Epithelial-specific Toll-like Receptor (TLR)5 Activation Mediates Barrier Dysfunction in Experimental Ileitis

Loris R. Lopetuso, MD, PhD, Ruo Jia, PhD,* Xiao-Ming Wang, MS,* Li-Guo Jia, PhD,† Valentina Petito, MS,* Wendy A. Goodman, PhD,* Jon B. Meddings, MD,‡ Fabio Cominelli, MD, PhD,† Brian K. Reuter, PhD,§ and Theresa T. Pizarro, PhD**

Background: A large body of evidence supports a central role of TLR5 and its natural ligand, flagellin, in Crohn's disease (CD), with the precise mechanism(s) still unresolved.

Methods: We investigated the role of flagellin/TLR5 in SAMP1/YitFc (SAMP) mice, a spontaneous model of Crohn's disease-like ileitis.

Results: Ileal *Tlr5* and serum antiflagellin IgG antibodies were increased in SAMP before the onset of inflammation and during established disease; these trends were abrogated in the absence of colonizing commensal bacteria. Irradiated SAMP receiving either wild-type (AKR) or SAMP bone marrow (BM) developed severe ileitis and displayed increased ileal *Tlr5* compared with AKR recipients of either SAMP or AKR bone marrow, neither of which conferred ileitis, suggesting that elevated TLR5 in native SAMP is derived primarily from a nonhematopoietic (e.g., epithelial) source. Indeed, ileal epithelial TLR5 in preinflamed SAMP was increased compared with age-matched AKR and germ-free SAMP. TLR5-specific ex vivo activation of SAMP ileal tissues decreased epithelial barrier resistance, indicative of increased permeability, and was accompanied by altered expression of the tight junction proteins, claudin-3, occludin, and zonula occludens-1.

Conclusions: Our results provide evidence that aberrant, elevated TLR5 expression is present in the ileal epithelium of SAMP mice, is augmented in the presence of the gut microbiome, and that TLR5 activation in response to bacterial flagellin results in a deficiency to maintain appropriate epithelial barrier integrity. Together, these findings represent a potential mechanistic pathway leading to the exacerbation and perpetuation of chronic gut inflammation in experimental ileitis and possibly, in patients with Crohn's disease.

(Inflamm Bowel Dis 2017;23:392–403)

Key Words: epithelial barrier function, epithelial permeability, TLR5, flagellin, gut microbiome, SAMP1/YitFc model of CD-like ileitis

Inflammatory bowel disease (IBD), such as Crohn's disease (CD) and ulcerative colitis, is a chronic, relapsing inflammatory disorder of the digestive tract resulting from a loss of homeostasis between the intestinal immune system and the gut microbiome in genetically-predisposed individuals.[1] Inappropriate mucosal immune responses, due to disruption of the intestinal epithelial barrier and/or dysregulated tolerance to the commensal flora, likely contribute to the development and perpetuation of IBD.[2–5]

Several lines of evidence have shown that the gut microbiome is crucial for the development of a normal mucosal immune system throughout the gastrointestinal (GI) tract, but can also play a central role in the pathogenesis of IBD.[6] In support of this concept, the majority of genetically-susceptible murine models of colitis do not develop significant inflammation when raised in a germ-free (GF) environment[7–10]; whereas in others, disease can be attenuated or completely abolished with antibiotic (ABX) treatment.[11,12] In this context, innate immune responses that recognize conserved microbial products, such as lipopolysaccharide (LPS) and peptidoglycan, are important for microbial–host interactions and intestinal homeostasis.[13] Critical to the host's sensing of microbes are members of the toll-like receptor (TLR) family that, alone or in combination, recognize a wide array of microbe-associated molecular patterns (MAMPs) on either pathogenic or commensal bacteria.[13] TLRs are expressed on cells within the gut mucosa, including intestinal epithelial cells (IECs), as well as on lamina propria macrophages and dendritic cells.[14–17] TLR2, -4, and -5 are the major cell-surface sensors of bacterial

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 November 24, 2016; Accepted December 15, 2016.

From the Departments of *Pathology, and †Medicine, Case Western Reserve University School of Medicine, Cleveland, Ohio; ‡Faculty of Medicine, University of Calgary, Health Sciences Centre, Calgary, Alberta, Canada; and §Centre of Excellence for Gastrointestinal Inflammation and Immunity Research, University of Alberta, Edmonton, Alberta, Canada.

Supported by grants from the National Institutes of Health: DK056762 (to TTP), DK091222 (to TTP/FC), DK055812 (to FC) and the Crohn's & Colitis Foundation of America: Career Development Award (to BKR and WAG), Research Fellowship Award (to LRL), Student Research Award (to RJ). We also acknowledge support from the Cores of the Cleveland Digestive Diseases Research Core Center (P30 DK097948).

The authors have no conflict of interest to disclose.

L. R. Lopetuso and R. Jia contributed equally.

Address correspondence to: Theresa T. Pizarro, PhD, Department of Pathology, Case Western Reserve University School of Medicine, 2103 Cornell Road, WRB 5534, Cleveland, OH 44106 (e-mail: theresa.pizarro@case.edu).

DOI 10.1097/MIB.0000000000001035

Published online 31 January 2017.

lipopeptides, including LPS and flagellins, whereas TLR3, -7, -8, and -9 detect nucleic acid motifs.[18]

Among the described TLRs, both human and murine studies have shown the importance of TLR5 and its ligand, the bacterial protein flagellin, in the regulation of innate and adaptive immune responses that are associated with IBD.[6,19–21] However, results from the currently published data are inconclusive regarding the precise role of TLR5, but open the possibilities to different mechanistic hypotheses. Commensal-derived flagellin has been identified as a dominant antigen in patients with CD,[19] and about 50% of CD patients usually have abnormally high levels of anti-flagellin antibodies that correlate with particular subtypes of severe disease.[20,21] In animal studies, infusion of intrarectal flagellin worsens the severity of dextran sulfate sodium-induced colitis,[22] and oral administration of *Toxoplasma gondii* induces a dramatic increase in the concentration of flagellin within the small intestine (in which flagellin is normally low), causing severe Th1-mediated ileitis.[23] Together, these findings suggest an association of TLR5 with IBD, particularly CD, and that exposure of flagellin to either basolaterally-expressed TLR5 or subepithelial TLR5-expressing cells, in the setting of a damaged mucosal barrier, may have the ability to initiate and/or perpetuate chronic intestinal inflammation.

Conversely, studies also document a downregulation of TLR5 in both animal models of colitis and patients with IBD.[24,25] As such, the role of flagellin/TLR5 in the pathogenesis of IBD is controversial (i.e., pathogenic versus protective) and the cell types that express TLR5, as well as the TLR5-dependent mechanisms that regulate gut permeability and homeostasis in response to the gut microbiome, remain unclear.

To gain more insight into how cell-specific expression and activation of TLR5 affects gut homeostasis and the potential development of chronic intestinal inflammation, we investigated the role of flagellin/TLR5 in ileitis-prone SAMP1/YitFc (SAMP) mice. The SAMP mouse strain represents a chronic model of IBD and provides an excellent system to study the contributions made by specific cell types to the development of enteritis before the onset of inflammation, during the initiation and acute phase of inflammation, and when chronic disease is established.[26] Additionally, since SAMP mice were derived from brother–sister mating of WT AKR mice (parental strain),[27] the phenotype occurs spontaneously as in the human condition, without chemical, genetic, or immunologic manipulation. Inflammation is fully developed by 10 to 12 weeks of age,[28] with Th1-type immune responses predominating early during the inductive phase,[29,30] and a mixed Th1/Th2 phenotype emerging as ileitis develops into chronic, established disease.[31,32]

In the present study, we found that TLR5 in whole ileal tissues and serum anti-flagellin IgG antibody levels were increased in SAMP before the onset of inflammation and during established disease, when compared with age-matched AKR mice. These trends were abrogated in the absence of colonizing commensal bacteria. Construction of reciprocal bone marrow chimeras (BMCs) suggested that elevated levels of TLR5 in SAMP are derived from the nonhematopoietic compartment, which was confirmed in isolated IECs that expressed increased TLR5 mRNA and protein levels. In addition, TLR5-specific activation in ex vivo-stimulated SAMP ileal tissues markedly decreased epithelial barrier resistance and altered expression of the tight junction (TJ) proteins, claudin-3 (Cldn3), occludin (Occl) and zonula occludens-1, compared with AKR controls. Taken together, our data suggest that, in the presence of the gut microbiome, elevated epithelial TLR5 expression is inherent to SAMP mice, resulting in the inability to maintain barrier function in response to bacterial flagellin, and represents a potential mechanistic pathway leading to the susceptibility of CD-like ileitis.

MATERIALS AND METHODS

Mice and Construction of BMCs

SAMP and AKR mice were provided by the Mouse Models Core of the Cleveland Digestive Diseases Research Core Center and bred in the Animal Resource Center at Case Western Reserve University (CWRU). Mice were maintained under specific pathogen-free (SPF) conditions, fed standard laboratory chow (Harlan Teklad, Indianapolis, IN), and kept on 12-hour light/dark cycles. Original AKR were purchased from The Jackson Laboratory (Bar Harbor, ME). GF SAMP were maintained at Taconic Farms (Albany, NY) and shipped in GF vessels for same- or next-day experimentation. Equal numbers of age-matched male and female mice were used in each experimental group whenever possible, with neither gender overrepresented.

BMCs were constructed from 10-week-old SAMP and AKR mice as previously described.[33,34] At 6 weeks posttransplant, BMCs were treated with either broad spectrum ABXs (ampicillin, neomycin, metronidazole, all at 1 g/mL; vancomycin, 0.5 g/mL) or vehicle for an additional 4-week period,[30] after which experimental mice were sacrificed.

Histologic Evaluation of Intestinal Inflammation

Distal 10 cm of ilea, or entire length of colon, from experimental mice were removed, flushed off fecal contents, opened longitudinally, and half of these tissues, further divided along the longitudinal axis, were placed in RNA*later* Stabilization Solution (Invitrogen, Carlsbad, CA) at 4°C for 24 hours, and stored at −20°C for later RNA extraction. The other half of the tissues were placed in Bouin's fixative (Ricca Chemical Company, Arlington, TX) for histologic assessment. Briefly, fixed tissues were embedded in paraffin, cut to 3 μm, and stained with H&E. Disease severity was evaluated by a trained gastrointestinal pathologist (J. R. Mize) in a blinded fashion, using established histologic scoring systems for ileitis[28] and colitis.[35] Images were obtained on an Axiophot microscope, captured on an Axiocam and assembled using Axiovision Release 4.5 (Carl Zeiss, Inc., Thornwood, NY).

In Vivo and Ex Vivo Permeability

Experimental mice were administered a sugar probe cocktail by orogastric gavage and in vivo small intestinal permeability was measured by urinary fractional excretion (FE) of lactulose to mannitol (Lac/Man FE ratio) and colonic permeability by urinary sucralose FE, using an established method.[33,36] Ex vivo permeability was measured by a previously described transepithelial electrical resistance (TEER) assay[33,37] performed on ilea from 4-week-old pre-inflammed SAMP and age-matched AKR controls, stimulated apically with either a TLR2 (Pam3Cys; Abcam, Cambridge, MA), TLR4 (LPS; Sigma, St. Louis, MO), or a TLR5 (FliC; Inotek Pharmaceuticals, Lexington, MA) agonist (all at 200 ng/mL) diluted in 3 mL Dulbecco's Modified Eagle Medium (DMEM, glutamine, nonessential amino acids, 100 U/mL penicillin, and 100 g/mL streptomycin, all from Invitrogen); media alone served as control. In selected experiments, FliC (200 ng/mL) and conditioned media from BMDMs (described below) were preincubated with and without an anti-FliC monoclonal antibody (1:10 for 18 hours; Inotek Pharmaceuticals) before application onto SAMP ileal tissues. TEER readings were taken using an EVOM voltmeter with an EndOhm chamber attachment (World Precision Instruments, Sarasota, FL) immediately after assembling transwell apparatuses, and after 1 hour incubation at 37°C. Baseline resistance readings were determined in transwells containing membrane inserts only and subtracted from sample values.

IEC Isolation and Purification

Freshly isolated ileal segments were chopped into 1-cm pieces and incubated in a solution containing DTT (Sigma) and EDTA (Life Technologies, Grand Island, NY). After a modification of the method described by Whitehead et al[38] and as previously performed by our group,[33,39,40] the specimens were then washed with 1× Hanks' balanced salt solution containing 15 mM Hepes, decanted, renewed with 10 mL of the 1× Hanks' balanced salt solution/Hepes solution, and shaken vigorously for 15 seconds. Intestinal villi and crypts were collected and the process was repeated 3 times. Resulting IECs were washed 3× with 10 mL of Hanks' balanced salt solution/Hepes solution and checked in prior and select experiments for purity, averaging 2% to 3% contamination of cells positive for CD45 (leukocyte common antigen), with no significant differences between mouse strains. Ion exchange chromatography (IEC) preps were then either cultured in complete media for 1 hour at 37°C in the presence or absence of FliC (200 ng/mL), or immediately lysed in Buffer RLT (RNeasy Mini Kit; Qiagen, Germantown, MD) or in Pierce RIPA Lysis and Extraction Buffer (ThermoFisher Scientific, Inc., Waltham, MA) containing 1× Halt Protease and Inhibitor Cocktail (Pierce), and stored at −20°C for later RNA isolation or Western blotting, respectively.

ELISA-based Binding Assay for Measuring Mouse Serum Anti-flagellin IgG

The 96-well microtiter plates were coated with 200 μL/well purified *Salmonella typhimurium* flagellin (1 mg/mL, Inotek Pharmaceuticals) brought up in coating buffer (#00-0000-12; eBioscience, Inc., San Diego, CA), and incubated overnight at 4°C. Excess coating solution was subsequently decanted, and plate washed 5× with 300 μL/well washing buffer (0.1% Tween 20 in PBS, pH 7.4), after which 200 μL/well blocking buffer (5% BSA, 0.1% Tween 20 in PBS, pH 7.4) was added and incubated for 2 hours at room temperature. After removal of blocking buffer, 200 μL/well of experimental mouse serum, diluted 1:500 (3% BSA, 0.1% Tween 20 in PBS pH 7.4) was added and incubated overnight at 4°C. Plates were then washed 5× with washing buffer, and 200 μL/well anti-mouse IgG conjugated with horseradish peroxidase (1:1000) was added, incubated at room temperature for 2 hours, and washed 3×. Finally, 200 μL of substrate was added to each well for 60 minutes at room temperature, after which plates were read at 450 nm on a standard ELISA plate reader. Results derived were converted from optical density to IgG concentration using an IgG standard curve with the formula of $y = Bmax \times X (kd + X)$.

Measurement of Luminal Bacterial Load

Luminal bacterial load in native SAMP and AKR mice was determined by homogenizing full-thickness pieces of ilea and jejuna (50–100 mg) (including contents) in sterile PBS and plating homogenates onto blood agar plates (trypticase soy agar containing 5% sheep's blood; Becton Dickinson, Franklin Lakes, NJ) in serial dilutions. Agar plates were incubated at 37°C for 24 hours. Plates containing between 10 and 100 colony-forming units (CFUs) were evaluated to determine bacterial numbers, with data expressed as CFU/g tissue.

Generation of Conditioned Media from BM-derived Macrophages (BMDMs)

Macrophages were derived from BM flushed from isolated femurs and tibia of SAMP and AKR mice, and cultured in Dulbecco's Modified Eagle Medium (Invitrogen) containing penicillin (100 U/mL), streptomycin (100 μg/mL), 10% (vol/vol) heat-inactivated fetal bovine serum, and 20% (vol/vol) L929 cell-conditioned medium at a density of 7.5×10^5 cells/cm^2 at 37°C in a 5% (vol/vol) CO_2 atmosphere for 7 days. Twenty-four hours before use, cells were harvested and plated into 24-well tissue culture plates at a density of 2×10^6/well in 500 μL of culture medium described above. BMDMs were stimulated with 200 ng/mL FliC for 4 hours, and supernatants collected and immediately used for TEER assay (as described above).

Total RNA Extraction and qRT-PCR

Ileal/colonic tissues were recovered from RNA*later* Stabilization Solution (Invitrogen), transferred to Matrix Lysing Tubes "D" (MP Biomedicals, Santa Ana, CA) containing RLT Buffer (RNeasy Mini Kit; Qiagen), and samples were homogenized in a Fast Prep 24 tissue homogenizer (MP Biomedicals). Total RNA from both tissue homogenates and isolated IEC preps was then isolated (RNeasy Mini Kits; Qiagen) and reverse-transcribed

(RNA-to-cDNA kit; Applied Biosystems, Forest City, CA), both according to manufacturer's instructions. qRT-PCR was performed as previously described, using primers for the murine TJ proteins, Cldn1 through 4, Occl,[33] and zonula occludens-1 sense (5′-CCTAAGACCTGTAACCATCT-3′) and antisense (5′-CTGATAGATATCTGGCTCCT-3′), and mouse TLR5 sense (5′-CCCGAAACAGGCTCAGGCTA-3′) and antisense (5′-CCAGGCACCAACCATCCTTC-3′), and control hypoxanthine-guanine phosphoribosyl transferase (mHGPRT)[33] on an Applied Biosystems Step Plus machine (Applied Biosystems). Reaction mixture consisted of 15% volume first-strand synthesis in a total volume of 20 μL that included Power SYBR Green core reagents (Applied Biosystems) and 500 nM final concentration of primers. Thermal cycling conditions were 95°C/10 min followed by 40 cycles of 95°C/15 s and 60°C/1 min. Target mRNA transcripts were normalized to mHGPRT and analysis of relative gene expression data was calculated using the $2^{-\Delta\Delta CT}$ method.[41] Data are reported as a relative fold-difference among groups within an experiment, with the control group set at arbitrarily as 1, as indicated in each figure legend.

Western Blot Analysis

Longitudinally-bisected sections from the distal 10 cm of ilea from experimental mice were placed in RIPA Lysis and Extraction Buffer containing 1× Halt Protease and Inhibitor Cocktail (all from Pierce, Thermo Fisher Scientific, Waltham, MA), and homogenized in a Fast Prep 24 tissue homogenizer (MP Biomedicals). Resulting ileal tissue homogenates as well as thawed IEC preparations (described above), were centrifuged (14,000 rpm for 20 min at 4°C), supernatants collected, and total protein extracts quantified using a modification of the Bradford colorimetric procedure (Bio-Rad Protein Assay; Bio-Rad Laboratories, Hercules, CA). Western blot analysis of total protein extracts was subsequently performed as described.[39] Briefly, samples were mixed 1:1 with Laemmli buffer and equally loaded (25 μg of total protein per lane), after denaturation, on a 10.5% to 14% Tris·HCl Criterion precast gel (Bio-Rad Laboratories). Samples were electrophoretically separated and transferred on a nitrocellulose membrane (Bio-Rad Laboratories). Blots were blocked in a 5% nonfat milk/Tris-buffered saline solution and incubated with specific anti-mouse primary antibodies: TLR5 IgG at a 1:800 dilution (clone EPR10373; Abcam), zonula occludens-1 and Occl IgG, both at a 1:100 dilution, Cldn3 IgG at a 1:100 dilution (all from Invitrogen), and β-actin IgG at a 1:5000 dilution (Abcam) overnight. After washing with Tris-buffered saline containing 0.1% Tween 20 (Sigma), either a polyclonal horseradish peroxidase-conjugated anti-rabbit or an anti-goat IgG at a 1:1000 dilution (R&D Systems, Minneapolis, MN) was used as a secondary detecting antibody. Blots were then developed by enhanced chemiluminescence Western blotting substrate (Pierce) according to manufacturer's instructions.

Densitometric analysis of Western blots was performed on scanned X-ray films using the ImageJ Java-based image processing program (NIH open access). Arbitrary densitometric values for target proteins were normalized to those measured for β-actin for each sample, with background noise subtracted. Data are reported as relative fold-difference either between or among groups within an experiment, with a comparator group set at arbitrarily as 1, as indicated in each figure legend.

Statistical Analysis

Data were analyzed using GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA). Selection of appropriate statistical tests was based on the variance and underlying distribution of data. Global effects between groups were first assessed using a one-way analysis of variance with Bonferroni correction for multiple comparisons. Based on the results of the *F*-test, individual *t*-test comparisons were adjusted for unequal variance (Welch's correction), as needed. For correlation analysis, the coefficient of determination (R^2) was used as a measure for correlation. Results are expressed as mean ± SEM and a probability of <0.05 was considered significant.

Ethical Considerations

All procedures performed on experimental mice were approved by the Institutional Animal Care and Use Committee at Case Western Reserve University and followed the American Association for Laboratory Animal Care guidelines.

RESULTS

TLR5 Is Expressed in Gut Tissues from Young, Uninflammed SAMP Mice and Is Increased During Established Disease Compared with AKR Controls

To evaluate the contribution of the major cell surface sensors of bacteria/bacterial products in the pathogenesis of SAMP ileitis, *Tlr2*, *Tlr4*, and *Tlr5* were measured in full thickness ileal and colonic tissues from SAMP mice at 4 weeks, before the onset of disease, and at 20 weeks, when ileitis is fully established (Fig. 1). Interestingly, *Tlr5* was the most abundantly expressed TLR when compared with *Tlr2* and *Tlr4*, and showed the greatest difference in magnitude versus age-matched AKR controls. During the preinflammatory state, *Tlr5* was increased both in SAMP ilea (1.8-fold increase, $P < 0.05$) and colons (1.9-fold increase, $P < 0.05$) compared with age-matched AKRs, whereas no differences in *Tlr2* and *Tlr4* were found. In older SAMP with established disease, *Tlr5* was markedly upregulated in tissues from the ilea (8.7-fold increase, $P < 0.01$) and colons (11.7-fold increase, $P < 0.01$) compared with age-matched AKR mice. During established disease, *Tlr4* was also increased in colons (2.9-fold increase, $P < 0.01$), but not in ilea compared with controls. No significant differences were measured in *Tlr2* in either the ilea or colons among all experimental groups (Fig. 1). These data show that TLR5 is one of the most abundantly expressed TLRs in the ilea and colons of young, uninflamed SAMP mice, and is potently

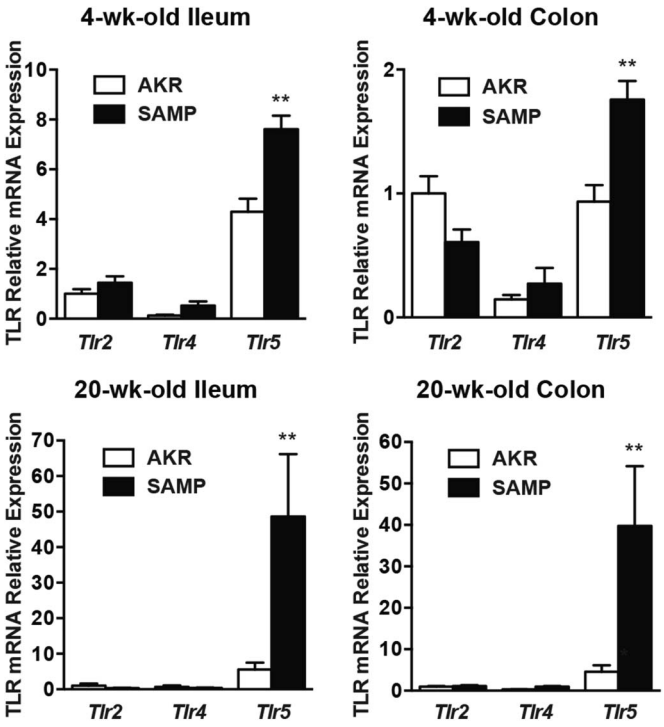

FIGURE 1. Tlr5 is increased in ilea and colons of young, uninflammed SAMP mice, and in older SAMP with established disease. mRNA transcripts of the major cell surface TLRs (i.e., TLR2, TLR4, and TLR5) were measured by qRT-PCR and normalized to *Hgprt* in full thickness ileal and colonic tissues from SAMP mice before the onset of disease (4 weeks of age) and during established ileitis (20 weeks of age), and compared with age-matched AKR controls. Results are expressed as relative mean ± SEM (N ≥ 6/group), with the value for *Tlr2* in 4-week-old AKR set arbitrarily as 1; $^{**}P < 0.01$ versus TLR5 in age-matched AKRs.

upregulated in older SAMP with established disease, versus age-matched AKR controls.

IEC-derived TLR5 and Circulating Anti-flagellin IgG Levels Are Increased Early During SAMP Ileitis, and Both Are Abrogated in the Absence of the Commensal Microflora

Our previous studies showed that a primary epithelial barrier defect is present early in the small intestine of 3-week-old SAMP mice, before the onset of ileal inflammation, that likely drives ileitis and persists even in a GF setting.[33] SAMP mice, however, still display ileal inflammation, albeit delayed and attenuated, when raised under GF conditions compared with age-matched SPF-raised SAMP,[35] indicating that importance of the interaction(s) between the commensal microflora and the intestinal epithelium in this model. We therefore evaluated epithelial-derived TLR5 and the impact of the gut microbiome on TLR5 expression during SAMP ileitis.

We first extended our prior findings and measured small intestinal epithelial permeability in 20-week-old SAMP raised in a GF environment and compared them with age-matched SAMP and AKR raised under SPF conditions. Experimental mice were given an orogastric gavage of a sugar probe cocktail 24 hours before urine collection and in vivo small intestinal epithelial permeability was measured by lactulose/mannitol FE, as previously described.[36] SAMP mice raised for 20 weeks in a GF setting had similar lactulose to mannitol FE ratios compared with age-matched SAMP raised under SPF conditions (0.33 ± 0.02 versus 0.34 ± 0.02, n.s.), despite having approximately 50% severity of disease (7.73 ± 1.53 versus 14.75 ± 1.11, $P < 0.01$) (Fig. 2A, left panel). Elevated lactulose to mannitol FE ratios, however, were measured in both GF- and SPF-raised SAMP relative to age-matched SPF AKR mice (0.19 ± 0.02, both $P < 0.0005$ versus GF and SPF SAMP), and indicated increased small intestinal permeability in SAMP that is independent of gut bacterial colonization (Fig. 2A, B). Conversely, no differences were detected in the FE of sucralose, an indicator of colonic permeability,[36] among the 3 aforementioned experimental groups (Fig. 2A, right panel), which all showed normal tissue morphology and no histologic signs of colitis, even at 20 weeks of age (Fig. 2A, B).

Interestingly, in freshly isolated ileal IECs, *Tlr5* was already markedly increased before the onset of inflammation in 4-week-old SPF SAMP compared with both age-matched SPF AKR (2.1-fold increase, $P < 0.05$) and GF SAMP (118.2-fold increase, $P < 0.001$) (Fig. 3A). These general trends were maintained during established disease with elevated *Tlr5* IEC in 20-week-old SPF SAMP compared with age-matched SPF AKR (3.7-fold increase, $P < 0.01$) and GF SAMP (3.4-fold increase, $P < 0.01$) (Fig. 3A). Moreover, a high positive correlation was observed between the ratio of *Tlr5* in SAMP/AKR ileal IEC and both age, as well as severity of ileitis ($R^2 = 0.99$) (Fig. 3B). At the protein level, similar results were observed, wherein Western blotting confirmed more abundant TLR5 protein in ileal IEC from pre-inflamed SAMP mice versus SAMP with established disease (67.3-fold increase) (see Fig. S1, Supplemental Digital Content 1, http://links.lww.com/IBD/B453). Also similar to trends observed at the mRNA level, although TLR5 protein levels were greater in IEC from older (versus younger) mice independent of strain, TLR5 protein in ileal IEC from SAMP were markedly greater than their respective age-matched AKR controls (see Fig. S1, Supplemental Digital Content 1, http://links.lww.com/IBD/B453). Importantly, circulating serum levels of anti-flagellin IgG were increased early, in 4-week-old SPF-raised SAMP compared with either age-matched SPF AKR or GF SAMP (1.98 ± 0.45 versus 0.78 ± 0.12 and 0.97 ± 0.05 ng/mL, respectively; $P < 0.01$ and $P < 0.05$), whereas no difference in anti-flagellin IgG serum levels was measured between SPF AKR and GF SAMP (Fig. 3C). Taken together, these data show that epithelial-derived TLR5 expression and circulating anti-flagellin IgG levels are increased early during SAMP ileitis, and both are abrogated in the absence of colonizing gut bacteria.

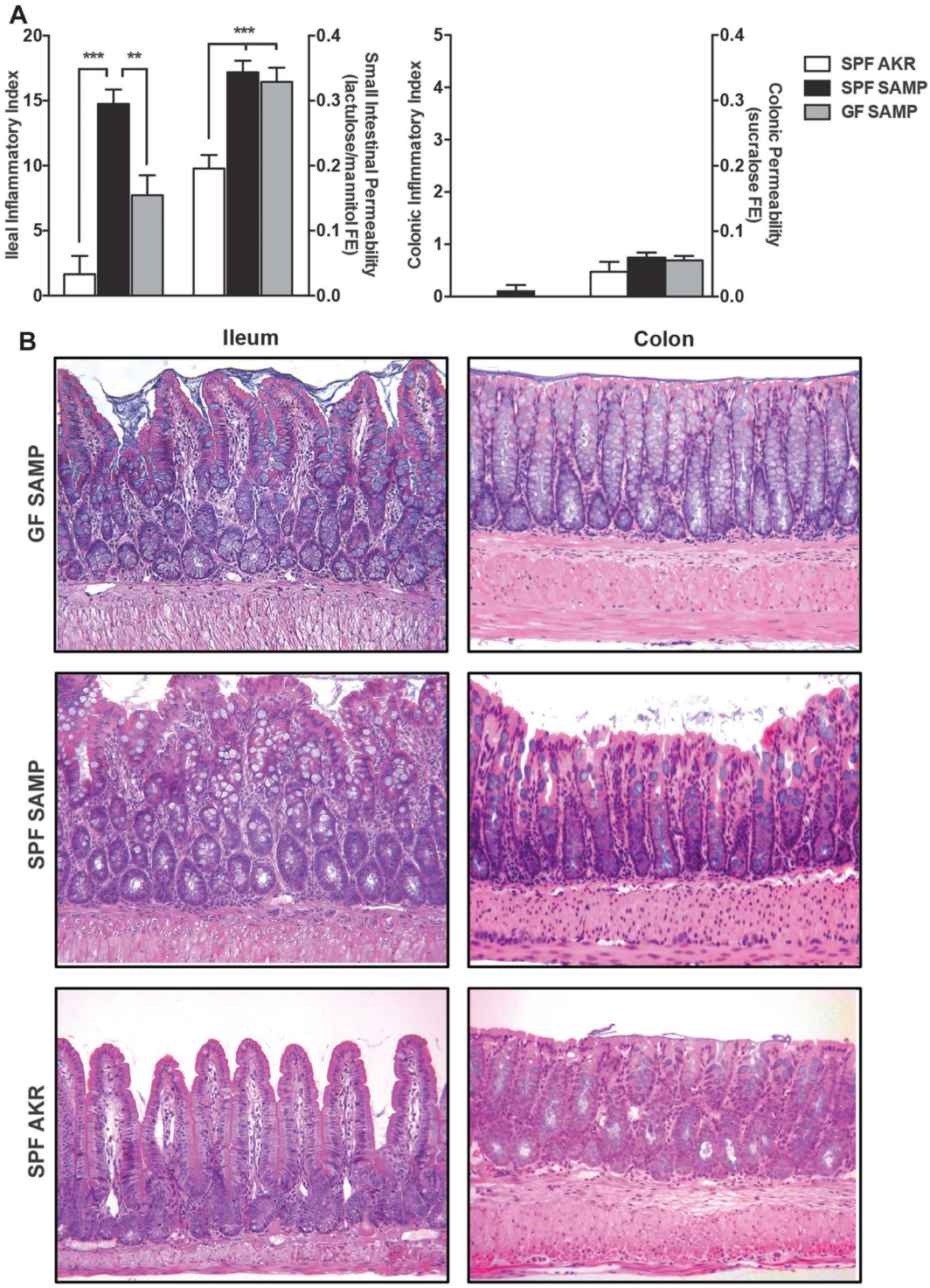

FIGURE 2. Colonization of commensal bacterial flora contributes to the onset and severity of SAMP ileitis, but does not affect increased small intestinal permeability inherent to native SAMP mice. A, Histologic evaluation and in vivo paracellular permeability of ileal/small intestinal tissues (N = 12–13, left panel) and of colonic tissues (N = 6–9, right panel) from 20-week-old GF SAMP and age-matched SPF-raised SAMP and AKR control mice. Results are expressed as mean ± SEM; $^{**}P < 0.01$ and $^{****}P < 0.0005$. B, Representative histology of ileal tissues from 20-week-old GF SAMP show dampened inflammation compared with age-matched SPF SAMP, whereas SPF AKR display normal intestinal architecture and no histologic signs of ileitis (left panels). No significant inflammation was observed in colonic tissues from either SPF AKR, SPF SAMP, or GF SAMP mice (right panels); Mag ×200 for all histology panels.

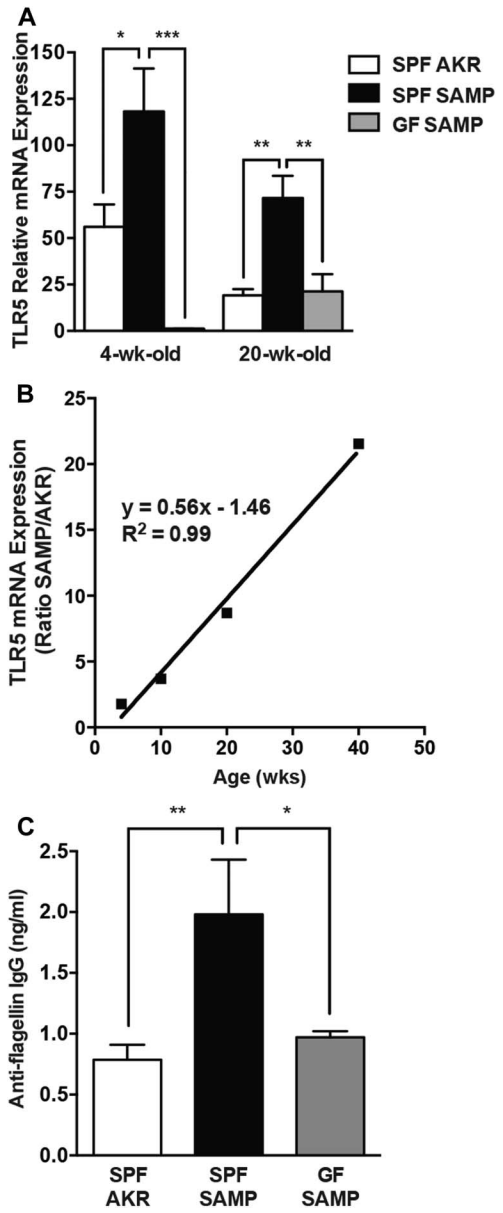

FIGURE 3. Tlr5 in ileal IEC and serum anti-flagellin IgG are markedly elevated in SPF SAMP mice compared with control AKR and GF SAMP. *Tlr5* was measured in freshly isolated IEC from ileal tissues of SPF SAMP, SPF AKR, and GF SAMP by qRT-PCR and normalized relative to *Hgprt*. A, *Tlr5* in 4-week-old SPF SAMP (N = 11) compared with both control SPF AKR (N = 10) and GF SAMP (N = 6), and in 20-week-old SPF SAMP with established disease (N = 9) compared with age-matched SPF AKR (N = 7) and GF SAMP (N = 6). Results are expressed as relative mean ± SEM, with the value for 4-week-old GF SAMP set arbitrarily as 1; $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$. B, The ratio of *Tlr5* in SAMP/AKR ilea was analyzed in 4-, 10-, 20-, and 40-week-old mice (N ≥ 6/group, with average taken for each time point and plotted). C, IgG antibodies against flagellin were measured by ELISA in serum samples from SAMP and AKR mice raised in conventionally-housed SPF conditions (N = 30 and N = 20, respectively), as well as SAMP mice raised under GF conditions (N = 15). Results are expressed as mean ± SEM; $^{*}P < 0.05$, $^{**}P < 0.01$.

BMCs With SAMP-, but Not AKR-derived Nonhematopoietic Compartments Express High Levels of TLR5 and Display Severe Ileitis that Is Dependent on the Commensal Bacterial Flora

To confirm the primary source of TLR5 and the importance of the gut microbiome in SAMP ileitis, reciprocal BMCs using SAMP and AKR mouse strains, as well as SAMP→SAMP and AKR→AKR controls, were constructed as previously described,[33,34] and subsequently treated with a cocktail of broad spectrum ABXs, consisting of ampicillin, vancomycin, neomycin, and metronidazole. After 4 weeks of ABX treatment, mice were sacrificed and histologic analysis was performed on ileal tissues (Fig. 4A, B). SAMP mice that received AKR BM, but not AKR mice that received SAMP BM, were severely inflammed, with overt villous blunting and infiltration of acute and chronic inflammatory cells (Fig. 4B), validating our earlier studies that SAMP ileitis is dependent on the host's nonhematopoietic compartment, and not donor BM.[33] ABX treatment significantly reduced the severity of disease in AKR BM→SAMP and SAMP BM→SAMP mice compared with untreated controls (6.05 ± 1.04 versus 18.00 ± 0.00 and 7.37 ± 1.10 versus 18.00 ± 0.00 total inflammatory index, respectively; $P < 0.001$). Conversely, no histologic evidence of ileitis was assessed in both untreated and ABX-treated AKR→AKR, as well as ABX-treated SAMP BM→AKR mice (Fig. 4A, B). Similar to native SAMP mice, *Tlr5* was markedly elevated in BM transplanted mice for which SAMP mice served as host recipients (AKR→SAMP and SAMP→SAMP) compared with controls (AKR→AKR, 11.3-fold increase, and SAMP→AKR, 22.3-fold increase, both $P < 0.05$, respectively). Also similar to native SAMP, no significant differences were observed in *Tlr2* and *Tlr4* among all experimental BMC groups (Fig. 4C).

Finally, luminal bacterial load in the ilea and jejuna of 4-week-old native SAMP and native AKR mice were measured (Fig. 4D). The number of bacterial CFUs was markedly increased in the ilea (by 3.8-fold), and to a lesser extent in the jejuna (by 2.7-fold), of SAMP compared with AKR mice (5.84 ± 1.59 versus 1.54 ± 0.42 and 0.52 ± 0.09 versus 0.19 ± 0.07 CFU × 10^8/g tissue, respectively; both $P < 0.05$). Overall, however, luminal bacterial load was significantly reduced in jejunal compared with ileal tissues independent of mouse strain (0.19 ± 0.07 versus 1.54 ± 0.42, by 87.7% in AKR and 0.52 ± 0.09 versus 5.84 ± 1.59 CFU × 10^8/g tissue in SAMP by 91.1%, $P < 0.01$ and $P < 0.001$, respectively) (Fig. 3D). Taken together, these data indicate that the mucosal surface of SAMP ilea is exposed to a greater bacterial load compared with AKR controls, early during the progression of ileitis, and that BMCs constructed with a pathogenic nonhematopoietic compartment (AKR→SAMP and SAMP→SAMP) display elevated *Tlr5* and severe ileitis that is abrogated by ABX treatment.

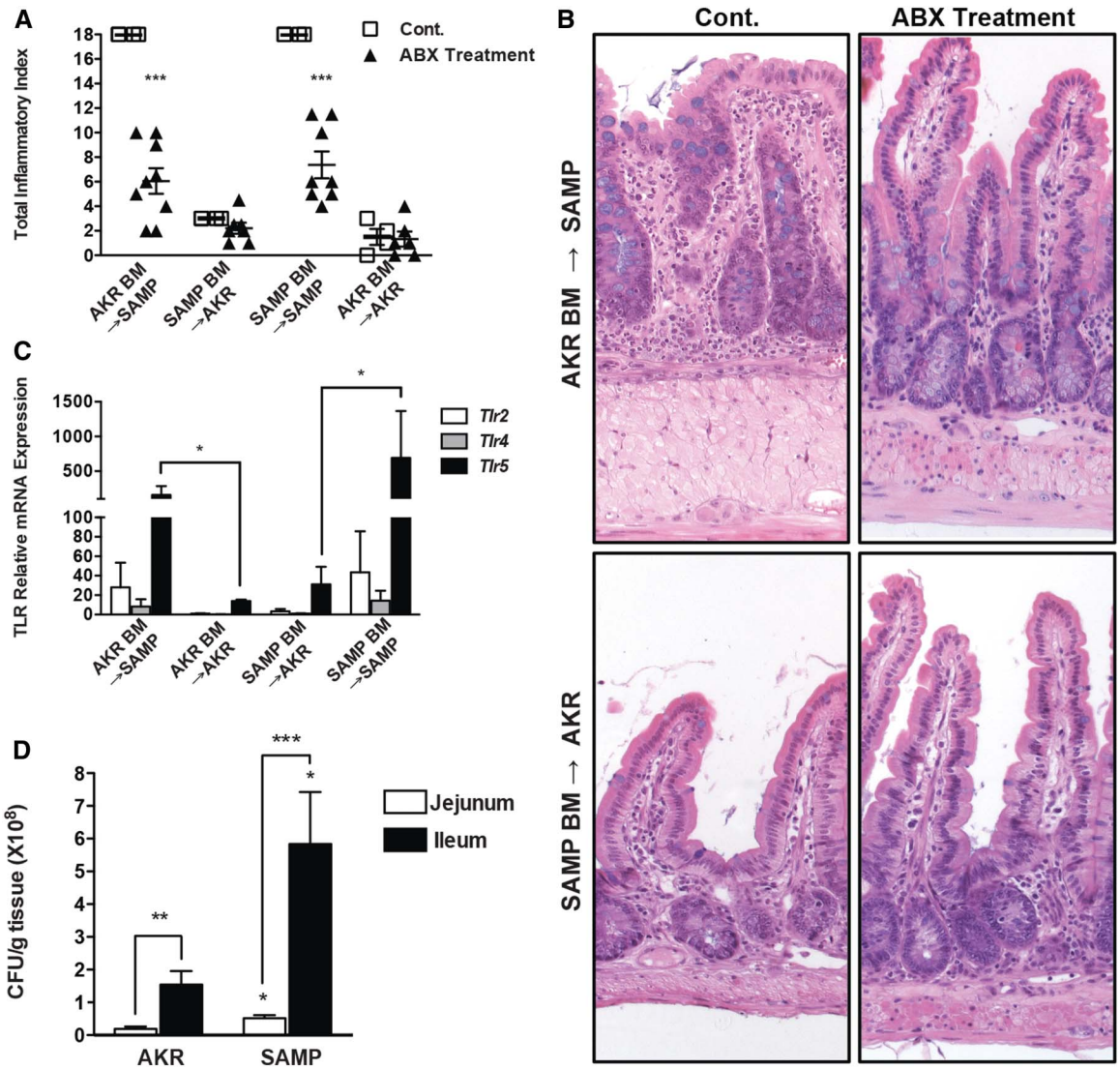

FIGURE 4. *Tlr5* is dependent on status of BM recipient hosts, with SAMP epithelium exposed to greater bacterial load than AKR controls. A, Histologic evaluation of disease severity in ileal tissues from reciprocal BMCs, AKR BM→SAMP (N = 6) and SAMP BM→AKR (N = 6), and controls, SAMP BM→SAMP (N = 8) and AKR→AKR (N = 6), and after treatment with broad spectrum ABXs: AKR BM→SAMP (N = 9), SAMP BM→AKR mice (N = 7), SAMP BM→SAMP mice (N = 8), and AKR→AKR (N = 6). Results are expressed as mean ± SEM; *$P < 0.001$ versus untreated BMCs. B, Representative histology of ilea from untreated AKR BM→SAMP display severe villous distortion and infiltration of acute and chronic inflammatory cells (upper left); after ABX-treatment, a marked reduction was observed in the severity of disease with restoration of normal villous architecture (upper right). Representative histology from both ABX-treated and -untreated SAMP BM→AKR show normal gut morphology with no signs of inflammation (bottom left and right panels, respectively); Mag ×200 for all panels. C, mRNA transcripts of TLRs were measured by qRT-PCR and normalized relative to *Hgprt* in full thickness ileal tissues from reciprocal BMCs and controls (N ≥ 5/group). Results are expressed as mean ± SEM, with the value for *Tlr2* in AKR BM→AKR set arbitrarily as 1; *$P < 0.05$. D, Measurement of luminal bacterial load in the ilea and jejuna of 4-week-old native SAMP (n = 5) and AKR (n = 6) mice. Data are expressed as mean ± SEM; *$P < 0.05$ versus age-matched AKR and **$P < 0.01$ and ***$P < 0.001$ comparing jejunal versus ileal tissues from AKR and SAMP mice, respectively.

TLR5 Activation Directly Acts on IEC and Results in Increased Epithelial Permeability

We next investigated the direct effects of TLR5 activation on epithelial barrier function in SAMP mice by measuring ileal epithelial permeability to charged ions using a previously described TEER assay.[33,37,40] We confirmed our previous findings that, although ilea from 4-week-old AKR mice maintained their baseline TEER after 1 hour in culture, ilea from age-matched SAMP showed a decrease in TEER during the same time period (ΔTEER at 1 h, 7.425 ± 1.870 versus −9.650 ± 2.493 $\Omega \cdot cm^2$, $P < 0.001$) (Fig. 5A), indicating that SAMP epithelium is unable to maintain an effective epithelial barrier, even

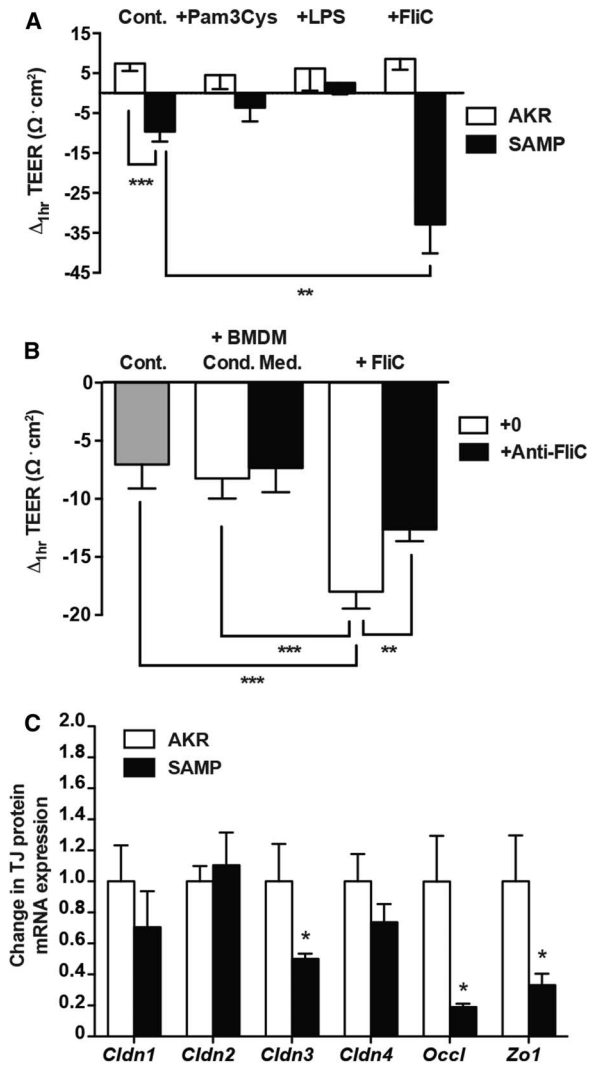

FIGURE 5. Apical application of a TLR5, but not a TLR2 or TLR4, agonist to SAMP ileal tissues directly acts on IECs to increase epithelial paracellular permeability. Ileal tissues from 4-week-old, pre-inflamed SAMP were apically-stimulated ex vivo with the TLR5 agonist, FliC, for 1 hour after which change in TEER (Δ_{1h} TEER) was measured to detect alterations in epithelial permeability (decrease in TEER indicates increased permeability). A, Δ_{1h} TEER comparing unstimulated controls measured in SAMP and AKR ilea to those stimulated with TLR2, TLR4, and TLR5 agonists (i.e., Pam3Cys, LPS, and FliC, respectively); N $\geq$ 7/group. B, Δ_{1h} TEER comparing unstimulated controls measured in SAMP ilea to those stimulated with conditioned media from FliC-stimulated BMDMs and FliC alone, and effects of preincubation of marrow-derived macrophages conditioned media and FliC with an anti-FliC antibody before application onto SAMP ileal tissues; N > 8/group. C, mRNA transcript levels of the TJ proteins, Cldn1–4, Occl, and Zo1, were measured by qRT-PCR and normalized relative to *Hgprt* in isolated ileal IEC preparations from pre-inflamed 4-week-old SAMP and AKR controls cultured for 1 hour in the presence or absence of FliC. Data are shown as relative (fold) differences in FliC-treated versus -untreated IEC (therefore, a value of 1 indicates no change) from SAMP versus age-matched AKRs; N = 6/group. All results are expressed as mean ± SEM; *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$.

as early as 4 weeks of age. Apical exposure of SAMP ilea to the TLR5 agonist, FliC (a subunit protein that polymerizes to form bacterial flagellin), but not to the TLR2 and 4 agonists, Pam3Cys and LPS, respectively, caused a further and substantial drop in TEER ($-32.871 \pm 7.272\ \Omega \cdot cm^2$, $P < 0.01$ versus unstimulated control SAMP), whereas TEER measured across AKR ilea did not significantly change on exposure to the same TLR agonists under the same conditions (Fig. 5A), suggesting that specific attributes of SAMP ileal epithelium, or factors produced preferentially by SAMP ilea, induce an increase in epithelial permeability on TLR5 activation with FliC. Preincubation of FliC with an anti-FliC antibody before application onto 4-week-old SAMP ileal tissues led to abrogation of the FliC-dependent reduction in TEER (-12.630 ± 1.009 versus $-17.99 \pm 1.440\ \Omega \cdot cm^2$, $P < 0.01$) (Fig. 5B), indicating the specificity of TLR5-dependent activation in causing an increase in epithelial permeability. We next determined whether FliC has either a direct effect on SAMP epithelium or an indirect effect, through soluble factors produced by TLR5 activation of hematopoietic-derived cells within the ileal lamina propria that ultimately leads to increased epithelial permeability. Conditioned media from TLR5-expressing SAMP BMDMs stimulated with FliC and preincubated with and without anti-FliC did not alter TEER compared with unstimulated controls when subsequently applied, apically, to SAMP ileal tissues (-8.250 ± 1.731 and -7.350 ± 2.067 versus $-7.050 \pm 20.58\ \Omega \cdot cm^2$, respectively; n.s.) (Fig. 5B), suggesting that TLR5 activation by FliC likely acts directly on SAMP ileal epithelium, inducing a reduction in TEER, indicative of increased epithelial permeability. Finally, TJ proteins in isolated IECs prepared from 4-week-old pre-inflamed SAMP and age-matched AKR ileal tissues after 1 hour culture in the absence or presence of FliC (therefore, measuring change in TJ protein mRNA expression) showed that *Occl*, and to a lesser extent *Zo1* and *Cldn3*, markedly decreased in SAMP compared with AKR controls (81%, 67%, and 50% decrease, respectively, $P < 0.05$), whereas IEC derived from AKR mice did not show significant changes in TJ protein mRNA expression on TLR5 activation (Fig. 5C). Together, these data provide evidence that TLR5, but not TLR2 or TLR4, activation of SAMP ilea specifically induces increased epithelial permeability by directly acting on IEC and by differentially modulating epithelial TJ protein expression.

DISCUSSION

Increasing evidence supports a critical involvement of flagellin/TLR5 in the pathogenesis of IBD, especially in CD,[19–21,24,25,42–44] but their exact roles remain controversial and the precise, downstream consequences of TLR5 activation in CD is currently unknown. Among bacterial antigens, flagellin is an interesting candidate to play a role in mucosal homeostasis and gut immune tolerance. Specifically, flagellin exists in significant quantities, originating from both the commensal flora as well as from pathogenic sources, and is a common bacterial antigen present on most motile bacteria in the gut.[45] Though its size and

structure vary between different species, bacterial flagellin remains highly conserved in regions recognized by TLR5 and is a potent and direct activator of the innate immune system.[46] Importantly, flagellin has been identified as a dominant antigen in IBD, and flagellin-specific antibodies have been reported to be elevated in IBD, particularly CD patients, whereas populations that negatively correlated with TLR5 expression were found to be less likely afflicted with CD.[19,47,48]

In the present study, similar trends were observed in SAMP mice when compared with WT AKR controls. Firstly, the luminal bacterial load was greater in the gut of SAMP compared with AKR, particularly in the ileum, and to a lesser extent in the jejunum, of young mice, facilitating early, increased exposure of bacteria and bacterial products to SAMP ilea. Although the precise etiology of this finding is unknown, several groups have reported marked goblet cell hyperplasia that results in abundant mucous production in SAMP and SAMP-related mouse strains,[29,49–51] which can promote adherence and trapping of bacteria/bacterial products to the surface of the ileal mucosa and subsequent activation of epithelial-expressing TLRs. Secondly, similar to patients with IBD, elevated serum anti-flagellin IgG antibodies were measured in SAMP compared with AKR controls, which may be the end result of the inherent, primary small intestinal epithelial barrier defect characteristic of young SAMP mice[33] that allows increased bacterial translocation across the epithelium, access to basolaterally-expressed TLR5 and initiation of proinflammatory innate immune response,[15,22,52] and eventual generation of antibodies against bacterial components, including flagellin. Thirdly, and also similar to patients afflicted with CD, TLR5 expression was increased in SAMP ilea, both in the preinflammatory state, as well as during established disease versus age-matched AKRs, with TLR5 representing one of the most abundantly expressed TLR cell surface sensor in SAMP ilea. Interestingly, a significant positive correlation with a strong linear trend was calculated showing that the ratio of TLR5 expression in SAMP/AKR ilea increased as the mice aged and ileitis became more severe, indicating a clear involvement of TLR5 not only at the onset, but also in the potential worsening, of disease.

In fact, several lines of evidence suggest that dysregulation of tolerance to intestinal TLR-stimulants and disruption of the epithelial barrier separating MAMPs from responsive, underlying tissues may contribute to the development or perpetuation of IBD.[5] For example, although it is well established that the presence of luminal MAMPs is not sufficient to initiate IBDs in animal models,[53–55] it has been shown that TLR-mediated detection of some luminal MAMPs can exacerbate existing disease.[54–56] Moreover, although the healthy gut contains appreciable quantities of soluble ligands for TLR2, TLR4, and TLR5, the relative abundance of stimulants within the gut microbiota for these receptors can increase dramatically during the course of experimental IBD.[23]

Our earlier studies, in fact, showed that the commensal bacterial flora, although not essential for the initiation of ileitis in the SAMP mouse strain, plays a paramount role in the progression and severity of gut inflammation characteristic of these mice.[30,33,35] Specifically, the small intestinal permeability defect that precedes ileitis in these mice,[33] as well as the ileitis phenotype itself, persists under GF conditions; however, the onset of disease is markedly delayed and the severity significantly dampened, lacking the proinflammatory Th2 immune component present in SPF-raised SAMP.[31,32,35] In the present study, our results provide evidence that the commensal microbiota also drives the expression of TLR5 on ilea IECs. In fact, TLR5 was significantly decreased in IEC isolated from young GF mice compared with SPF SAMP, but even when compared with age-matched SPF-raised AKR mice. In addition, serum anti-flagellin IgG levels were reduced in the absence of commensal bacteria in GF SAMP and were similar to levels in healthy AKR control mice.

Our ex vivo experiments showed that, when activated, TLR5, but not TLR2 and TLR4, is able to cause a significant decrease in transepithelial resistance in ilea from young preinflammatory SAMP mice, and that blockade of TLR5 agonist activity can restore normal gut barrier resistance, confirming specificity of the TLR5-dependent increase in epithelial paracellular permeability. These effects seemed to act directly on SAMP ileal epithelium by modulation of steady state mRNA transcript levels of TJ proteins, including *Occl* and *Zo1*, and to a lesser extent *Cldn3*, which all decreased in response to flagellin when compared with age-matched AKR controls. Of note, no significant differences were observed in protein levels of the aforementioned TJ proteins after TLR5 activation (see Fig. S2, Supplemental Digital Content 2, http://links.lww.com/IBD/B454). However, these measurements may simply reflect the relative brevity of the ex vivo TEER assay (1 h), during which changes in protein levels are not likely detected, whereas conformational changes in epithelial TJ protein complexes, leading to increased permeability, cannot be ruled out. These data, along with the observation that *Tlr5* is markedly elevated in BMCs for which SAMP mice serve as host recipients, independent of donor BM, and that these same BMCs constructed from the nonhematopoietic compartment (e.g., epithelium) of SAMP mice develop severe ileitis, suggest that flagellin/TLR5 activation likely plays a proinflammatory, pathogenic role in SAMP CD-like ileitis.

Although our data differ from previous work that indicates a primary protective role for flagellin/TLR5 activation in IBD,[15,42,57] there are certainly important differences to consider, particularly when comparing SAMP mice with the TLR5 deficient ($Tlr5^{-/-}$) model. Aside from the inherent epithelial barrier defect, SAMP mice display multifactorial predisposing factors to CD-like ileitis, including a polygenic predisposition, innate and adaptive immune dysregulation, and localized susceptibility to the terminal ileum,[26] which are features that closely resembles the human condition. By comparison, $Tlr5^{-/-}$ mice that develop spontaneous colitis were generated by deletion of a single gene (i.e., *Tlr5*) in otherwise healthy mice; a scenario that does not occur in the human setting, specifically in patients with IBD, who do not display either isolated or complete TLR5 loss-of-function. In addition, $Tlr5^{-/-}$ mice develop colitis versus the ileal-specific

disease in SAMP. Since vast differences exist between the colon and the ileum, including but not restricted to, their general anatomy, various cellular components that make up their mucosal immune systems, the composition/load of the microflora, as well as the abundance and cellular localization of TLRs, the possibility exists that TLR5 activation may have very different, and even dichotomous, downstream effects in these 2 gut locations.

In summary, our studies provide evidence that flagellin and TLR5 are important mediators in the pathogenesis of CD-like ileitis in SAMP mice. Although the small intestinal permeability defect characteristic of SAMP mice does not seem to depend on the expression of TLR5 and/or the presence of flagellin, ileal luminal bacterial load is markedly increased and upregulation of TLR5 occurs rapidly in the epithelium of young SAMP mice, before the onset of histologic evidence of ileal inflammation, and is dependent on colonization of the commensal flora. The permissive nature of the inherent epithelial barrier defect allows initial access of bacteria and bacterial products, including flagellin, to epithelial-expressed TLR5, whose basolateral localization has been well documented by several groups.[15,22,58] Direct epithelial activation of TLR5 leads to dysregulated TJ protein expression and to further increased permeability, events that perpetuate bacterial/antigen translocation, as well as the initiation and amplification of inflammation. Although our results suggest that factors produced by TLR5-expressing macrophages after exposure to flagellin likely do not directly affect epithelial paracellular permeability, the possibility exists that these mediators have the ability to augment the inflammatory response through both innate and adaptive mucosal immune responses. For example, recent studies have shown that lamina propria dendritic cells have the ability to rapidly produce IL-22[59] and IL-23[60] in response to bacterial flagellin, and may be important in promoting downstream Th17-mediated immune responses. Taken together, our data suggest a direct role of flagellin and TLR5 activation in promoting epithelial barrier dysfunction and propose a potential mechanistic pathway that contributes to the exacerbation and progression of experimental CD-like ileitis.

ACKNOWLEDGMENTS

The authors thank Sharon B. Hoang and James ("Rusty") R. Mize, MD for their contributions to histology/capturing of histologic images, and histopathologic evaluation of intestinal tissues, respectively.

REFERENCES

1. Bamias G, Corridoni D, Pizarro TT, et al. New insights into the dichotomous role of innate cytokines in gut homeostasis and inflammation. *Cytokine*. 2012;59:451–459.
2. Xavier RJ, Podolsky DK. Unravelling the pathogenesis of inflammatory bowel disease. *Nature*. 2007;448:427–434.
3. Jostins L, Ripke S, Weersma RK, et al. Host-microbe interactions have shaped the genetic architecture of inflammatory bowel disease. *Nature*. 2012;491:119–124.
4. Khor B, Gardet A, Xavier RJ. Genetics and pathogenesis of inflammatory bowel disease. *Nature*. 2011;474:307–317.
5. Pastorelli L, De Salvo C, Mercado JR, et al. Central role of the gut epithelial barrier in the pathogenesis of chronic intestinal inflammation: lessons learned from animal models and human genetics. *Front Immunol*. 2013;4:280.
6. Peloquin JM, Nguyen DD. The microbiota and inflammatory bowel disease: insights from animal models. *Anaerobe*. 2013;24:102–106.
7. Mombaerts P, Mizoguchi E, Grusby MJ, et al. Spontaneous development of inflammatory bowel disease in T cell receptor mutant mice. *Cell*. 1993;75:274–282.
8. Sadlack B, Merz H, Schorle H, et al. Ulcerative colitis-like disease in mice with a disrupted interleukin-2 gene. *Cell*. 1993;75:253–261.
9. Kuhn R, Lohler J, Rennick D, et al. Interleukin-10-deficient mice develop chronic enterocolitis. *Cell*. 1993;75:263–274.
10. Blumberg R, Powrie F. Microbiota, disease, and back to health: a metastable journey. *Sci Transl Med*. 2012;4:137rv137.
11. Madsen KL, Doyle JS, Tavernini MM, et al. Antibiotic therapy attenuates colitis in interleukin 10 gene-deficient mice. *Gastroenterology*. 2000;118:1094–1105.
12. Kang SS, Bloom SM, Norian LA, et al. An antibiotic-responsive mouse model of fulminant ulcerative colitis. *PLoS Med*. 2008;5:e41.
13. Akira S, Uematsu S, Takeuchi O. Pathogen recognition and innate immunity. *Cell*. 2006;124:783–801.
14. Cario E, Podolsky DK. Differential alteration in intestinal epithelial cell expression of toll-like receptor 3 (TLR3) and TLR4 in inflammatory bowel disease. *Infect Immun*. 2000;68:7010–7017.
15. Gewirtz AT, Navas TA, Lyons S, et al. Cutting edge: bacterial flagellin activates basolaterally expressed TLR5 to induce epithelial proinflammatory gene expression. *J Immunol*. 2001;167:1882–1885.
16. Abreu MT, Arnold ET, Thomas LS, et al. TLR4 and MD-2 expression is regulated by immune-mediated signals in human intestinal epithelial cells. *J Biol Chem*. 2002;277:20431–20437.
17. Hershberg RM. The epithelial cell cytoskeleton and intracellular trafficking. V. Polarized compartmentalization of antigen processing and Toll-like receptor signaling in intestinal epithelial cells. *Am J Physiol Gastrointest Liver Physiol*. 2002;283:G833–G839.
18. Aderem A, Ulevitch RJ. Toll-like receptors in the induction of the innate immune response. *Nature*. 2000;406:782–787.
19. Lodes MJ, Cong Y, Elson CO, et al. Bacterial flagellin is a dominant antigen in Crohn disease. *J Clin Invest*. 2004;113:1296–1306.
20. Dubinsky MC, Kugathasan S, Mei L, et al. Increased immune reactivity predicts aggressive complicating Crohn's disease in children. *Clin Gastroenterol Hepatol*. 2008;6:1105–1111.
21. Targan SR, Landers CJ, Yang H, et al. Antibodies to CBir1 flagellin define a unique response that is associated independently with complicated Crohn's disease. *Gastroenterology*. 2005;128:2020–2028.
22. Rhee SH, Im E, Riegler M, et al. Pathophysiological role of Toll-like receptor 5 engagement by bacterial flagellin in colonic inflammation. *Proc Natl Acad Sci U S A*. 2005;102:13610–13615.
23. Erridge C, Duncan SH, Bereswill S, et al. The induction of colitis and ileitis in mice is associated with marked increases in intestinal concentrations of stimulants of TLRs 2, 4, and 5. *PLoS One*. 2010;5:e9125.
24. Ortega-Cava CF, Ishihara S, Rumi MA, et al. Epithelial toll-like receptor 5 is constitutively localized in the mouse cecum and exhibits distinctive down-regulation during experimental colitis. *Clin Vaccin Immunol*. 2006;13:132–138.
25. Stanislawowski M, Wierzbicki PM, Golab A, et al. Decreased Toll-like receptor-5 (TLR-5) expression in the mucosa of ulcerative colitis patients. *J Physiol Pharmacol*. 2009;60(suppl 4):71–75.
26. Pizarro TT, Pastorelli L, Bamias G, et al. SAMP1/YitFc mouse strain: a spontaneous model of Crohn's disease-like ileitis. *Inflamm Bowel Dis*. 2011;17:2566–2584.
27. Matsumoto S, Okabe Y, Setoyama H, et al. Inflammatory bowel disease-like enteritis and caecitis in a senescence accelerated mouse P1/Yit strain. *Gut*. 1998;43:71–78.
28. Rivera-Nieves J, Bamias G, Vidrich A, et al. Emergence of perianal fistulizing disease in the SAMP1/YitFc mouse, a spontaneous model of chronic ileitis. *Gastroenterology*. 2003;124:972–982.
29. Kosiewicz MM, Nast CC, Krishnan A, et al. Th1-type responses mediate spontaneous ileitis in a novel murine model of Crohn's disease. *J Clin Invest*. 2001;107:695–702.

30. Bamias G, Marini M, Moskaluk CA, et al. Down-regulation of intestinal lymphocyte activation and Th1 cytokine production by antibiotic therapy in a murine model of Crohn's disease. *J Immunol.* 2002;169:5308–5314.
31. Bamias G, Martin C, Mishina M, et al. Proinflammatory effects of TH2 cytokines in a murine model of chronic small intestinal inflammation. *Gastroenterology*. 2005;128:654–666.
32. De Salvo C, Wang XM, Pastorelli L, et al. IL-33 drives eosinophil infiltration and pathogenic type 2 helper T-cell immune responses leading to chronic experimental ileitis. *Am J Pathol.* 2016;186:885–898.
33. Olson TS, Reuter BK, Scott KG, et al. The primary defect in experimental ileitis originates from a nonhematopoietic source. *J Exp Med.* 2006;203:541–552.
34. Corridoni D, Kodani T, Rodriguez-Palacios A, et al. Dysregulated NOD2 predisposes SAMP1/YitFc mice to chronic intestinal inflammation. *Proc Natl Acad Sci U S A*. 2013;110:16999–17004.
35. Bamias G, Okazawa A, Rivera-Nieves J, et al. Commensal bacteria exacerbate intestinal inflammation but are not essential for the development of murine ileitis. *J Immunol.* 2007;178:1809–1818.
36. Meddings JB, Gibbons I. Discrimination of site-specific alterations in gastrointestinal permeability in the rat. *Gastroenterology*. 1998;114:83–92.
37. El Asmar R, Panigrahi P, Bamford P, et al. Host-dependent zonulin secretion causes the impairment of the small intestine barrier function after bacterial exposure. *Gastroenterology*. 2002;123:1607–1615.
38. Whitehead RH, VanEeden PE, Noble MD, et al. Establishment of conditionally immortalized epithelial cell lines from both colon and small intestine of adult H-2Kb-tsA58 transgenic mice. *Proc Natl Acad Sci U S A.* 1993;90:587–591.
39. Pastorelli L, Garg RR, Hoang SB, et al. Epithelial-derived IL-33 and its receptor ST2 are dysregulated in ulcerative colitis and in experimental Th1/Th2 driven enteritis. *Proc Natl Acad Sci U S A.* 2010;107:8017–8022.
40. Corridoni D, Pastorelli L, Mattioli B, et al. Probiotic bacteria regulate intestinal epithelial permeability in experimental ileitis by a TNF-dependent mechanism. *PLoS One*. 2012;7:e42067.
41. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods*. 2001;25:402–408.
42. Gewirtz AT, Vijay-Kumar M, Brant SR, et al. Dominant-negative TLR5 polymorphism reduces adaptive immune response to flagellin and negatively associates with Crohn's disease. *Am J Physiol Gastrointest Liver Physiol.* 2006;290:G1157–G1163.
43. Meena NK, Ahuja V, Meena K, et al. Association of TLR5 gene polymorphisms in ulcerative colitis patients of north India and their role in cytokine homeostasis. *PLoS One*. 2015;10:e0120697.
44. Bank S, Andersen PS, Burisch J, et al. Polymorphisms in the toll-like receptor and the IL-23/IL-17 pathways were associated with susceptibility to inflammatory bowel disease in a Danish Cohort. *PLoS One*. 2015;10:e0145302.
45. Winstanley C, Morgan JA. The bacterial flagellin gene as a biomarker for detection, population genetics and epidemiological analysis. *Microbiology*. 1997;143(pt 10):3071–3084.
46. Hayashi F, Smith KD, Ozinsky A, et al. The innate immune response to bacterial flagellin is mediated by Toll-like receptor 5. *Nature*. 2001;410:1099–1103.
47. Sitaraman SV, Klapproth JM, Moore DA III, et al. Elevated flagellin-specific immunoglobulins in Crohn's disease. *Am J Physiol Gastrointest Liver Physiol.* 2005;288:G403–G406.
48. Duchmann R, Kaiser I, Hermann E, et al. Tolerance exists towards resident intestinal flora but is broken in active inflammatory bowel disease (IBD). *Clin Exp Immunol.* 1995;102:448–455.
49. Vidrich A, Buzan JM, Barnes S, et al. Altered epithelial cell lineage allocation and global expansion of the crypt epithelial stem cell population are associated with ileitis in SAMP1/YitFc mice. *Am J Pathol.* 2005;166:1055–1067.
50. Reuter BK, Pizarro TT. Mechanisms of tight junction dysregulation in the SAMP1/YitFc model of Crohn's disease-like ileitis. *Ann N Y Acad Sci.* 2009;1165:301–307.
51. McNamee EN, Wermers JD, Masterson JC, et al. Novel model of TH2-polarized chronic ileitis: the SAMP1 mouse. *Inflamm Bowel Dis.* 2010;16:743–752.
52. Thagia I, Shaw EJ, Smith E, et al. Intestinal epithelial suppressor of cytokine signaling 3 enhances microbial-induced inflammatory tumor necrosis factor-alpha, contributing to epithelial barrier dysfunction. *Am J Physiol Gastrointest Liver Physiol.* 2015;308:G25–G31.
53. Lange S, Delbro DS, Jennische E, et al. The role of the Lps gene in experimental ulcerative colitis in mice. *APMIS.* 1996;104:823–833.
54. Hotta T, Yoshida N, Yoshikawa T, et al. Lipopolysaccharide-induced colitis in rabbits. *Res Exp Med (Berl).* 1986;186:61–69.
55. Sanders CJ, Yu Y, Moore DA III, et al. Humoral immune response to flagellin requires T cells and activation of innate immunity. *J Immunol.* 2006;177:2810–2818.
56. Obermeier F, Dunger N, Deml L, et al. CpG motifs of bacterial DNA exacerbate colitis of dextran sulfate sodium-treated mice. *Eur J Immunol.* 2002;32:2084–2092.
57. Vijay-Kumar M, Sanders CJ, Taylor RT, et al. Deletion of TLR5 results in spontaneous colitis in mice. *J Clin Invest.* 2007;117:3909–3921.
58. Sun J, Fegan PE, Desai AS, et al. Flagellin-induced tolerance of the Toll-like receptor 5 signaling pathway in polarized intestinal epithelial cells. *Am J Physiol Gastrointest Liver Physiol.* 2007;292:G767–G778.
59. Chassaing B, Ley RE, Gewirtz AT. Intestinal epithelial cell toll-like receptor 5 regulates the intestinal microbiota to prevent low-grade inflammation and metabolic syndrome in mice. *Gastroenterology*. 2014;147:1363–1377.e1317.
60. Kinnebrew MA, Buffie CG, Diehl GE, et al. Interleukin 23 production by intestinal CD103(+)CD11b(+) dendritic cells in response to bacterial flagellin enhances mucosal innate immune defense. *Immunity*. 2012;36:276–287.