrp3*$^{-/-}$*Nod2*$^{-/-}$ double knockout 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, which 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-knockout 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 knockout 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 knockout compared with WT mice: *Lactobacillus* and *Clostridium perfringens* groups in *Nod2*$^{-/-}$, *Pglyrp3*$^{-/-}$, and *Pglyrp3*$^{-/-}$*Nod2*$^{-/-}$ mice; *Eubacterium rectale*, *Clostridium 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*$^{-/-}$ 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 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 weeks to deplete their intestinal microflora. At the end of 3 weeks 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 non-promoting (WT) stools (Fig. 4 and ref. 22). Culture supernatants were then assayed for different cytokines and chemokines. Stools from *Pglyrp3*$^{-/-}$ mice induced higher production of CCL-2, CXCL-9, and IL-6 in cells from WT, but not from *Nod2*$^{-/-}$ colon cells and fragments (Fig. 5A) and higher production of CCL-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*$^{-/-}$ mice are more pro-inflammatory than stools from WT mice (22) and show that induction of these pro-inflammatory chemokines and cytokines is significantly higher on *Nod2*$^{+/+}$ than on *Nod2*$^{-/-}$ background. The results in Fig. 5 also show that although the responses of cells from *Nod2*$^{-/-}$ mice are generally lower than from WT mice (as expected), the cells from *Nod2*$^{-/-}$ 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*$^{-/-}$ mice are differentially responsive to stools from WT and *Pglyrp3*$^{-/-}$ mice. For example, CXCL-10 production was not reduced in colon from *Nod2*$^{-/-}$ mice, compared with WT mice (Fig. 5A), and bone marrow macrophages from both WT and *Nod2*$^{-/-}$ mice produced IL-6 in response to stools only from *Pglyrp3*$^{-/-}$ 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*$^{-/-}$*Nod2*$^{-/-}$ compared with *Pglyrp3*$^{-/-}$ mice (Fig. 3 and Table S1).

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: (i) ATP is produced by gut microflora; (ii) ATP is known to increase sensitivity to T-cell-mediated colitis by increasing the numbers of inflammatory Th17 cells in the intestine (54); and (iii) we had increased expression of Th17 markers, CCL-2, CXCL-1, and CXCL-5 in the colon of DSS-treated *Pglyrp3*$^{-/-}$ and *Pglyrp3*$^{-/-}$*Nod2*$^{-/-}$ mice (Fig. 3).

Pglyrp3$^{-/-}$ and *Pglyrp3*$^{-/-}$*Nod2*$^{-/-}$ mice had significantly higher levels of ATP in the stools than WT and *Nod2*$^{-/-}$ mice (Fig. 6A). Thus, we next tested whether elevated ATP increases the sensitivity to DSS-colitis in *Nod2*$^{-/-}$ and WT mice. We used *Nod2*$^{-/-}$ mice for these experiments to recreate the *Nod2*$^{-/-}$ genetic background of *Pglyrp3*$^{-/-}$*Nod2*$^{-/-}$ mice and one aspect of *Pglyrp3* deficiency (higher ATP) and we used WT mice as controls. *Nod2*$^{-/-}$ and WT mice were treated with DSS to induce colitis, gavaged daily into the stomach with the stable ATP analog, α,β-ATP or PBS (control), and assayed for the development of colitis. *Nod2*$^{-/-}$ and WT mice treated with α,β-ATP showed significantly higher mortality, significantly greater loss in body weight, and significantly higher stool scores, compared with PBS-treated *Nod2*$^{-/-}$ and WT mice, respectively (Fig. 6B). Furthermore, *Nod2*$^{-/-}$ mice treated with α,β-ATP had significantly higher mortality, significantly greater loss in body weight, and significantly higher stool scores, than WT mice treated with α,β-ATP. These results indicate that increased levels of ATP promote DSS-induced colitis in *Nod2*$^{-/-}$ and WT mice, and that *Nod2* deficiency further enhances the promoting effect of ATP on the severity of colitis. Our results also suggest that the increased sensitivity to DSS-colitis in *Pglyrp3*$^{-/-}$*Nod2*$^{-/-}$ mice can be partially explained by the elevated ATP in the intestine and that *Pglyrp3* deficiency contributes the increased ATP, whereas *Nod2* deficiency enhances sensitivity to ATP and severity of colitis.

To further understand the role of elevated colon ATP levels in the increased sensitivity to DSS-colitis of *Pglyrp3*$^{-/-}$*Nod2*$^{-/-}$ mice, we next compared the levels of inflammatory T cells in the colon lamina propria of DSS treated *Nod2*$^{-/-}$ mice in the presence and absence of the stable ATP analog, α,β-ATP. Again, we used *Nod2*$^{-/-}$ mice for these experiments to recreate *Nod2*$^{-/-}$ genetic background of *Pglyrp3*$^{-/-}$*Nod2*$^{-/-}$ mice and one aspect of *Pglyrp3* deficiency (higher ATP). The numbers of $CD3^+$, $CD4^+$, $CD4^+TGF\beta^+$, $CD4^+IL\text{-}4^+$ (Th2), and $CD4^+IL\text{-}17^+$ (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 and D). There was no significant difference in $CD4^+$ cells expressing $Foxp3^+$ (Fig. 6C), which is the marker for the anti-inflammatory regulatory T (Treg) cells. 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 Table S1).

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 the elevated ATP and that *Nod2*$^{-/-}$ mice respond to 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. 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*$^{-/-}$ > *Pglyrp3*$^{-/-}$*Nod2*$^{-/-}$ and *Pglyrp3*$^{-/-}$*Nod2*$^{-/-}$ > *Pglyrp3*$^{-/-}$ bone marrow chimeric mice, as well as control mice, which received bone marrow cells from the same strain of mice: *Pglyrp3*$^{-/-}$ > *Pglyrp3*$^{-/-}$ and *Pglyrp3*$^{-/-}$*Nod2*$^{-/-}$ > *Pglyrp3*$^{-/-}$*Nod2*$^{-/-}$. Chimerism was confirmed by PCR of genomic DNA and showed that blood cells from *Pglyrp3*$^{-/-}$*Nod2*$^{-/-}$ > *Pglyrp3*$^{-/-}$ mice contained only *Nod2* KO allele, whereas blood cells from *Pglyrp3*$^{-/-}$ > *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*$^{-/-}$ 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*$^{-/-}$*Nod2*$^{-/-}$ > *Pglyrp3*$^{-/-}$) predisposes *Pglyrp3*$^{-/-}$ mice to very severe colitis, more severe than the lack of *Nod2* in both bone marrow-derived and structural cells (*Pglyrp3*$^{-/-}$*Nod2*$^{-/-}$ > *Pglyrp3*$^{-/-}$*Nod2*$^{-/-}$) or the lack of *Nod2* only in structural cells (*Pglyrp3*$^{-/-}$ > *Pglyrp3*$^{-/-}$*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

Inflammatory bowel disease 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 of 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 increased 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-knockout 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-knockouts, 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 caspases 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 pro-inflammatory 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 *Pglyrps* 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, as 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, CXCL-10, CXCL-11, and CCL-2 in DSS-colitis in *Pglyrp*-deficient mice (22). CCR-7, TNF-α, IL-1β, and IL-1f6 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 knockout 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-treated *Pglyrp3*$^{-/-}$*Nod2*$^{-/-}$ mice may be in response to the altered gut microflora due to *Pglyrp3* deficiency. IL-1f6 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-1f6 expression is increased in a *Nod*$^{-/-}$ but not a *Nod*$^{+/+}$ 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 form WT, *Nod2*$^{-/-}$, *Pglyrp3*$^{-/-}$, or *Pglyrp3*$^{-/-}$*Nod2*$^{-/-}$ mice demonstrate that *Pglyrp3* deficiency, but not *Nod2* deficiency, contributes colitis-promoting microflora to the double-knockout 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 Courtier-Maillard *et al*., who used co-housed WT mice (43).

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 of time. We did not use F2 littermates in our experiments because a change to colitis-predisposing microflora due a *Pglyrp*-deficiency does not happen immediately or over a few weeks, but is established over more than one generation (Dziarski, unpublished data). Therefore, all mice used in our experiments were derived from knockout strains that were established after backcrossing to the same WT stock at least six 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 due to different parents and different cages. Stools for microflora testing were collected at three time points over a 5-year 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-year 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 cells in the colon lamina propria. Extracellular levels of ATP are tightly controlled and elevated amounts serve as a danger signal that modulates immune functions (54, 62, 63). High extracellular ATP induces differentiation of lamina propria 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 Treg cells (66). ATP in the colon may be released from injured epithelial cells or from gut bacteria, which are known to secrete large amount of ATP (67). The most likely source of elevated ATP in *Pglyrp3*$^{-/-}$ and *Pglyrp3*$^{-/-}$*Nod2*$^{-/-}$ mice is the altered microbiota and not injured epithelial cells, because elevated 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 lamina propria Treg cells, and increased differentiation of lamina propria 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 Treg cells in the colon lamina propria compared with PBS-treated mice. Thus, in our model of ulcerative colitis, 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 Treg cells. Thus, our data suggest that a *Pglyrp3* deficiency results in elevated 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* due to the lack of *Nod2* expression in bone marrow-derived cells combined with the expression of *Nod2* in structural (radio-resistant) cells in a *Pglyrp3*-deficient 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 cells-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 microflora-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 due to a combination of factors, including higher apoptosis, altered gut microbiota, and increased ATP in the colon. *Pglyrp3* deficiency contributes colitispredisposing intestinal microflora and increased intestinal ATP, whereas *Nod2* deficiency contributes higher apoptosis and higher sensitivity to increased ATP and to microflora.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Julie Cook for maintaining and breeding our mice.

This work was supported by USPHS Grants R01AI028797 and R01AI073290 from NIH Abbreviations: CD, Crohn's disease; DSS, dextran sodium sulfate; IBD, inflammatory bowel disease; LP, lamina propria; Nod, nucleotide-binding oligomerization domain; Pglyrp, peptidoglycan recognition protein; qRT-PCR, quantitative real time RT-PCR; Treg, regulatory T; UC, ulcerative colitis; WT, wild type.

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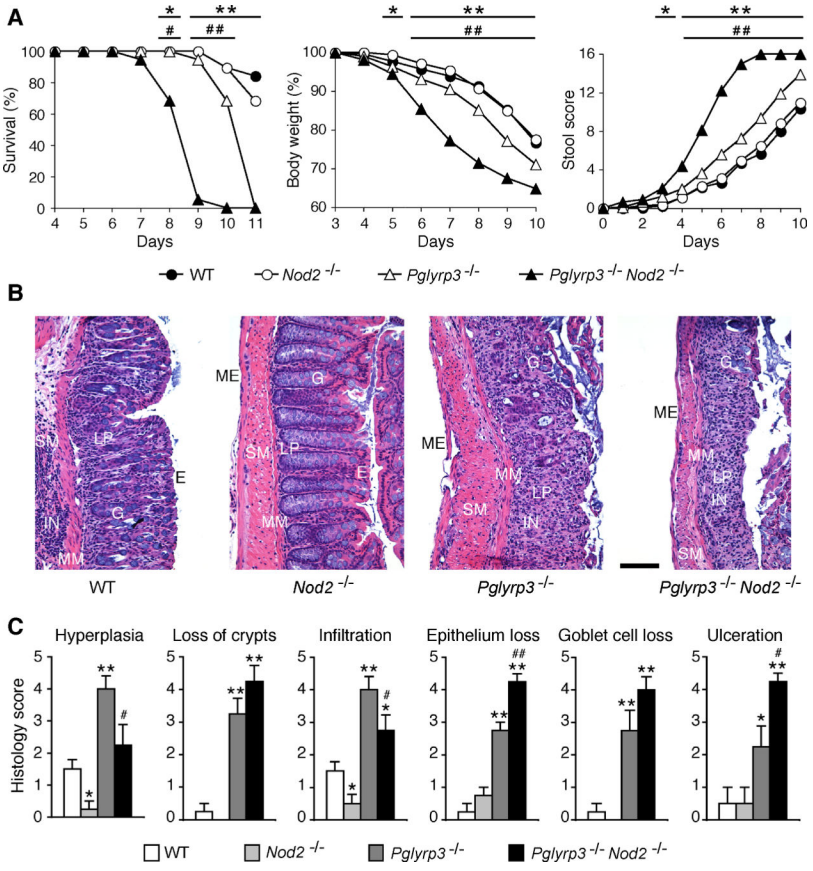

Figure 1. *Pglyrp3*$^{-/-}$*Nod2*$^{-/-}$ mice are more susceptible to DSS-induced colitis than *Pglyrp3* and *Nod2* single-deficient mice

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 hematoxylin-eosin stained sections of the colon were (**B**) qualitatively and (**C**) quantitatively evaluated for severity of colitis. Epithelial cells (E), lamina propria (LP), goblet cells (G), muscularis mucosa (MM), submucosa (SM), muscularis externa (ME), and inflammatory cell infiltrations (IN) are indicated; size bar = 100 μm. The data are (**A**) mean ± SEM of 19-20 mice/group; and (**B**) representative images or (**C**) mean ± SEM from 4 mice/group. Significance of differences between *Pglyrp3*$^{-/-}$*Nod2*$^{-/-}$ and WT mice is indicated by asterisks (*); and between *Pglyrp3*$^{-/-}$*Nod2*$^{-/-}$ and *Pglyrp3*$^{-/-}$ is indicated by the number sign (#); *, #, $P \leq 0.05$; **, ##, $P \leq 0.005$.

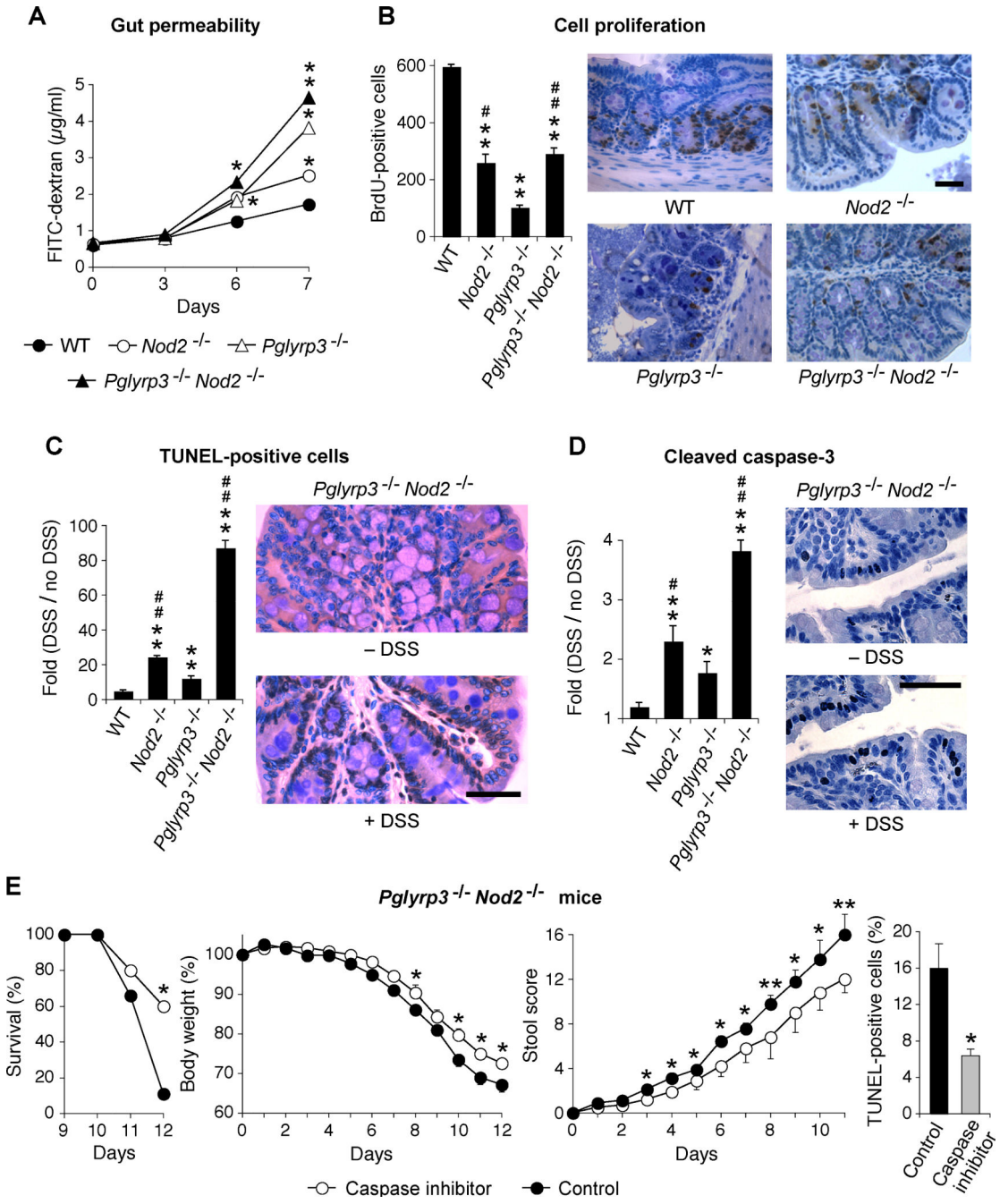

Figure 2. ***Pglyrp3*$^{-/-}$*Nod2*$^{-/-}$ mice have higher permeability and increased apoptosis in the colon**

(**A** to **D**) WT, *Nod2*$^{-/-}$, *Pglyrp3*$^{-/-}$, and *Pglyrp3*$^{-/-}$*Nod2*$^{-/-}$ mice were treated with 5% DSS and (**A**) gavaged with FITC-dextran on days 0, 3, 6, and 7 and assayed for FITC-dextran in the serum; (**B**) 4 days later injected with BrdU and colon sections were stained for BrdU; (**C** and **D**) 4 days later colon sections were stained for (**C**) TUNEL; or (**D**) cleaved caspase-3. (**E**) *Pglyrp3*$^{-/-}$ *Nod2*$^{-/-}$ mice were treated with 5% DSS and gavaged daily with the caspase inhibitor Q-VD-Oph or with PBS for the control group and development of colitis was monitored by survival, change in body weight, and stool and rectal bleeding scores or 4 days

later colon sections were stained for TUNEL. The data are mean ± SEM of (**A**) 12, (**B**, **C,** and **D**) 4 to 6, and (**E**) 9 to 10 mice/group for colitis and 6 mice/group for TUNEL staining; representative histological images are shown in (**B-D**). Significance of differences for (**A** to **D**) knockout versus WT mice or (**E**) inhibitor treated versus control group is indicated by asterisks (*); and between *Pglyrp3*$^{-/-}$*Nod2*$^{-/-}$ or *Nod2*$^{-/-}$ versus *Pglyrp3*$^{-/-}$ is indicated by the number sign (#); *, #, $P \leq 0.05$; **, ##, $P \leq 0.005$.

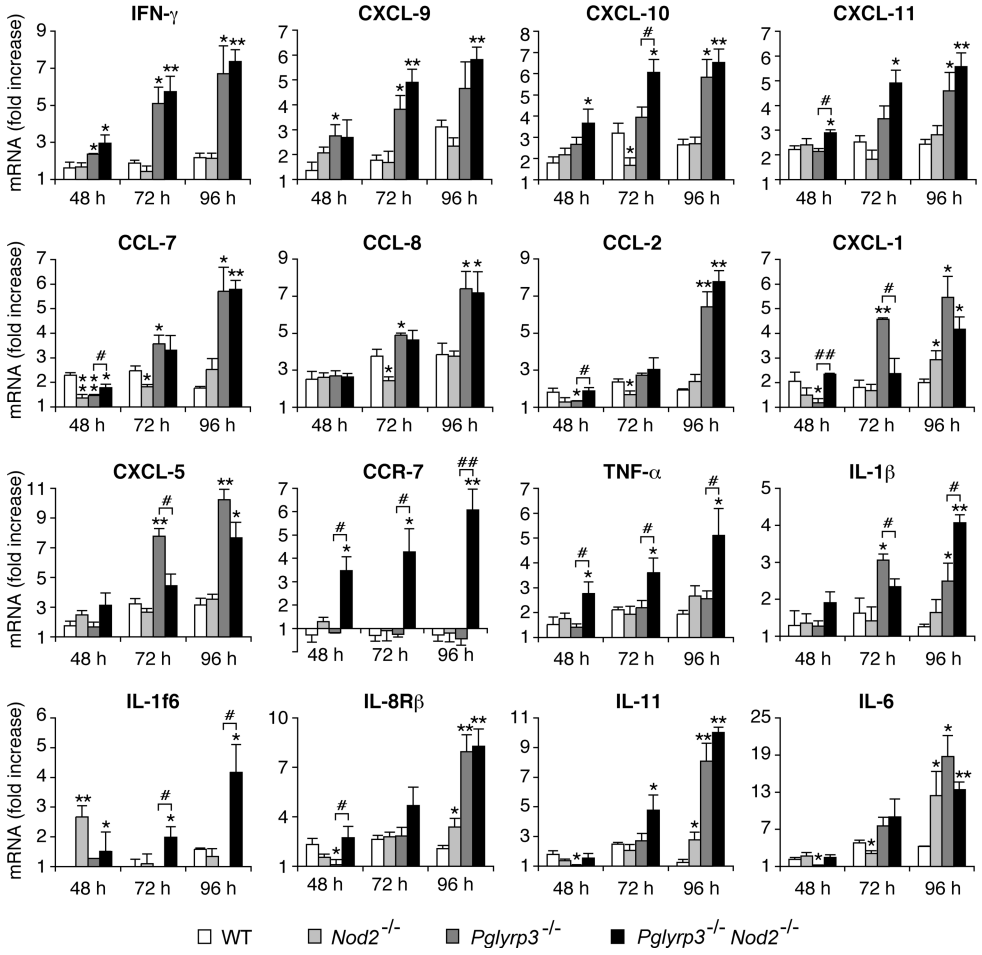

Figure 3. *Pglyrp3*$^{-/-}$*Nod2*$^{-/-}$ mice express higher levels of chemokine and cytokine mRNA in the colon following DSS treatment

WT, *Nod2*$^{-/-}$, *Pglyrp3*$^{-/-}$, and *Pglyrp3*$^{-/-}$*Nod2*$^{-/-}$ mice were treated with 5% DSS for indicated times and expression of inflammatory genes in the colon was measured by qRT-PCR. The results are means ± SEM of the ratio of the amount of mRNA in DSS-treated to untreated mice from 9 mice per group, 3 mice pooled per array. Significance of differences between knockouts and WT mice is indicated by asterisks (*) and between *Pglyrp3*$^{-/-}$ *Nod2*$^{-/-}$ and *Pglyrp3*$^{-/-}$ is indicated by the number sign (#); *, #, $P \leq 0.05$; **, ##, $P \leq 0.005$. The data are selected from the 96 inflammatory gene expression array shown in supplemental Table S1.

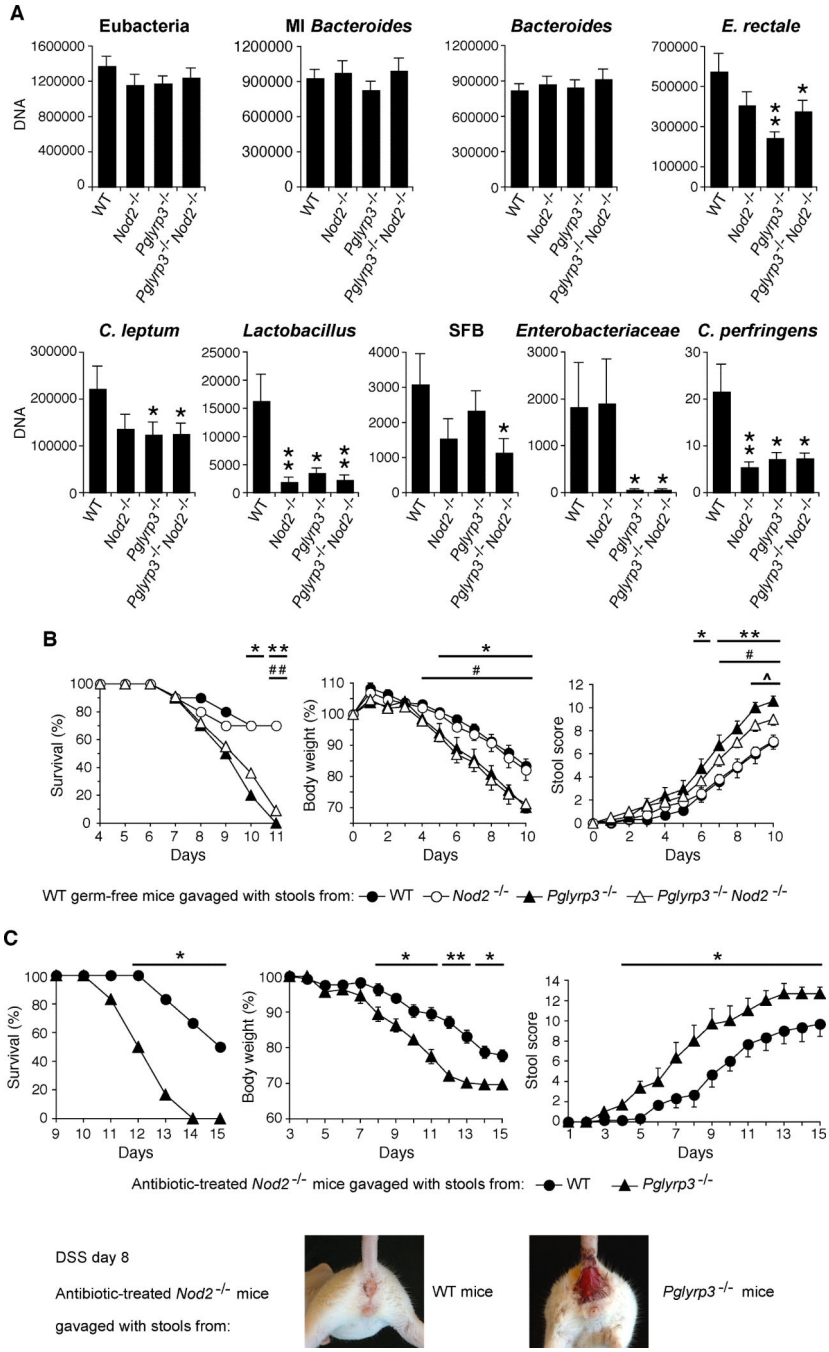

Figure 4. *Pglyrp3*$^{-/-}$*Nod2*$^{-/-}$ mice have changes in intestinal bacterial flora, however only *Pglyrp3*-dependent changes contribute to the increased sensitivity to colitis in *Pglyrp3*$^{-/-}$ *Nod2*$^{-/-}$ mice

(**A**) Changes in the composition of bacterial flora in the stools of WT and knockout mice in all *Eubacteria* and the indicated bacterial groups, measured by qPCR; (**B**) WT germ-free mice were treated with 4% DSS and gavaged daily with stool homogenates from conventionally raised WT, *Nod2*$^{-/-}$, *Pglyrp3*$^{-/-}$, or *Pglyrp3*$^{-/-}$*Nod2*$^{-/-}$ mice or (**C**) *Nod2*$^{-/-}$ mice were pre-treated with antibiotics for 3 weeks and then gavaged daily with stool homogenates from conventionally raised WT or *Pglyrp3*$^{-/-}$ and treated with 4% DSS.

Development of colitis in **B** and **C** was evaluated by measuring survival, body weight, and stool score. Gross rectal bleeding in mice gavaged with stools from *Pglyrp3*$^{-/-}$ mice but not from WT mice is shown in (**C**). The results are means ± SEM of (**A**) 18 mice/group and (**B and C)** 6 mice/group from 2 experiments. Significance of differences for (**A**) *, knockout versus WT; (**B)** *, *Pglyrp3*$^{-/-}$ versus *Nod2*$^{-/-}$ and WT; #, *Pglyrp3*$^{-/-}$*Nod2*$^{-/-}$ versus *Nod2*$^{-/-}$ and WT; ^, *Pglyrp3*$^{-/-}$*Nod2*$^{-/-}$ versus *Pglyrp3*$^{-/-}$; (**C**) *, *Pglyrp3*$^{-/-}$ versus WT; *, #, ^, $P \leq 0.05$; **, ##, ^^, $P \leq 0.005$.

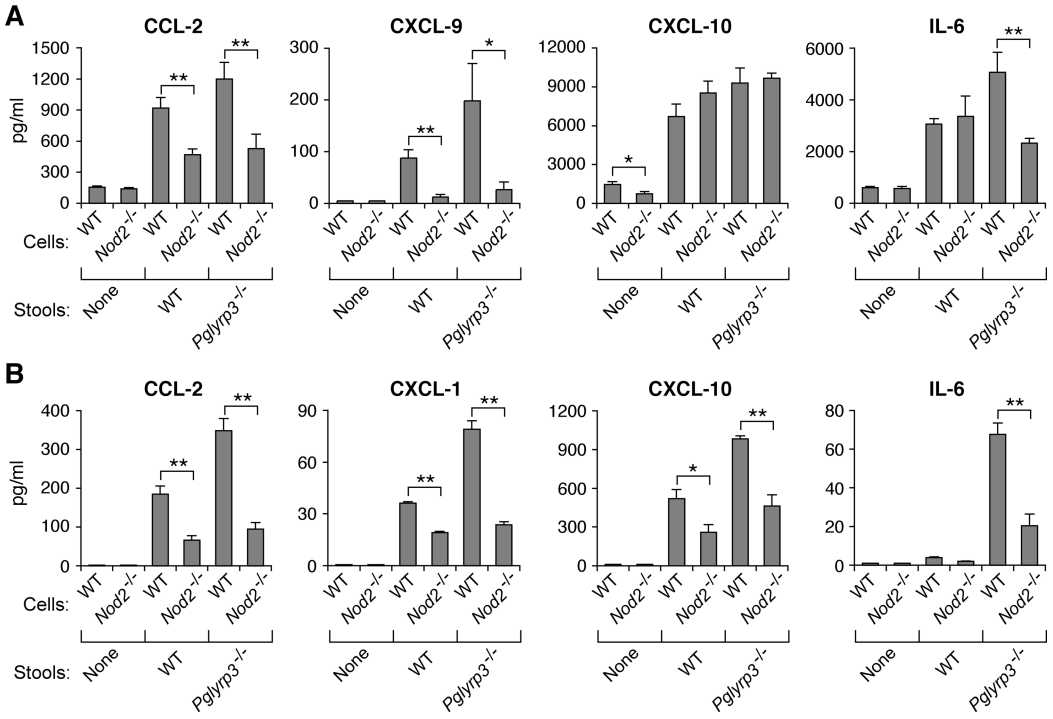

Figure 5. WT (*Nod*$^{+/+}$) but not *Nod2*$^{-/-}$ macrophages and colon cells produce more chemokines and cytokines in response to stools from *Pglyrp3*-deficient mice

(**A**) Colon cells and colon fragments or (**B**) bone marrow-derived macrophages from WT or *Nod2*$^{-/-}$ mice were stimulated with stools from WT or *Pglyrp3*$^{-/-}$ mice or with PBS (no stimulant). Supernatants were assayed for the indicated chemokines or cytokines. The results are means ± SEM (N = 6 experiments); significance of differences for *Nod2*$^{-/-}$ versus WT cells; *, $P \leq 0.05$; **, $P \leq 0.005$.

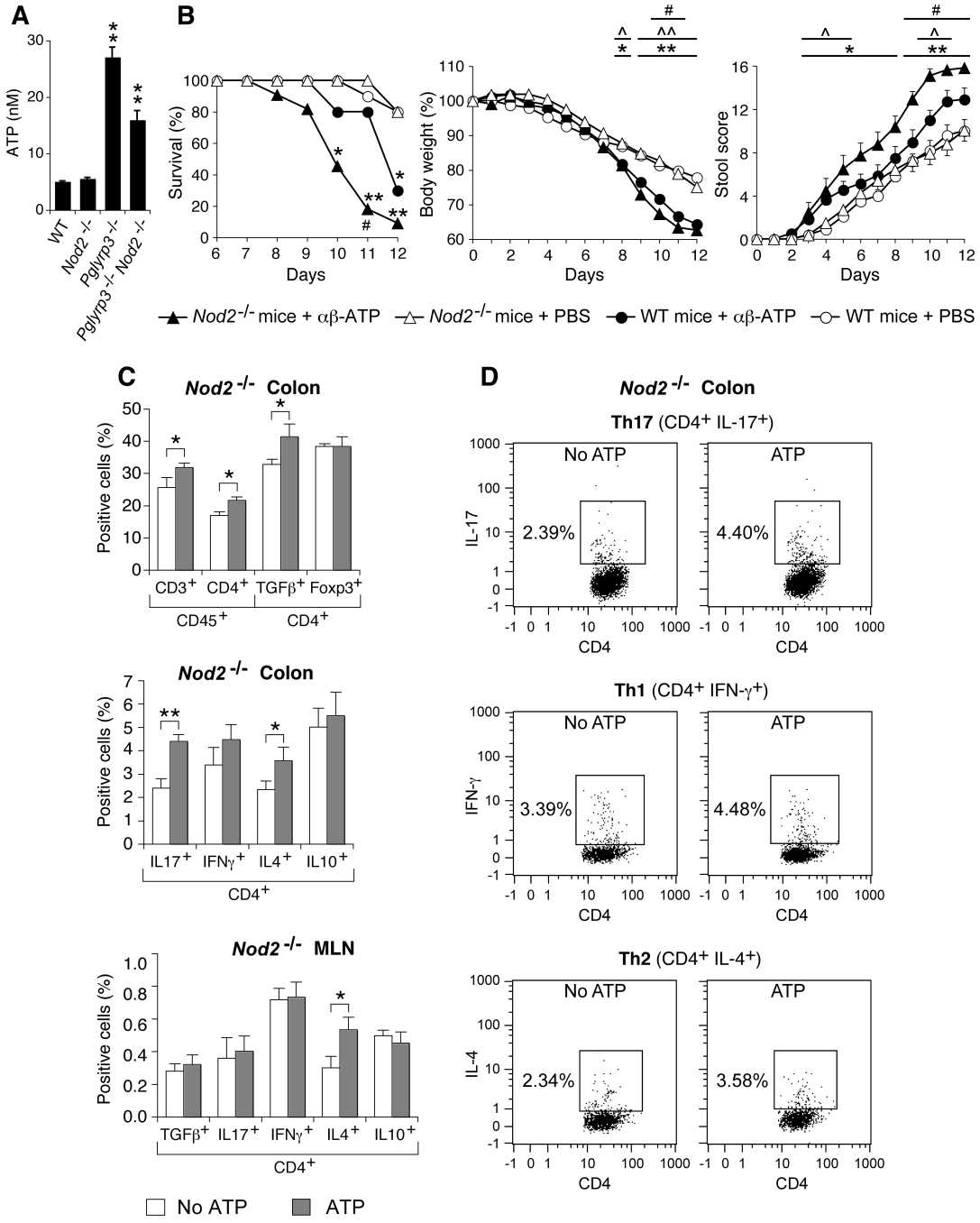

Figure 6. *Pglyrp3*$^{-/-}$*Nod2*$^{-/-}$ and *Pglyrp3*$^{-/-}$ mice have higher levels of ATP in the colon, and intestinal ATP preferentially increases sensitivity of *Nod2*$^{-/-}$ mice to DSS-colitis

(**A**) Amounts of ATP in the stools of WT, *Nod2*$^{-/-}$, *Pglyrp3*$^{-/-}$, and *Pglyrp3*$^{-/-}$*Nod2*$^{-/-}$ mice. (**B**) WT and *Nod2*$^{-/-}$ mice treated with 5% DSS and gavaged with the ATP analog, α,β-ATP, have lower survival and body weight, and higher stool scores than PBS-treated mice, and *Nod2*$^{-/-}$ mice are more sensitive to ATP than WT mice. (**C**) Increased percentages of the indicated cell types in colon lamina propria and mesenteric lymph nods (MLN) in *Nod2*$^{-/-}$ mice, gated for CD45 or CD4. (**D**) Representative dot plots for Th17,

Th1, and Th2 cells for the data shown in (**C**). The results are means ± SEM of (**A**) 6-8, (**B**) 10-11, and (**C**) 6-7 mice/group from 2 experiments. Significance of differences for (**A**) knockouts *versus* WT; (**B**) *, **, ATP- *versus* PBS-treated *Nod2*$^{-/-}$ mice; ^, ^^, ATP- versus PBS-treated WT mice; #, *Nod2*$^{-/-}$ *versus* WT ATP-treated mice; or (**C**) ATP *versus* PBS; *, ^, #, $P \leq 0.05$; **, ^^, $P \leq 0.001$.

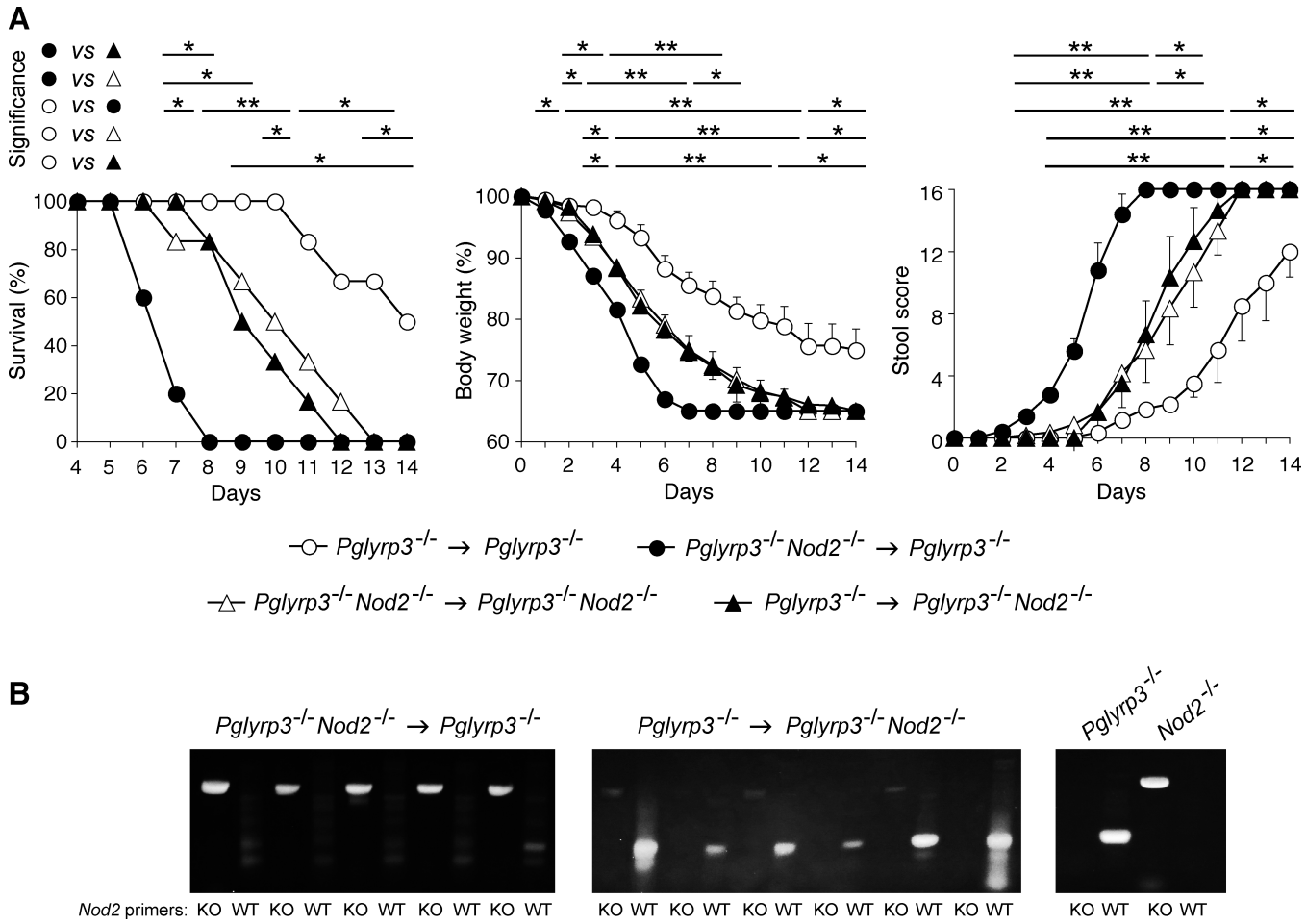

Figure 7. Lack of *Nod2* in bone marrow-derived cells combined with presence of *Nod2* in structural cells predisposes *Pglyrp3*$^{-/-}$ mice to severe colitis

(**A**) Chimeric (*Pglyrp3*$^{-/-}$ > *Pglyrp3*$^{-/-}$*Nod2*$^{-/-}$ and *Pglyrp3*$^{-/-}$*Nod2*$^{-/-}$ > *Pglyrp3*$^{-/-}$) and transplanted control (*Pglyrp3*$^{-/-}$ > *Pglyrp3*$^{-/-}$ and *Pglyrp3*$^{-/-}$*Nod2*$^{-/-}$ > *Pglyrp3*$^{-/-}$ *Nod2*$^{-/-}$) mice were treated with 4% DSS in drinking water and development of colitis was evaluated. DSS-treated mice were monitored over time for survival, change in body weight, and stool and rectal bleeding. The results are means ± SEM of 5-6 mice/group. Statistically significant differences between the indicated groups are shown as *, $P \leq 0.05$; **, $P \leq 0.005$. (**B**) Presence of *Nod2* KO (1 kb amplified fragment) or *Nod2* WT (300 bp amplified fragment) alleles was assessed by PCR of genomic DNA from blood cells of chimeric (*Pglyrp3*$^{-/-}$*Nod2*$^{-/-}$ > *Pglyrp3*$^{-/-}$ and *Pglyrp3*$^{-/-}$ > *Pglyrp3*$^{-/-}$*Nod2*$^{-/-}$) or non-transplanted *Pglyrp3*$^{-/-}$ and *Nod2*$^{-/-}$ mice.