Toll-interacting Protein Modulates Colitis Susceptibility in Mice

Michel H. Maillard, MD, PhD, Hristina Bega, MSc,* Holm H. Uhlig, MD, PhD,† Nicolas Barnich, PhD,‡,§ Teddy Grandjean, MSc,‖,¶,**,†† Mathias Chamaillard, PhD,‖,¶,**,†† Pierre Michetti, MD,*,‡‡ and Dominique Velin, PhD**

Background: The intestinal epithelium accommodates with a myriad of commensals to maintain immunological homeostasis, but the underlying mechanisms regulating epithelial responsiveness to flora-derived signals remain poorly understood. Herein, we sought to determine the role of the Toll/interleukin (IL)-1 receptor regulator Toll-interacting protein (Tollip) in intestinal homeostasis.

Methods: Colitis susceptibility was determined after oral dextran sulfate sodium (DSS) administration or by breeding *Tollip*$^{-/-}$ on an *IL-10*$^{-/-}$ background. The intestinal flora was depleted with 4 antibiotics before DSS exposure to assess its contribution in colitis onset. Bone marrow chimeras were generated to identify the cellular compartment, whereby Tollip may negatively regulate intestinal inflammation in response to DSS. Tollip-dependent epithelial barrier functions were studied in vitro by using *Tollip*-knockdown in Caco-2 cells and in vivo by immunohistochemistry and fluorescein isothiocyanate-labeled dextran gavage.

Results: Genetic ablation of *Tollip* did not lead to spontaneous intestinal inflammatory disorders. However, Tollip deficiency aggravated spontaneous disease onset in IL-10$^{-/-}$ mice and increased susceptibility to DSS colitis. Increased colitis severity in *Tollip*-deficient mice was not improved by bacterial flora depletion using broad-spectrum antibiotics. In addition, DSS exposure of bone marrow chimeric mice revealed a protective role for Tollip in nonhematopoietic cells. Knockdown of *Tollip* in epithelial cells led to exaggerated NFκ-B activity and proinflammatory cytokine secretion. Finally, DSS-treated *Tollip*$^{-/-}$ mice showed enhanced intestinal permeability and increased epithelial apoptosis when compared with wild-type controls, a finding that coincided with tight junction alterations on injury.

Conclusion: Overall, our data show an essential role for Tollip on colitis susceptibility in mice.

(Inflamm Bowel Dis 2014;20:660–670)

Key Words: toll-interacting protein, colitis, epithelium

In the intestinal tract, signals between the luminal content and the mucosa are central for educating, shaping, and maintaining intestinal homeostasis.[1,2] These signals are in part mediated by a family of molecules called pattern recognition receptors (PRR) that include the Toll-like receptors (TLR) family. TLR signaling occurs mostly through the adaptor MyD88 (myeloid differentiation primary response gene 88), but MyD88-independent pathways also exist.[3] Depending on the experimental setting and the receptor studied, TLR signals have been shown to be either protective or detrimental in experimental models of colitis. Indeed, although *Tlr2*-, *Tlr4*-, or *MyD88*-deficient mice are more sensitive to colitis induced by dextran sulfate sodium (DSS),[4] absence of TLR4 expression confers resistance to trinitrobenzene sulfate-induced colitis.[5] TLR signals also play a role in some models of spontaneous colitis as in interleukin (IL)-10$^{-/-}$ mice where housing in a germ-free environment is sufficient to protect mice from colitis. Consistently, IL-10$^{-/-}$ mice bred on a MyD88$^{-/-}$ background are protected from colitis.[6,7]

Thus, adequate immune responses toward endogenous bacteria contribute to gut homeostasis and rely on well-balanced proinflammatory and antiinflammatory PRR signals. Accordingly, a variety of normal host mechanisms have evolved to avoid overzealous PRR signals and protect the host from uncontrolled microbiota-triggered inflammatory reactions.[8] For example, genetic loss of the Toll-IL-1R signaling regulator SIGIRR (single immunoglobulin IL-1 receptor-related molecule) was linked to defects in epithelial homeostasis, which renders mice more susceptible to DSS-induced colitis.[9] Similarly, loss of the E3 ligase-editing

Supplemental digital content is available for this article. Direct URL citations appear in the printed text and are provided in the HTML and PDF versions of this article on the journal's Web site (www.ibdjournal.org).

Received for publication January 6, 2014; Accepted January 21, 2014.

From the *Service of Gastroenterology and Hepatology, Department of Medicine, Centre Hospitalier Universitaire Vaudois and University of Lausanne, Lausanne, Switzerland; †Translational Gastroenterology Unit, Division of Experimental Medicine, University of Oxford, John Radcliffe Hospital, Oxford, United Kingdom; ‡M2iSH, UMR1071 Inserm, Université d'Auvergne, USC-INRA 2018, Clermont-Ferrand, France; §Institut Universitaire de Technologie en Génie Biologique, Aubière, France; ‖Univ Lille Nord de France, Lille, France; ¶Institut Pasteur de Lille, Center for Infection and Immunity of Lille, Lille, France; **Centre National de la Recherche Scientifique, Unité Mixte de Recherche 8204, Lille, France; ††Institut National de la Santé et de la Recherche Médicale, U1019, Team 7, Equipe FRM, Lille, France; and ‡‡La Source-Beaulieu, Lausanne, Switzerland.

Funding was provided by the European Crohn's and Colitis Organisation (ECCO), a career development grant from the University of Lausanne and the San Salvatore Foundation to M. H. Maillard, and by the Swiss National Foundation Grant 3200B0-120717 to D. Velin. M. Chamaillard was financially supported by grants from the Fondation pour la Recherche Médicale (Equipe FRM 2009) and the FEDER/Région Nord-Pas-de-Calais.

The authors have no conflicts of interest to disclose.

Reprints: Michel H. Maillard, MD, PhD, Service of Gastroenterology and Hepatology, Department of Medicine, Centre Hospitalier Universitaire Vaudois and University of Lausanne, CHUV-BH10, Rue du Bugnon 46, 1011 Lausanne, Switzerland (e-mail: michel.maillard@chuv.ch).

Copyright © 2014 Crohn's & Colitis Foundation of America, Inc.

DOI 10.1097/MIB.0000000000000006

Published online 25 February 2014.

Downloaded from https://academic.oup.com/ibdjournal/article/20/4/660/4579016 by guest on 05 December 2022

Copyright © 2014 Crohn's & Colitis Foundation of America, Inc. Unauthorized reproduction of this article is prohibited.

enzyme A20 or IRAK-M (IL-1 receptor-associated kinase M), 2 other inhibitors of NF-κB and TLR signaling, renders mice more susceptible to experimental colitis.[10,11]

The Toll-interacting protein (Tollip) is an ubiquitously expressed 274 amino acid protein that binds to IRAK (IL-1 receptor-associated kinase) and inhibits its phosphorylation following IL-1 receptor engagement.[12] Notably, signals downstream of TLR2 and TLR4 are also negatively regulated by Tollip, an effect that was associated with direct interaction of Tollip to these receptors.[13] Intestinal epithelial cells (IECs) and antigen-presenting cells constitutively or inducibly express Tollip.[14,15] Previous exposure of IECs to a TLR ligand (such as lipopolysaccharide) is thought to induce a hyporesponsive state to the second challenge with the same or another TLR ligand by selectively limiting proinflammatory responses through upregulation of Tollip.[15] Interestingly, the amount of Tollip expression seems to directly correlate with the luminal bacterial load in vivo with highest *Tollip* expression found in healthy colonic mucosa.[15] IECs from patients with Crohn's disease (CD) fail to upregulate *Tollip* expression, suggesting that Tollip might be implicated in controlling chronic colitis development.[7,16] However, unlike SIGIRR, A20 or IRAK-M, the regulatory role of Tollip in intestinal homeostasis remains unknown in vivo.

MATERIALS AND METHODS

Mice

Wild type (WT) and IL-10$^{-/-}$ mice on a C57Bl/6J background were both purchased from Harlan (Ad Horst, the Netherlands) and bred under specific pathogen-free conditions together with *Tollip*$^{-/-}$ mice at the Centre Hospitalier Universitaire Vaudois. All animal procedures were approved by the State Veterinary Office (authorization no. 1748.1). *Tollip*$^{-/-}$ mice were generously provided by Kimberly Burns (Institut de Biochimie, Epalinges, Switzerland) and backcrossed over 10 generations on a C57Bl/6J background. To obtain *Tollip*$^{-/-}$/IL-10$^{-/-}$ mice, *Tollip*$^{-/-}$ mice were bred with IL-10$^{-/-}$ mice. Mice were screened by polymerase chain reaction (PCR) on tail DNA using specific primers for *Tollip* and *IL-10* as previously described.[17]

Induction of Colitis

Two- to 3-month-old *Tollip*$^{+/+}$ or *Tollip*$^{-/-}$ female mice (C57Bl/6J) were given 1.5% (wt/vol) DSS (MW 47,000; TdB Consultancy, Uppsala, Sweden) in drinking water ad libitum for 7 days and were either killed or returned to tap water until day 10. Mice were weighed and clinically monitored daily. At day 4 or 5, fecal blood scores were obtained using fecal occult blood tests (Colorectal test; Axonlab, Baden, Switzerland) as previously described.[18]

Tissue Sampling

Mice were killed and the dissected colons were carefully rinsed with 0.9% NaCl. A 1-cm section of distal colon was fixed in 4% (wt/vol) buffered formalin for subsequent paraffin embedding and histological analysis. The remaining part of the colon was blotted dry and frozen in liquid nitrogen. Frozen colon samples were thoroughly ground using a cooled mortar and pestle, weighed, and stored at −80°C for subsequent RNA isolation.

Histological Scoring of Colitis

Formalin-fixed colons from DSS- or water-exposed WT or *Tollip*$^{-/-}$ mice were H&E stained, and each sample was graded from 0 to 4 according to the score of Wirtz.[19] Similarly, H&E stained colons from IL-10$^{-/-}$ and *Tollip*$^{-/-}$/IL-10$^{-/-}$ mice were graded from 0 to 4 for colitis severity as previously described.[20] Histological scores of colon sections were analyzed by an independent person (H.U.) who was blinded to the experimental labeling groups.

Analysis of *Tollip* Expression in Primary Cells and Cell Lines

Murine colonic epithelial (MC38) and endothelial (H5V) cell lines were used and cultured with Dulbecco's modified eagle medium supplemented with 10% fetal calf serum and with penicillin/streptomycin. The HEPA 1.6 and P815 cell lines were also used to analyze gene expression in murine hepatocytes and mastocytes, respectively. Thioglycollate-elicited peritoneal macrophages and granulocytes were collected, and dendritic cells were derived from bone marrow. CD4$^+$ and CD8$^+$ T cells were negatively isolated from spleen of C57BL6/J mice, according to the manufacturer's instructions (Dynal; Invitrogen, Cergy-Pontoise, France). Isolated RNA from primary cells and cell lines were reverse transcribed with the High-capacity complementary DNA Archive kit (Applied Biosystems, Foster City, CA), according to the manufacturer's instructions. The resulting complementary DNA (equivalent to 5 ng of total RNA) was amplified using the SYBR Green real-time PCR kit and detected on a Stratagene Mx3005P (Agilent Technologies, Basel, Switzerland). Reverse-transcription PCR (RT-PCR) was performed with the following primers for *Tollip* (sense: 5′GCCTCAGCATCACTGTGGTA3′; antisense: 5′GTCCAAGCTATGCGGTCATC3′) that were designed using Primer express software, version 1.0 (Applied Biosystems, Life Technologies, Zug, Switzerland). On completion of the PCR amplification, a DNA melting curve analysis was carried out to confirm the presence of a single amplicon. *Actb* was used as an internal reference gene to normalize the transcript levels.

Preparation of Bone Marrow Chimeras

WT or *Tollip*$^{-/-}$ female 3-month-old mice were lethally irradiated (900 rads) and subsequently injected intravenously with donor bone marrow cells (10^7 cells per recipient mouse). Mice were then clinically followed for 3 months. Irradiated and reconstituted mice received enrofloxacin (Baytril; Bayer, Leverkusen, Germany) at 0.25 mg/mL in their drinking water for 2 weeks to prevent posttransplant bacterial complications. Efficacy of bone marrow reconstitution was controlled by PCR analysis of chimeras' bone marrow DNA at the time of being killed.

Quantitative RT-PCR

Total RNA was isolated using RNeasy Plus Mini kit (Qiagen, Hombrechtikon, Switzerland). Sample quality was tested

Downloaded from https://academic.oup.com/ibdjournal/article/20/4/660/4579016 by guest on 05 December 2022

Copyright © 2014 Crohn's & Colitis Foundation of America, Inc. Unauthorized reproduction of this article is prohibited.

on agarose gels, and absence of genomic DNA was assessed by PCR using primers specific for the housekeeping gene GAPDH (sequences available on request). Total RNA samples (1 μg) were then reverse transcribed using the ThermoScript RT-PCR system (Invitrogen Life Technologies, Carlsbad, CA) according to the manufacturer's protocol, and oligo-dT as primers. PCR amplification was performed on a MyiQ iCycler (Bio-Rad, Hercules, CA) using the iQ SYBR Green Supermix (Bio-Rad) and primers specific for GAPDH, interferon [IFN]-γ, IL-1β, IL-6, KC, MIP-2, and IL-17 (sequences available on request). Primers for *Tollip* were purchased from Qiagen. For each individual sample, messenger RNA (mRNA) quantification was determined by normalizing the number of mRNA copies obtained for the gene of interest per million of mRNA copies obtained for GAPDH. Relative mRNA expression levels were finally calculated by normalizing individual data to the mean value obtained with the control group of untreated or water-exposed mice.

Determination of Myeloperoxidase Activity

Myeloperoxidase activity in 1 μg total proteins was determined as previously described.[20]

Fecal Bacterial Quantification by 16s ribosomal DNA Analysis

Total DNA from about 10 mg of frozen feces/mouse was extracted using the QIAamp Stool DNA extraction kit according to manufacturer's instructions (Qiagen, Courtaboeuf, France). Absolute numbers of bacteria were determined by quantitative PCR analysis on a Stratagene Mx3005P (Agilent Technologies) from serial dilutions of a plasmid construct containing a 16s ribosomal DNA construct shared by all bacteria (sequence available on request). On completion of the PCR amplification, a DNA melting curve analysis was carried out to confirm the presence of a single amplicon.

Epithelial Permeability

Age- and sex-matched WT or *Tollip*$^{-/-}$ 2- to 3-month-old female littermates were exposed to DSS 1.5% for 5 days and subsequently orally fed with 160 μL fluorescein isothiocyanate (FITC)-Dextran (concentration 50 mg/mL, 8 mg per mouse, Sigma, Buchs, Switzerland). After 4 hours, serum was isolated from peripheral blood after cardiac puncture. Fluorescence was determined using 100 μL of serum in a transparent plastic 96 wells flat-bottom plate for reading by a multifunction plate spectrofluorimeter (480 nm excitation and 530 nm emission). A standard curve was generated using serum from an untreated mouse as blank and serum supplemented with increasing concentrations of FITC-Dextran (0–10 mg FITC/mL serum).

Cell Culture, siRNA Transfection and Stimulation

Caco-2 cells were obtained from the American Type Culture Collection (Manassas, VA) and grown until 90% to 100% confluent in complete medium as described previously.[21] Cells were then transfected 8 hours after seeding with a cationic lipid (Lipofectamine 2000; Invitrogen, Cergy-Pontoise, France) according to the manufacturer's protocols. Two target sequences for *Tollip* and control siRNA were purchased from Qiagen (sequences available on request). Cell monolayers were in vitro stimulated with 0.1, 1, and 10 μg/mL of peptidoglycan or lipopolysaccharide (obtained from Sigma, saint-Quentin Falavier, France. LPS: catalog n° L3024, from *Escherichia coli* O111:B4, purified by ion-exchange chromatography, TLR ligand tested; PGN: catalog n° 69,554, from *Bacillus subtilis*) for 24 hours, and cell supernatants were collected for IL-8 determination by enzyme-linked immunosorbent assay according to the manufacturer's recommendation (R&D, Lille, France).

Immunohistochemical Analysis of Cleaved Caspase-3 and Zona Occludens-1

Four-micrometer paraffin-embedded colonic sections were deparaffinised and rehydrated with xylol and ethanol 100% to 70% before exposure to 10 mM sodium citrate (pH 6.0) for 10 minutes at 89°C for antigens retrieval. Slides were then quenched with H_2O_2 for 10 minutes and blocked with normal goat serum 5% in TBST 1x. The rabbit anti-human (cross reactive with mouse) cleaved caspase-3 antibody (cell signaling, 1:100) was used as primary antibody followed by a biotinylated anti-rabbit antibody (diluted 1/250; Vector Laboratories, Burlingame, CA). We used the avidin-biotinylated horseradish peroxidase complex as the third step (Vectastain ABC Kit; Vector Laboratories). For the zona occludens-1 staining, tris-EDTA pH 9.0 at 89°C was used for antigen retrieval, and sections were exposed to 0.2% sapponin in phosphate-buffered saline for permeabilization. The rat anti-human (cross reactive with mouse) Zona occludens-1 antibody (diluted: 50; Santa Cruz, Heidelberg, Germany) was used as primary antibody followed by a goat anti-rat Alexa 488 nm antibody (diluted 1:500; Molecular Probes, Life Technologies).

BrdU Labeling

BrdU (Sigma) was intraperitoneally injected (1.5 mg per mouse in 750 μL phosphate-buffered saline) 1 hour before being killed. Colons were then harvested, fixed with 4% (wt/vol) buffered formalin, and paraffin-embedded. Five-micrometer tissue section was deparaffinized and antigen retrieved using the Retrievagen (pH 6.0) according to manufacturer's recommendations (BD Biosciences, Allschwill, Switzerland). Immunostaining of BrdU was performed using a mouse biotinylated monoclonal anti-BrdU antibody (diluted 1/30; Zymed, Zug, Switzerland), followed by avidin-biotinylated horseradish peroxidase complex (Vectastain ABC Kit; Vector Laboratories Burlingame, CA). BrdU^{+} cells were visualized using diaminobenzidine tetrahydrochloride substrate kit for peroxidase (Vector Laboratories). Mayer's hematoxylin was used as a nuclear counterstain.

RESULTS

Unchallenged *Tollip*$^{-/-}$ Mice Do Not Develop Spontaneous Colitis

Given the known regulatory role of Tollip on Toll-IL-1R-mediated signaling pathway, we first thought to determine

Downloaded from https://academic.oup.com/ibdjournal/article/20/4/660/4579016 by guest on 05 December 2022

Copyright © 2014 Crohn's & Colitis Foundation of America, Inc. Unauthorized reproduction of this article is prohibited.

whether Tollip deficiency was sufficient to induce pathological inflammatory responses in the gut. Histological analysis of *Tollip*$^{-/-}$ mice at various ages showed no gross abnormalities on small IEC morphology, villous architecture, crypt length, and Paneth cell numbers (Fig. A and B, Supplemental Digital Content 1, http://links.lww.com/IBD/A417). Likewise, we failed to find any change in goblet cell number or stigmata of inflammation in the colon (Fig. C, Supplemental Digital Content 1, http://links.lww.com/IBD/A417). Consistently, intestinal or colonic epithelial proliferation was also found unchanged when compared with that in controls (Fig. D and E, Supplemental Digital Content 1, http://links.lww.com/IBD/A417). Thus, *Tollip* deficiency does not interfere with gut mucosal homeostasis under steady-state condition.

Tollip$^{-/-}$ Mice Are More Susceptible to Chronic and Acute Colitis

We next thought to assess whether Tollip deficiency might impact experimental colitis outcome. We first bred *Tollip*$^{-/-}$ mice on an *IL-10*$^{-/-}$ background, as colitis in this model is known to depend on TLR signals.[6] Tollip deficiency significantly aggravated chronic colitis onset in IL-10$^{-/-}$ mice as demonstrated by severe inflammatory changes in colons from 2 month-old *Tollip*$^{-/-}$/IL-10$^{-/-}$ mice compared with IL-10$^{-/-}$ littermates (Fig. 1A). Consistently, IL-17 and IFN-γ expression was significantly increased in colons from *Tollip*$^{-/-}$/IL-10$^{-/-}$ mice than IL-10$^{-/-}$ mice (Fig. 1B).

To further address whether Tollip might also regulate acute inflammatory responses on injury, we challenged *Tollip*$^{-/-}$ mice with DSS. An enhanced mortality of *Tollip*$^{-/-}$ mice was observed on injury with either 2% or 2.5% DSS, whereas none of similarly treated control mice succumbed. On challenge with DSS 1.5%, none of the animals died but *Tollip*$^{-/-}$ mice lost more weight than WT controls as soon as day 5 of DSS exposure (Fig. 1C). Consistently, *Tollip*$^{-/-}$ mice showed increased rectal bleeding (data not shown), enhanced histological scores of colitis (Fig. 1D), and elevated proinflammatory cytokines expression (IL-1β and IL-6) (Fig. 1E) when compared with that in similarly treated control animals. Altogether, our findings demonstrate that Tollip plays a protective role against chronic and acute colitis development.

Enhanced Colitis Sensitivity in Tollip-deficient Mice Is Not Improved on Broad-spectrum Antibiotic Treatment

The gut microbial content is thought to maintain mucosal homeostasis as evidenced by the link between dysbiosis and the pathogenesis of CD.[22] Most experimental and spontaneous models of colitis in mice depend on gut microbial composition as judged by reduced disease risk in the absence of any commensals.[7] To address whether DSS-induced colitis susceptibility in *Tollip*$^{-/-}$ mice is influenced by gut bacterial load, *Tollip*$^{+/+}$ and *Tollip*$^{-/-}$ mice were treated with 4 broad-spectrum antibiotics (ampicillin, vancomycin, neomycin sulfate, and metronidazole [AVNM]) for 1 month before and during a 7-day DSS 1.5% exposure[4] (Fig. 2A). As expected, fecal bacteria load was barely detectable in AVNM-treated *Tollip*$^{+/+}$ and *Tollip*$^{-/-}$ mice before DSS exposure (Fig. 2B). In DSS-treated animals that were not exposed to antibiotics, increased colitis susceptibility was associated with an approximate 2 log decrease in total fecal bacterial content in *Tollip*$^{-/-}$ compared with *Tollip*$^{+/+}$ mice. In contrast, we failed to observe any difference in fecal bacterial load of untreated *Tollip*$^{+/+}$ and *Tollip*$^{-/-}$ mice (Fig. 2B). Only mild histological signs of inflammation were observed on antibiotic treatment before DSS injury. As previously reported,[4,23] antibiotics-treated *Tollip*$^{+/+}$ mice developed significant but mild inflammatory changes in their colon on DSS exposure. In contrast, antibiotics-treated *Tollip*$^{-/-}$ mice had significantly greater scores of colitis (Fig. 2C, D) and expressed even more IL-6 mRNA but not IL-1β (Fig. 2E) than similarly treated control animals. Altogether, these data suggest that depletion of intestinal bacterial load does not improve disease severity in *Tollip*$^{-/-}$ mice.

Expression of *Tollip* in Nonhematopoietic Cells Is Required for Protection Against DSS-induced Colitis

Although Tollip was reported to be ubiquitously expressed,[12] quantitative PCR determination of Tollip expression in various cell types revealed a predominant expression in colonic epithelial cells (Fig. 3A). We thus asked whether colitis susceptibility is modulated by Tollip expression in hematopoietic versus nonhematopoietic cells. To do so, bone marrow chimeras were generated. Three months after bone marrow reconstitution, a specific defect in Tollip expression in hematopoietic (*Tollip*$^{-/-}$ bone marrow → WT recipient) or nonhematopoietic cells (WT bone marrow → *Tollip*$^{-/-}$ recipient) was confirmed by PCR analyses on tail DNA and bone marrow DNA. Unfortunately, we were unable to study *Tollip*$^{-/-}$ → *Tollip*$^{-/-}$ chimeras as all recipient mice died a few days after bone marrow transplantation. We nevertheless challenged the 3 remaining chimera groups with DSS for 7 days (Fig. 3A). As expected, WT → WT chimeras lost body weight starting at day 6 of DSS exposure (Fig. 3B). Interestingly, body weight loss in *Tollip*$^{-/-}$ → WT chimeras was comparable with WT → WT control mice suggesting that a hematopoietic-specific deficiency in *Tollip* expression is not sufficient to confer disease risk (Fig. 3B). In contrast, WT → *Tollip*$^{-/-}$ chimeras had a significant increase in body weight loss compared with WT → WT mice starting at day 5 of DSS exposure (Fig. 3B). These results were further supported by increased rectal bleeding scores (Fig. 3C), enhanced histological scores of colitis (Fig. 3D, E), and elevated transcript levels of IL-1β and IL-6 (Fig. 3F) in WT → *Tollip*$^{-/-}$ chimeras relative to WT → WT controls. Altogether, these data show that Tollip deficiency in nonhematopoietic cells is sufficient to render mice more susceptible to acute colitis.

Loss of *Tollip* Expression in Epithelial Cells Result in Enhanced Proinflammatory Response to Bacterial Products

Our data using chimeric mice suggest that the absence of Tollip in nonhematopoietic cells is mandatory to modulate disease

Downloaded from https://academic.oup.com/ibdjournal/article/20/4/660/4579016 by guest on 05 December 2022

Copyright © 2014 Crohn's & Colitis Foundation of America, Inc. Unauthorized reproduction of this article is prohibited.

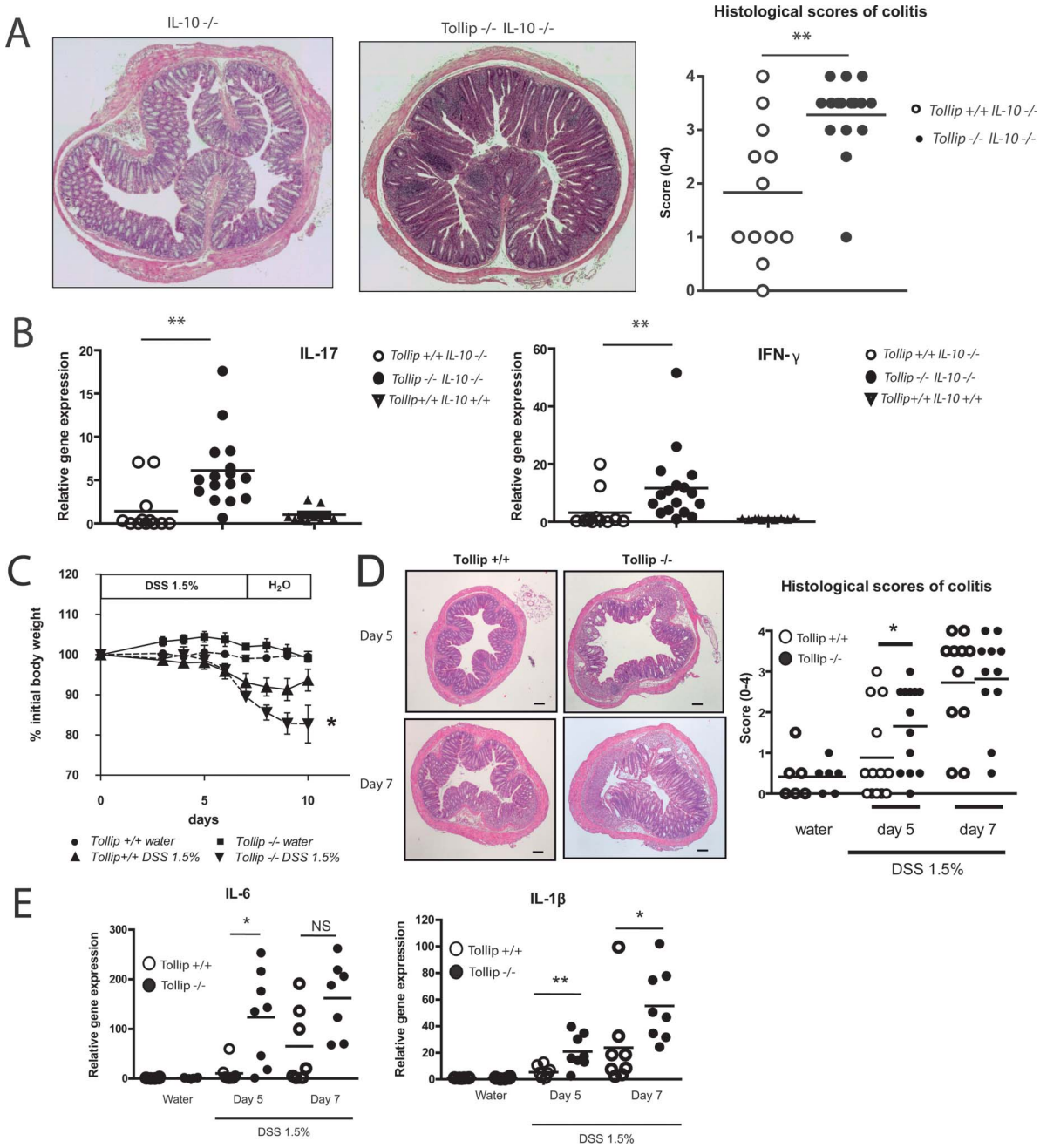

FIGURE 1. Tollip deficiency aggravates immune-mediated colitis and chemical injury. A, Representative H&E stained sections from colons of 2-month-old IL-10$^{-/-}$ or *Tollip*$^{-/-}$/IL-10$^{-/-}$ mice showing marked crypt hyperplasia and a more pronounced cellular infiltrate in the lamina propria of *Tollip*$^{-/-}$/IL-10$^{-/-}$ mice. Right panel: Blindly determined histological scores of colitis in IL-10$^{-/-}$ (white circles) or *Tollip*$^{-/-}$/IL-10$^{-/-}$ mice (black circles) at 2 (2 mo) of age. Scores obtained for 2-month-old sex-matched WT mice are shown as a negative control. Each circle represents 1 individual mouse. Horizontal bars represent means. Shown are data combined from several independent experiments. B, RT-qPCR analysis of expression of proinflammatory genes in 2-month-old IL-10$^{-/-}$ or *Tollip*$^{-/-}$/IL-10$^{-/-}$ mice. C, Evolution of the average weight loss over time in WT (triangles, solid line, n = 5) or *Tollip*$^{-/-}$ (inverted triangles, dashed line, n = 5) on exposure to DSS 1.5% compared with WT (dots, solid line, n = 5) or *Tollip*$^{-/-}$ mice (squares, dashed line, n = 5) kept under tap water. Shown are average ± SEM of 1 of at least 5 independent experiments. D, Representative histological changes in colons from mice exposed to DSS at days 5 and 7. Scale bars: 100 μm. Graphic: Histological scores of colitis of WT (white circles) or *Tollip*$^{-/-}$ (black circles) mice at day 0, 5, and 7 of DSS 1.5% exposure. Each dot represents 1 individual mouse. Horizontal bars represent means. E, RT-qPCR analysis of expression of proinflammatory genes at days 5 and 7 in WT (white circles) or *Tollip*-deficient mice (black circles). Data have been normalized to GAPDH expression and then to mean values for the water group. Statistical *P* values have been determined using the Mann–Whitney test. *$P < 0.05$. RT-qPCR, quantitative RT-PCR.

Copyright © 2014 Crohn's & Colitis Foundation of America, Inc. Unauthorized reproduction of this article is prohibited.

Downloaded from https://academic.oup.com/ibdjournal/article/20/4/660/4579016 by guest on 05 December 2022

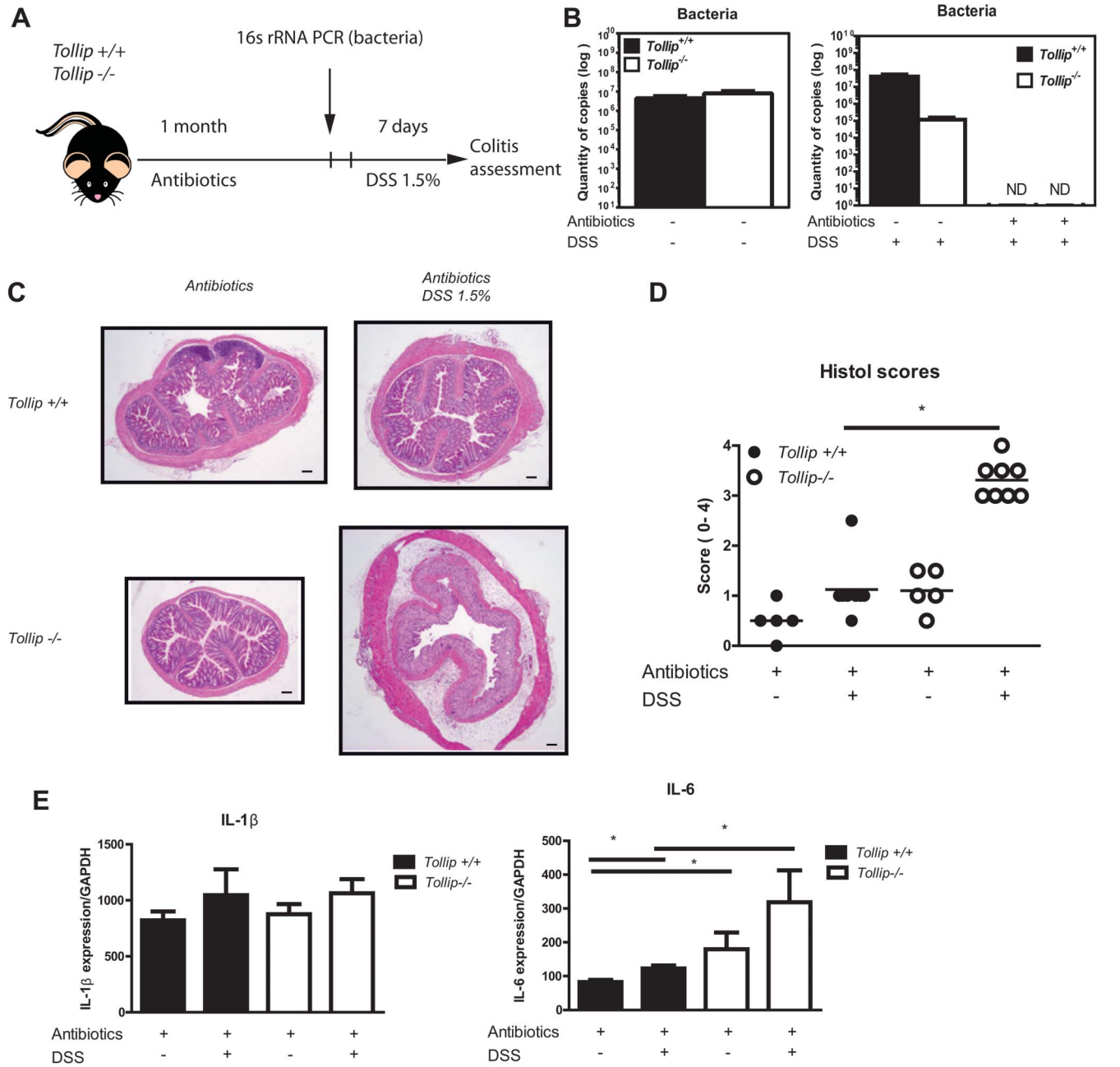

FIGURE 2. Gut flora depletion with antibiotics is not sufficient to improve disease severity in DSS-exposed *Tollip*$^{-/-}$ mice. A, *Tollip*$^{+/+}$ or *Tollip*$^{-/-}$ mice were treated with ampicillin (1 g/L), vancomycin (500 mg/L), neomycin sulfate (1 g/L), and metronidazole (1 g/L) (AVNM) in drinking water for 1 month before exposure to DSS 1.5% for 7 days. Antibiotics were kept during the DSS challenge to avoid recolonization. B, Quantitative PCR determination of 16s ribosomal DNA frequencies of total bacteria in unchallenged or antibiotics ± DSS-exposed *Tollip*$^{+/+}$ or *Tollip*$^{-/-}$ mice. Shown are mean ± SEM of 5 to 8 mice per group. C and D, Representative H&E stainings (C) and blindly determined histological scores of colitis (D) in *Tollip*$^{+/+}$ or *Tollip*$^{-/-}$ mice exposed to antibiotics ± DSS 1.5%. E, RT-qPCR analysis of expression of the proinflammatory IL-1β and IL-6 genes at day 7 of DSS 1.5% exposure in WT (black bars) or *Tollip*-deficient mice (white bars). Statistical *P* values have been determined using the Mann–Whitney test. *$P < 0.05$. RT-qPCR, quantitative RT-PCR.

risk in mice. Hence, we evaluated the impact of Tollip deficiency in IECs on bacterial product stimulation. To this end, we knocked down *Tollip* expression in the human colon carcinoma cell line Caco-2 using the siRNA technology. As shown in Figure 4A, *Tollip* expression was markedly reduced using this approach. As expected, stimulation of Caco-2 cells with lipopolysaccharide (TLR4 agonist) or with peptidoglycan (TLR2 and NOD2 agonist[19]) exposed to control siRNA led to a dose-dependent induction of IL-8 secretion (Fig. 4B). Importantly, stimulation of Tollip knocked-down Caco-2 cells led to a significant increase in IL-8

Copyright © 2014 Crohn's & Colitis Foundation of America, Inc. Unauthorized reproduction of this article is prohibited.

Downloaded from https://academic.oup.com/ibdjournal/article/20/4/660/4579016 by guest on 05 December 2022

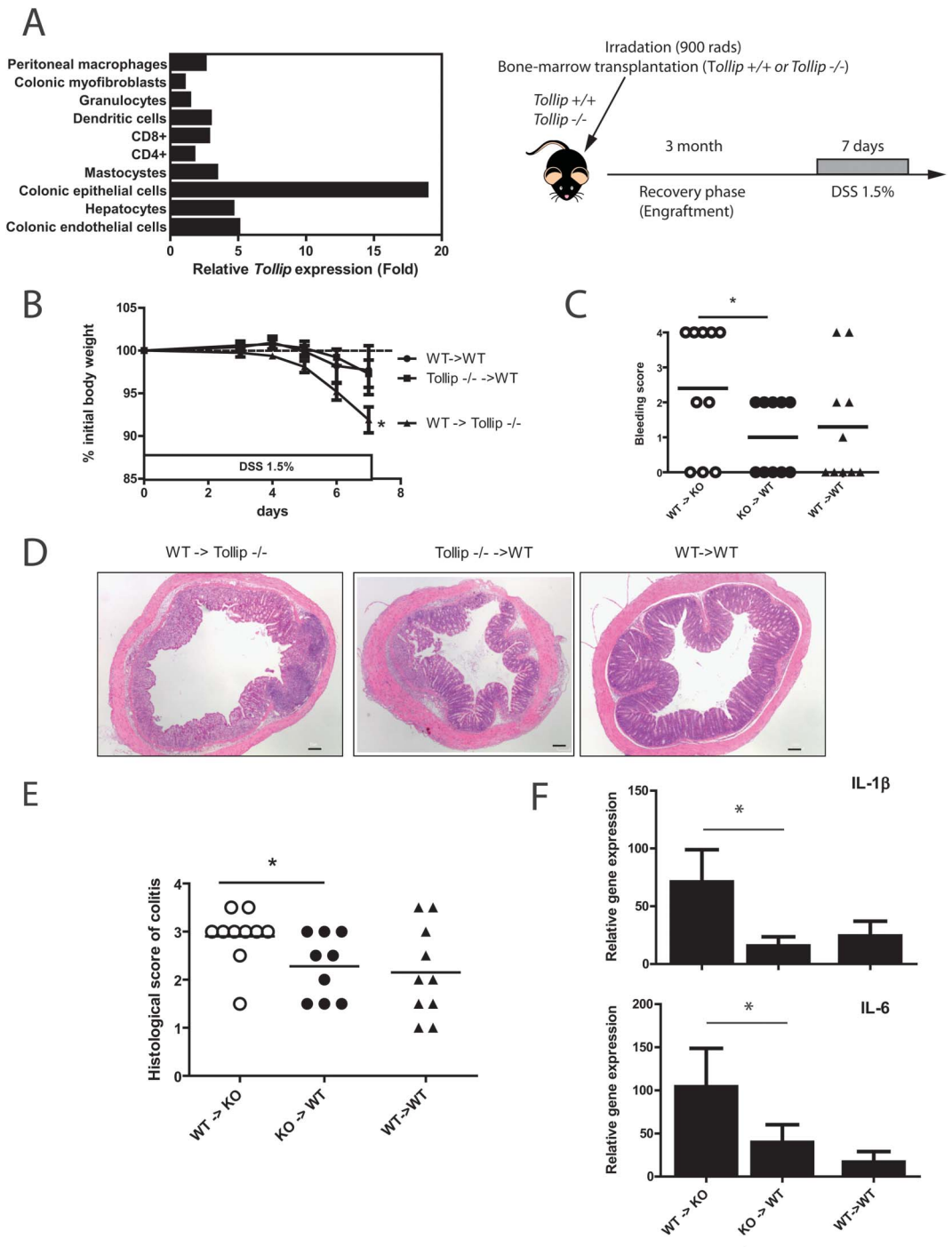

FIGURE 3. Bone marrow-derived *Tollip* expression is not sufficient to restore colitis susceptibility. A, Left Panel: Quantitative PCR determination of Tollip expression in various cell types (see material and methods for cell isolation techniques). *Actb* was used as an internal reference gene to normalize the transcript levels. Right panel: WT or *Tollip*$^{-/-}$ mice were irradiated to deplete endogenous bone marrow cells and transplanted with WT or *Tollip*$^{-/-}$ bone marrow cells. Resulting chimeric mice were challenged with DSS 1.5% to assess colitis susceptibility. B, % weight change over time on DSS exposure of WT→WT, WT→KO, KO→WT chimeras. Shown are mean ± SEM of 2 independent experiments combined (n = 9–10 mice per group). C, Mean ± SEM rectal bleeding scores at day 5 of DSS exposure. D and E, Representative histological changes and blind determination of histological scores of colitis at day 7 of DSS exposure. F, RT-qPCR analysis of expression of proinflammatory genes at day 7 of DSS exposure. Data have been normalized to GAPDH expression and then to mean values for the water group. Statistical *P* values have been determined using the Mann–Whitney test. *$P < 0.05$ ** $P < 0.01$. RT-qPCR, quantitative RT-PCR.

Copyright © 2014 Crohn's & Colitis Foundation of America, Inc. Unauthorized reproduction of this article is prohibited.

Downloaded from https://academic.oup.com/ibdjournal/article/20/4/660/4579016 by guest on 05 December 2022

secretion (Fig. 4B). We next addressed whether Tollip deficiency also led to increased epithelial responsiveness to pathogen-associated molecular patterns and increased KC (murine equivalent of IL-8) expression in vivo. Although KC expression in DSS-exposed *Tollip*$^{+/+}$ did not differ from that in unexposed controls, expression was significantly increased in DSS-exposed *Tollip*$^{-/-}$ mice (Fig. 4C). In contrast, we only found a nonsignificant trend for increased MIP-2 expression in DSS-exposed *Tollip*$^{-/-}$ mice (Fig. 4C). Importantly, increased KC expression conicided with a marked increase in colonic myeloperoxidase activity, indirectly reflecting increased innate immune cell abundance in the lamina propria (Fig. 4C).

Thus, Tollip deficiency in IECs leads to excessive bacterial product responsiveness and subsequent increased cytokine secretion, suggesting that epithelial expression of *Tollip* may negatively regulate intestinal inflammatory responses to microbial-derived signals.

Tollip Deficiency Enhances Epithelial Permeability on Injury

We next asked whether increased susceptibility to DSS-induced colitis might correlate with abnormal epithelial permeability in *Tollip*$^{-/-}$ mice. To this end, we orally fed mice with FITC-labeled dextran 5 days after DSS exposure and measured the FITC-signal in blood as readout of dextran uptake. In line with our previous findings, no differences were found in naive animals, but there was a significant increase in serum concentrations of FITC-dextran in DSS-exposed *Tollip*$^{-/-}$ mice compared with controls (Fig. 4D).

To further address whether Tollip deficiency might affect tight junction integrity, we analyzed zona occludens-1 distribution, a tight junction marker, in IECs from *Tollip*$^{-/-}$ and *Tollip*$^{+/+}$ animals before and after DSS exposure. Before injury, we failed to detect any histological differences on tight junction integrity between genotypes. However, in contrast to *Tollip*$^{+/+}$ animals, we found a diffuse and unpolarized zona occludens-1 staining in IECs from *Tollip*$^{-/-}$ mice after DSS exposure, which was the most obvious in the vicinity of epithelial ulcers (Fig. 4E).

We next explored whether increased colitis susceptibility in *Tollip*$^{-/-}$ mice may correlate with increased IEC apoptosis. We therefore stained colonic sections from DSS-exposed *Tollip*$^{+/+}$ and *Tollip*$^{-/-}$ mice for the cleaved caspase 3.[24] Baseline numbers of apoptotic cells per section were similar between unchallenged *Tollip*$^{+/+}$ and *Tollip*$^{-/-}$ mice (Fig. 5B–E). However, the number of cleaved caspase-3 positive cells was significantly enhanced at day 7 of DSS exposure in *Tollip*$^{-/-}$ mice when compared with *Tollip*$^{+/+}$ animals (Fig. 5B). In contrast, immunohistochemical analysis of BrdU expressing epithelial cell numbers per crypts in small intestines and colons from DSS-challenged mice did not show any significant difference in epithelial proliferation between genotypes (Fig. 5C, D). Altogether, these data suggest that Tollip is required to maintain epithelial barrier integrity on injury.

DISCUSSION

The gut mucosa is continuously exposed to a wide variety of microbial-derived products and environmental antigens. TLRs engagement are key events that allow a symbiotic crosstalk and maintenance of the epithelial barrier on either physical, chemical, or immune stresses.[16] Although a number of reports have established that activation of TLR pathways are critically implicated in gut homeostasis, it remains unclear how regulation of these signals is occurring in vivo.[25] Our data show that lack of Tollip does not lead to spontaneous colitis but rather increases colonic susceptibility to chronic colitis and acute chemical injury. Increased susceptibility to DSS-induced colitis was essentially due to lack of *Tollip* expression in nonhematopoietic cells and did not depend per se on gut bacterial content. Within the colonic epithelium, absence of *Tollip* expression was associated with excessive proinflammatory cytokine secretion, elevated mucosal permeability, and increased epithelial apoptosis. Altogether, these data demonstrate that Tollip plays a key role in maintaining mucosal homeostasis and regulating epithelial responses to stresses.

In this report, we show that Tollip deficiency increases susceptibility to chronic colitis in IL-10$^{-/-}$ mice (Fig. 1). Increased colitis susceptibility has also been documented in IL-10$^{-/-}$ mice lacking the TLR regulator IRAK-M.[26] Since colitis in IL-10$^{-/-}$ mice can be prevented by genetic ablation of MyD88, our data suggest that excessive TLR activation could be a mechanism for increased chronic colitis susceptibility. Alternatively, Tollip might directly control the differentiation/proliferation of proinflammatory Th17 cells as recent data showed that SIGIRR was involved in such signaling pathway.[9] Altogether, these data show that TLR/IL-1R inhibition plays a major role in preventing susceptibility to chronic TLR-dependent colitis.

Likewise, *Tollip*$^{-/-}$ mice are more sensitive to DSS (Fig. 1). Indeed, as compared with WT mice, *Tollip*$^{-/-}$ mice have increased body weight loss, increased histological scores of colitis, and increased colonic content of proinflammatory cytokines. In addition, our data demonstrate that increased sensitivity to DSS is due to Tollip deficiency in nonhematopoietic cells and point toward a tolerogenic role for Tollip in epithelial cells. In vivo, excessive NF-κB signaling in epithelial cells has been associated with enhanced epithelial apoptosis and increased colitis susceptibility.[27] This is illustrated in mice expressing an epithelium-specific constitutively active IKK2 mutant leading to exaggerated NF-κB signals.[28,29] These mice were shown to spontaneously develop mild colitis, increased sensitivity to DSS-induced colitis, and increased epithelial apoptosis.[28,29] Consistent with these reports, we now demonstrate that Tollip deficiency leads to excessive DSS-induced epithelial apoptosis and increased colitis sensitivity. Similar findings were also reported on other Toll/IL-1R negative regulators such as SIGIRR or A20. Indeed, SIGIRR deficient mice are more sensitive to DSS-induced colitis, and this could be restored by epithelial-specific SIGIRR expression.[9] Likewise, conditional knockout of A20 in the gut epithelium led to increased DSS-induced colitis susceptibility and excessive epithelial apoptosis.[10] Collectively, our data show that Tollip plays a protective role in IECs on DSS challenge. Our observations together with those made on PRR/IL-1R[16,30] or other Toll-/IL-1R-signaling inhibitors,[9,10] reinforce the notion that excessive or impaired signaling through these

Downloaded from https://academic.oup.com/ibdjournal/article/20/4/660/4579016 by guest on 05 December 2022

Copyright © 2014 Crohn's & Colitis Foundation of America, Inc. Unauthorized reproduction of this article is prohibited.

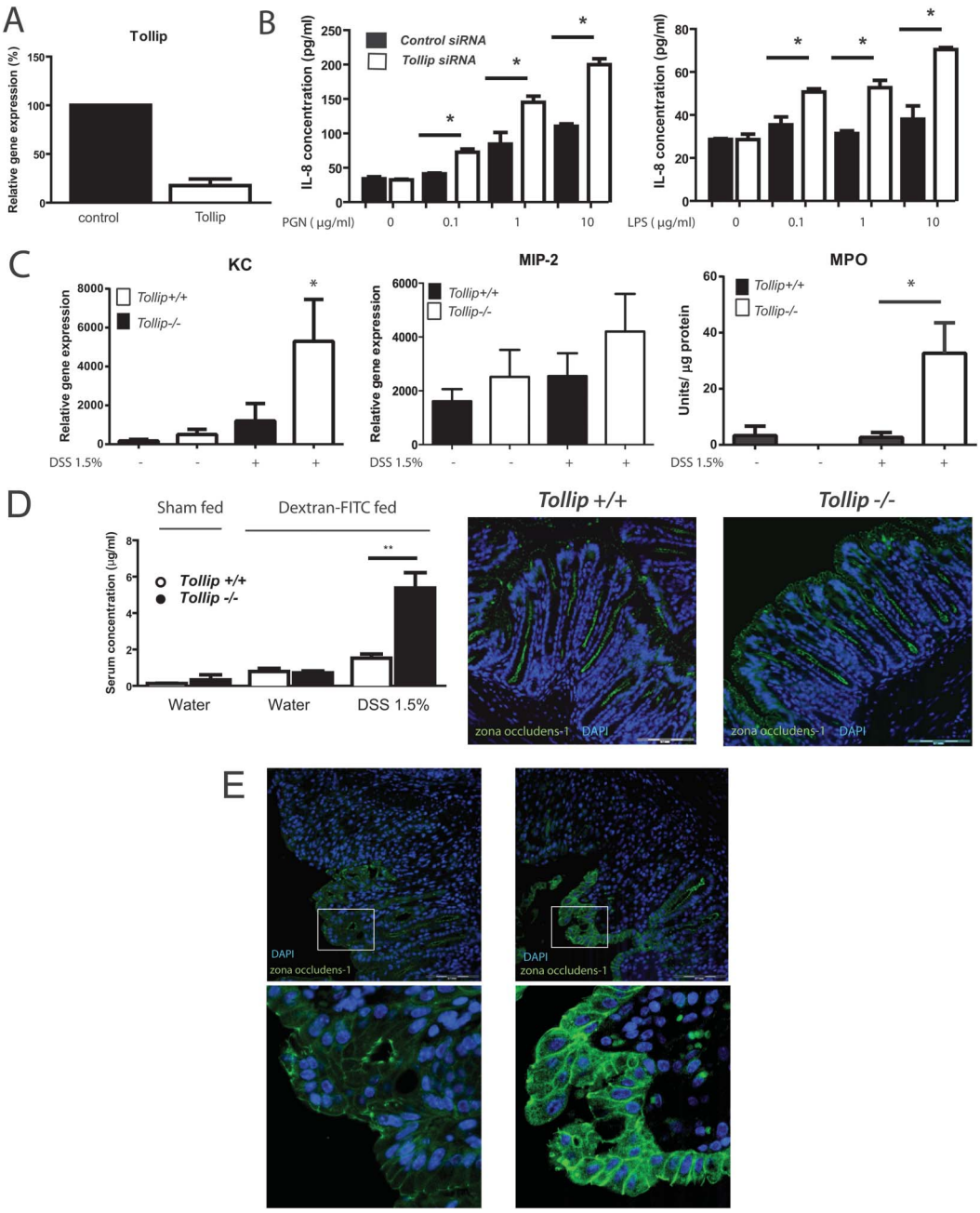

FIGURE 4. *Tollip* regulates epithelial NFκB activity on microbial-derived signals and barrier function on injury. A, Quantitative PCR analysis of *Tollip* expression in Caco-2 cells after control (black) or *Tollip* (white) siRNA transfection. B, IL-8 concentration (enzyme-linked immunosorbent assay) in culture supernatant of control (black bars) or *Tollip* (white bars) siRNA transfected Caco-2 cells stimulated with 0 to 10 µg/mL peptidoglycan (PGN; left panel) or lipopolysaccharide (LPS; right panel). Shown are mean ± SEM of 1 out of 3 independent experiments performed in triplicates. C, Quantitative PCR analysis of IL-8 and MIP-2 expression in the colon of unchallenged versus DSS-exposed *Tollip*$^{+/+}$ or *Tollip*$^{-/-}$ mice. Myeloperoxidase activity in the lamina propria of unchallenged versus DSS-exposed *Tollip*$^{+/+}$ or *Tollip*$^{-/-}$ mice. *$P < 0.05$, Mann–Whitney test comparing *Tollip*$^{-/-}$ mice under DSS to WT controls (water). D, *Tollip* deficiency leads to increased epithelial permeability. WT (white bars) and *Tollip*$^{-/-}$ (black bars) mice were exposed to DSS 1.5% for 5 days (or water) and then orally fed with FITC-dextran (or saline as control) to determine epithelial permeability. Shown are concentrations obtained in the serum of 9 to 12 mice in each group 4 hours after gavage. Shown are the combined data from 3 independent experiments. Right panels: Representative immunofluorescence appearance of zona occludens-1 (green) distribution in unchallenged (upper panels) WT (left) and *Tollip*$^{-/-}$ (right) colons. D, Immunofluorescent appearance of zona occludens-1 staining in mice exposed to DSS 1.5% for 5 days. DAPI was used as counterstain for nuclei and is shown in blue. Magnification: 20×. Scale bars, 10 µm. Bottom 2 panels represent a zoomed portion of the top 2 panels (white box).

Copyright © 2014 Crohn's & Colitis Foundation of America, Inc. Unauthorized reproduction of this article is prohibited.

Downloaded from https://academic.oup.com/ibdjournal/article/20/4/660/4579016 by guest on 05 December 2022

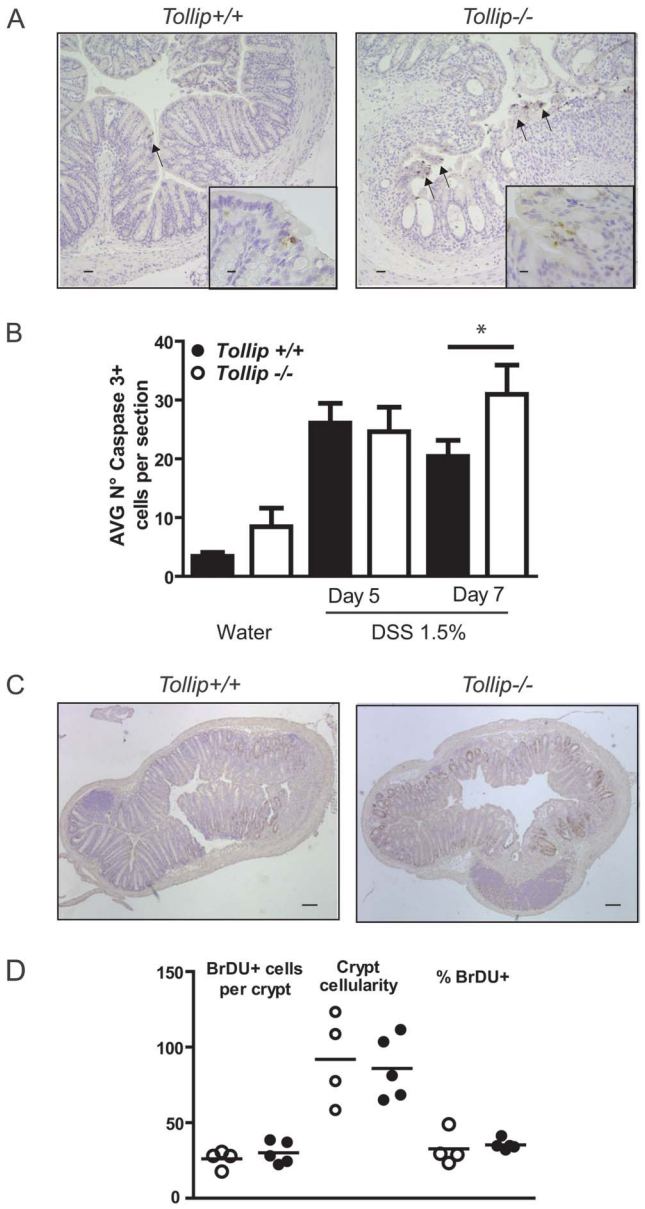

FIGURE 5. Tollip deficiency leads to excessive epithelial apoptosis on DSS exposure. A and B, Representative immunohistochemical determination (DSS day 7, magnification 10×. Scale bar: 100 μm. Insets: magnification 40×, Scale bar: 10 μm) and quantification (B) of the number of cleaved caspase-3 positive cells per section. Cleaved caspase-3 positivity stained braun (arrows) and hematoxylin was used to counterstain nuclei in purple. For each mouse, only cross-sectional and fully circular sections were taken into account, and 3 to 6 sections were evaluated per mouse. Shown are mean ± SD from 3 independent experiments combined. Statistical *P* values have been determined using the Mann–Whitney test. NS, Nonsignificant; $*P < 0.05$; $**P < 0.01$. C, Representative immunohistochemical determination of BrDU uptake in colons from *Tollip*$^{+/+}$ (left) and *Tollip*$^{-/-}$ mice after 5 days of DSS exposure (DSS day 10, magnification 4×, scale bars: 10 μm). BrDU positivity stained braun (arrows) and hematoxylin was used to counterstain nuclei in purple. D, Quantification of the number of BrDU^{+} cells per crypt adjacent to ulcers, crypt cellularity and % BrDU positive cells per crypt. White dots: *Tollip*$^{+/+}$. Black dots: *Tollip*$^{-/-}$. Horizontal bars represent mean per group.

receptors, have detrimental consequences on IEC-sensitivity to stresses and colitis susceptibility.

On DSS exposure, we found that *Tollip*-deficient animals experienced epithelial barrier defects that lead to enhanced permeability (Fig. 4). Although the clear mechanism underlying those observations remains to be determined, we speculate that this might be related to excessive epithelial responsiveness to bacterial signals and/orIL-1β. Indeed, tight junctions and epithelial integrity have been shown to be regulated by IL-1β.[31] Here, we found that IL-1β is more abundant in the lamina propria of DSS-exposed *Tollip*-deficient mice. Given the known role of Tollip in the negative regulation of IL-1 receptor signaling,[12,32] increased colonic IL-1β in *Tollip*-deficient animals might lead to excessive epithelial stimulation and exaggerated tight junction destabilization.

We have observed that lack of Tollip in epithelial cells in vitro leads to increased IL-8 secretion in response to several bacterial-derived signals. In vivo, colonic KC expression was increased in *Tollip*$^{-/-}$ mice when exposed to DSS. Such high KC production may favor mucosal neutrophil recruitment, a critical step in early mucosal injury processes that ultimately lead to tissue destruction and ulcer formation. Consistently, we also report an increase in myeloperoxidase activity in the colonic lamina propria of DSS-challenged *Tollip*$^{-/-}$ mice when compared with controls. Consistently, Tollip was previously shown to inhibit neutrophil recruitment to the inflamed lung mucosa through IL-8.[33]

Even if the composition of the gut microbiota is thought to modulate colitis severity, pretreatment of *Tollip*$^{-/-}$ mice with a cocktail of 4 antibiotics (AVNM) failed to protect at-risk animals from excessive DSS-induced intestinal damage. A recent report demonstrated that AVNM treatment of C57BL/6J mice before acute oral DSS challenge leads to the accumulation and systemic spread of a multidrug-resistant *E. coli* O21:H+ strain.[23] This strain mediates systemic sepsis leading to excessive death rates in comparison with AVNM-untreated controls. Importantly, this IL-1β driven immunopathological process is dependent on intact Naip5-Nlrc4 inflammasome signals as mice deficient for both Naip5 and Nlrc4 were protected from sepsis.[23] Given the known inhibitory role of Tollip in IL-1R signaling, it is tempting to speculate that enhanced susceptibility to DSS colitis despite AVNM treatment in *Tollip*$^{-/-}$ mice might in fact be due to an IL-1β driven immunopathological response to translocating bacteria. Alternatively, AVNM preconditioning might have selected for other facultative pathogens, such as enterococci, that could contribute to colitis development in at-risk inidividuals. Further studies are needed to determine whether increased sensitivity to DSS-induced injury in AVNM-treated *Tollip*$^{-/-}$ mice is directly related to exaggerated DSS-induced inflammasome activation[34] and/or nonbacterial components of the microbiota such as fungi.[35]

In this report, we show that Tollip is implicated in the regulation of several key aspects of intestinal mucosal homeostasis that might be of relevance to CD or other human intestinal pathologies. This assumption is supported by 2 clinical reports in

Downloaded from https://academic.oup.com/ibdjournal/article/20/4/660/4579016 by guest on 05 December 2022

Copyright © 2014 Crohn's & Colitis Foundation of America, Inc. Unauthorized reproduction of this article is prohibited.

which IECs from CD patients failed to upregulate *Tollip* expression.[7,16] Moreover, Tollip and other TLR inhibitors were recently linked to celiac disease,[36] providing a rational for further exploring the role of *Tollip* in human inflammatory diseases.

In conclusion, we have shown that Tollip deficiency affects susceptibility to acute chemical injury and chronic immune-mediated colitis despite any steady-state propensity to pathological inflammation. Our work, together with previous reports on other IL-1R and TLR inhibitors, shows that regulation of Toll-/IL-1R-signaling is instrumental in controlling gut epithelial responses to stresses and promoting recovery from acute injuries.

ACKNOWLEDGMENTS

The authors would like to thank Prof. Giuseppe Pantaleo, Prof. Jean-Pierre Kraehenbuhl, Prof. Darius Moradpour, and Prof. Gian Dorta for their constant support and critical inputs on the project. The authors also thank Catherine Pythoud and Daniel Bachmann for their outstanding technical assistance.

REFERENCES

1. Cario E. Toll-like receptors in inflammatory bowel diseases: a decade later. *Inflamm Bowel Dis.* 2010;16:1583–1597.
2. Elinav E, Henao-Mejia J, Flavell RA. Integrative inflammasome activity in the regulation of intestinal mucosal immune responses. *Mucosal Immunol.* 2013;6:4–13.
3. Kawai T, Akira S. The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. *Nat Immunol.* 2010;11: 373–384.
4. Rakoff-Nahoum S, Paglino J, Eslami-Varzaneh F, et al. Recognition of commensal microflora by toll-like receptors is required for intestinal homeostasis. *Cell*. 2004;118:229–241.
5. Shi D, Das J, Das G. Inflammatory bowel disease requires the interplay between innate and adaptive immune signals. *Cell Res.* 2006;16: 70–74.
6. Rakoff-Nahoum S, Hao L, Medzhitov R. Role of toll-like receptors in spontaneous commensal-dependent colitis. *Immunity*. 2006;25: 319–329.
7. Nell S, Suerbaum S, Josenhans C. The impact of the microbiota on the pathogenesis of IBD: lessons from mouse infection models. *Nat Rev Microbiol.* 2010;8:564–577.
8. Shibolet O, Podolsky DK. TLRs in the Gut. IV. Negative regulation of Toll-like receptors and intestinal homeostasis: addition by subtraction. *Am J Physiol Gastrointest Liver Physiol.* 2007;292:G1469–G1473.
9. Xiao H, Gulen MF, Qin J, et al. The Toll-interleukin-1 receptor member SIGIRR regulates colonic epithelial homeostasis, inflammation, and tumorigenesis. *Immunity*. 2007;26:461–475.
10. Vereecke L, Sze M, Mc Guire C, et al. Enterocyte-specific A20 deficiency sensitizes to tumor necrosis factor-induced toxicity and experimental colitis. *J Exp Med.* 2010;207:1513–1523.
11. Berglund M, Melgar S, Kobayashi KS, et al. IL-1 receptor-associated kinase M downregulates DSS-induced colitis. *Inflamm Bowel Dis.* 2010; 16:1778–1786.
12. Burns K, Clatworthy J, Martin L, et al. Tollip, a new component of the IL-1RI pathway, links IRAK to the IL-1 receptor. *Nat Cell Biol.* 2000;2:346–351.
13. Zhang G, Ghosh S. Negative regulation of toll-like receptor-mediated signaling by Tollip. *J Biol Chem.* 2002;277:7059–7065.
14. Melmed G, Thomas LS, Lee N, et al. Human intestinal epithelial cells are broadly unresponsive to Toll-like receptor 2-dependent bacterial ligands: implications for host-microbial interactions in the gut. *J Immunol.* 2003; 170:1406–1415.
15. Otte JM, Cario E, Podolsky DK. Mechanisms of cross hyporesponsiveness to Toll-like receptor bacterial ligands in intestinal epithelial cells. *Gastroenterology*. 2004;126:1054–1070.
16. Abreu MT. Toll-like receptor signalling in the intestinal epithelium: how bacterial recognition shapes intestinal function. *Nat Rev Immunol.* 2010; 10:131–144.
17. Didierlaurent A, Brissoni B, Velin D, et al. Tollip regulates proinflammatory responses to interleukin-1 and lipopolysaccharide. *Mol Cell Biol.* 2006;26:735–742.
18. Bernasconi E, Favre L, Maillard MH, et al. Granulocyte-macrophage colony-stimulating factor elicits bone marrow-derived cells that promote efficient colonic mucosal healing. *Inflamm Bowel Dis.* 2010;16: 428–441.
19. Travassos LH, Girardin SE, Philpott DJ, et al. Toll-like receptor 2-dependent bacterial sensing does not occur via peptidoglycan recognition. *EMBO Rep.* 2004;5:1000–1006.
20. Qualls JE, Kaplan AM, van Rooijen N, et al. Suppression of experimental colitis by intestinal mononuclear phagocytes. *J Leukoc Biol.* 2006;80: 802–815.
21. Barnich N, Aguirre JE, Reinecker HC, et al. Membrane recruitment of NOD2 in intestinal epithelial cells is essential for nuclear factor-{kappa} B activation in muramyl dipeptide recognition. *J Cell Biol.* 2005;170: 21–26.
22. Cerf-Bensussan N, Gaboriau-Routhiau V. The immune system and the gut microbiota: friends or foes? *Nat Rev Immunol.* 2010;10:735–744.
23. Ayres JS, Trinidad NJ, Vance RE. Lethal inflammasome activation by a multidrug-resistant pathobiont upon antibiotic disruption of the microbiota. *Nat Med.* 2012;18:799–806.
24. Martinon F, Tschopp J. Inflammatory caspases and inflammasomes: master switches of inflammation. *Cell Death Differ.* 2007;14:10–22.
25. Wullaert A, Bonnet MC, Pasparakis M. NF-kappaB in the regulation of epithelial homeostasis and inflammation. *Cell Res.* 2011;21: 146–158.
26. Biswas A, Wilmanski J, Forsman H, et al. Negative regulation of Toll-like receptor signaling plays an essential role in homeostasis of the intestine. *Eur J Immunol.* 2011;41:182–194.
27. Jabbari A, Harty JT. The generation and modulation of antigen-specific memory CD8 T cell responses. *J Leukoc Biol.* 2006;80:16–23.
28. Guma M, Stepniak D, Shaked H, et al. Constitutive intestinal NF-kappaB does not trigger destructive inflammation unless accompanied by MAPK activation. *J Exp Med.* 2011;208:1889–1900.
29. Vlantis K, Wullaert A, Sasaki Y, et al. Constitutive IKK2 activation in intestinal epithelial cells induces intestinal tumors in mice. *J Clin Invest.* 2011;121:2781–2793.
30. Chen GY, Shaw MH, Redondo G, et al. The innate immune receptor Nod1 protects the intestine from inflammation-induced tumorigenesis. *Cancer Res.* 2008;68:10060–10067.
31. Al-Sadi RM, Ma TY. IL-1beta causes an increase in intestinal epithelial tight junction permeability. *J Immunol.* 2007;178:4641–4649.
32. Brissoni B, Agostini L, Kropf M, et al. Intracellular trafficking of interleukin-1 receptor I requires Tollip. *Curr Biol.* 2006;16:2265–2270.
33. Habibzay M, Saldana JI, Goulding J, et al. Altered regulation of Toll-like receptor responses impairs antibacterial immunity in the allergic lung. *Mucosal Immunol.* 2012;5:524–534.
34. Bauer C, Duewell P, Mayer C, et al. Colitis induced in mice with dextran sulfate sodium (DSS) is mediated by the NLRP3 inflammasome. *Gut.* 2010;59:1192–1199.
35. Sokol H, Conway KL, Zhang M, et al. Card9 mediates intestinal epithelial cell restitution, T-helper 17 responses, and control of bacterial infection in mice. *Gastroenterology*. 2013;145:591–601 e593.
36. Kalliomaki M, Satokari R, Lahteenoja H, et al. Expression of microbiota, Toll-like receptors, and their regulators in the small intestinal mucosa in celiac disease. *J Pediatr Gastroenterol Nutr.* 2012;54:727–732.

Downloaded from https://academic.oup.com/ibdjournal/article/20/4/660/4579016 by guest on 05 December 2022

Copyright © 2014 Crohn's & Colitis Foundation of America, Inc. Unauthorized reproduction of this article is prohibited.