**Colitis-Associated Variant of TLR2 Causes Impaired Mucosal Repair Because of TFF3 Deficiency**

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**BACKGROUND & AIMS:** Goblet cells (GC) facilitate mucosal protection and epithelial barrier repair, yet the innate immune mechanisms that selectively drive GC functions have not been defined. The aim of this study was to determine whether Toll-like receptor (TLR) 2 and modulation of GC-derived trefoil factor (TFF) 3 are functionally linked in the intestine. **METHODS:** GC modulation was assessed using quantitative real-time polymerase chain reaction analysis (qRT-PCR), Western blotting, and confocal microscopy. Dextran sulfate sodium (DSS) colitis was induced in wild-type, TFF3<sup>−/−</sup>, and TLR2<sup>−/−</sup> mice. Recombinant TLR2 ligand or TFF3 peptide were orally administered after DSS termination. Caco-2 cells overexpressing full-length TLR2 or mutant TLR2-R753Q were tested for TFF3 synthesis and functional-related effects in a wounding assay. **RESULTS:** Data from in vitro (Ls174T) and ex vivo models of murine and human GC reveal that TLR2 activation selectively induces synthesis of TFF3. In vivo studies using TFF3<sup>−/−</sup> or TLR2<sup>−/−</sup> mice demonstrate the ability for oral treatment with a TLR2 agonist to confer antiapoptotic protection of the intestinal mucosa against inflammatory stress-induced damage through TFF3. Recombinant TFF3 rescues TLR2-deficient mice from increased morbidity and mortality during acute colonic injury. Severe ulcerative colitis (UC) has recently been found to be associated with the R753Q polymorphism of the TLR2 gene. The relevance of the observed functional effect of TLR2 in regulating GC is confirmed by the finding that the UC-associated TLR2-R753Q variant is functionally deficient in the ability to induce TFF3 synthesis, thus leading to impaired wound healing. **CONCLUSIONS:** These data demonstrate a novel function of TLR2 in intestinal GC that links products of commensal bacteria to innate immune protection of the host via TFF3.
In this study, we provide evidence of an essential molecular link between innate immunity and host-protective GC function. We show that the benefit of commensal-host interaction in the intestine is through TLR2-mediated induction of the GC-product TFF3, which critically confers antiapoptotic protection of the intestinal mucosa against inflammatory stress-induced damage. Of note, patients affected with ulcerative colitis (UC) can develop extensive colonic disease, a condition characterized by mucosal inflammation and ulceration. This severe phenotype has recently been associated with innate immune dysfunction through the R753Q polymorphism of the TLR2 gene, but the underlying pathophysiology remained so far unresolved. The relevance of our findings is confirmed by showing that the R753Q mutant of TLR2 resulted in reduced TFF3 and impaired healing, thus establishing the mechanistic link to disease pathogenesis. These findings provide a new strategy for developing therapeutic approaches in intestinal injuries.

Materials and Methods

Reagents and Antibodies

Synthetic lipopeptide Pam3Cys-SKKKx3HCl (PCSK; lot No. L08/02) was obtained from EMC Microcollections GmbH (Tübingen, Germany). Recombinant TFF3 (rTFF3) peptide was kindly provided by The GI Company, Framingham, MA. Rabbit polyclonal and mouse monoclonal antisera generated against rat TFF3 have recently been described. Polyclonal antibody to murine MUC2 and monoclonal antibody to pancytokeratin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), and polyclonal antibodies to cleaved/total caspases 7, 8, and 9 were obtained from Cell Signaling (Danvers, MA). ZO-1 polyclonal antibody was from Zymed-Invitrogen (Frankfurt, Germany). Horseradish peroxidase-conjugated anti-rabbit and anti-mouse antibodies were from Amersham (Munich, Germany). All other reagents were obtained from Sigma-Aldrich (Hamburg, Germany), unless otherwise specified.

Cells

Caco-2, IEC-6, and Ls174T cells (American Type Culture Collection) were cultured as previously described or as recommended by the manufacturer, respectively.

Mice

TLR2+/− (Thr2mlKir; >F10 [C57BL6/J]) with wild-type (WT) (TLR2+/+) controls (C57BL6/J) and TFF3+/− (Tff3mlDkpy; >F7 [129S2/SvPaF]) with WT (TFF3+/+) controls (129S2/SvPaF) have previously been described. Further details can be obtained online from The Jackson Laboratory (Bar Harbor, ME). Representative allele-specific genotyping is provided in Supplementary Figure 1. Mice were housed under strict specific pathogen-free conditions (Helicobacter species-, MNV-free) at the Central Animal Facility, University Hospital of Essen, Germany. Protocols were in compliance with German law for use of live animals and approved by the Institutional Animal Care and Use Committee at the University Hospital of Essen and the responsible district government. For more information, please see Supplementary Materials.

3D-Human Intestinal Mucosa-Like Culture Model of Biopsies

Tissue samples were obtained from healthy patients undergoing complete colonoscopy for regular colon cancer screening examinations and/or polypectomy at the Endoscopy Unit (head: M. Rünzi, MD), Kliniken Essen-Süd. Informed consent was obtained from all patients before the procedure, and the protocol was approved by the Human Studies Committee, Kliniken Essen-Süd, Essen, Germany. For processing of biopsy specimens, please see Supplementary Materials.

Organ Culture of Murine Small Intestine

Organ culture of murine small intestine was performed as previously described.

Induction of Colitis and Treatment

Please see Supplementary Materials.

Histologic and Morphometric Analysis

Please see Supplementary Materials.

Immunohistochemistry

Please see Supplementary Materials.

Confocal Immunofluorescence Microscopy

Please see Supplementary Materials.

Analysis of Apoptosis in Colonic Specimens

Please see Supplementary Materials.

Protein Analysis by Immunoblotting and Cytokine Array

Please see Supplementary Materials.

RNA Extraction and Real-Time Polymerase Chain Reaction Analysis

Please see Supplementary Materials.

Plasmid Constructs and Cell Transfection

Please see Supplementary Materials.

Restitution (Migration) in an in Vitro Model of Wounding

Please see Supplementary Materials.

Statistical Analysis

Differences between means were calculated using the 2-tailed, unpaired t test (GraphPad Prism, version 4.03; GraphPad Software, San Diego, CA). P values of <.05 were considered as significant. All data are ex-
pressed as the means ± SEM (n ≥ 3 independent experiments or as indicated).

**Results**

**TLR2 Stimulates TFF3 Synthesis in Intestinal GC**

We first studied the effects of TLR2 in regulating intestinal GC function. We initially focussed on the human GC-like cell line Ls174T and found that it constitutively expressed TLR2 messenger RNA (mRNA), which was enhanced by stimulation with the synthetic TLR2 ligand PCSK at 4 hours (Figure 1A). We then examined whether the major GC products, TFF3 and/or MUC2, were transcriptional and translational targets of TLR2. Stimulation of Ls174T cells with PCSK yielded increased steady-state TFF3 mRNA within 20 minutes (Figure 1A) and maximal activation after 30 minutes with a median effective concentration (EC)_{50} (Supplementary Figure 2) comparable with dose response values recently reported in enterocytes^{12} or splenocytes.^{18} These results are consistent with previous findings that TFF3 acts as an “immediate-early” gene.\(^{19}\) Increase of TFF3 mRNA in Ls174T cells in response to PCSK was followed by induction of TFF3 dimer secretion into the culture superna-

![Figure 1](image_url)

**Figure 1.** TLR2 induces TFF3 production in murine and human GCs in vitro and in vivo. (A) Increase of TLR2 mRNA (240 minutes) and TFF3 mRNA (30 minutes) expression, but not MUC2 mRNA, in GC-like Ls174T cells by PCSK stimulation (20 \(\mu\)g/mL), as determined by real-time RT-PCR analysis. Results are shown in relation to mRNA expression for the housekeeping gene Gapdh and normalized to unstimulated Ls174T cells. Data are presented as means ± SEM (n = 2 or 3 independent experiments): *P < .05; **P < .005. (B) Induction of TFF3 peptide secretion in Ls147T cells by PCSK (20 \(\mu\)g/mL) after 24 hours of stimulation, as determined by Western blotting. (C) Up-regulation of TFF3 peptide (FITC: green) in PCSK-treated human primary GC in an ex vivo culture model of colonic pinch biopsy specimens, as assessed by confocal immunofluorescence (original magnification, 63×/1.3, oil, scan zoom 2.0). Seventy-one separate images (0.29 \(\mu\)m spacing) were collected through the region of interest, and 3D-reconstruction was performed. Nuclei were counterstained with DAPI (red) and IEC with anti-cytokeratin (beige). (D) Increase of intracellular and secreted TFF3 peptide production after 24 hours of ex vivo culture of murine WT (C57BL6/J), but not TLR2\(^{-/-}\), small intestines in the presence of PCSK (20 \(\mu\)g/mL), as determined by Western blotting.
tant (Figure 1B), with similar kinetics as previously described for other stimuli of the TFF3 peptide. To validate the results obtained from the immortalized cell line in primary human GC, we established a novel intestinal mucosa-like culture model of human colonic pinch biopsy specimens in the presence or absence of PCSK. As assessed by confocal immunofluorescence using 3-dimensional (3D) reconstruction, TFF3 peptide expression was markedly enhanced in PCSK-treated human primary GC but attenuated in untreated (Figure 1C). To confirm the functional dependence between TLR2 signaling and PCSK-induced TFF3 synthesis, we cultured small intestines from WT and TLR2\(^{-/-}\) mice with or without PCSK ex vivo and assessed levels of cellular and secreted TFF3 peptide. As shown in Figure 1D, murine primary small intestinal WT-GC produced substantially more TFF3 after PCSK stimulation compared with untreated GC. In contrast, small intestinal TLR2\(^{-/-}\) GC expressed significantly less TFF3, which was not influenced by PCSK, suggesting that PCSK-induced TFF3 synthesis directly requires TLR2.

Next, we determined whether in vivo TLR2 activation may modulate GC function in the intestine. Oral PCSK treatment of healthy mice induced GC proliferation and increased GC size in small intestines (data not shown) and colons via TLR2 (Supplementary Table 1A), correlating with enhanced TFF3 peptide production (data not shown). To investigate whether TLR2 may also induce TFF3 under conditions of inflammatory stress-induced damage, we used DSS-colitis, a well-established model of acute intestinal mucosal injury. Indeed, PCSK treatment led to rapid colonic GC regeneration (Figure 2, left; Supplementary Table 1B), as evidenced by enhanced TFF3 peptide production (Figure 2, right) during the post-DSS healing phase, which correlated with inhibition of inflammatory destruction of the intestinal mucosa. However, expression levels of TFF3 mRNA were elevated in all DSS mice during the recovery phase (day 12), regardless of PCSK administration (Supplementary Figure 3A), possibly reflecting TLR2-mediated differences in mRNA/protein stability or posttranscriptional regulation of TFF3 during GC regeneration. In contrast, TLR2 stimulation did not induce MUC2 gene transcription or protein expression in vitro and in vivo (Figure 1A, Supplementary Figure 3A and B).

**Antiapoptotic Efficacy of Treatment With TLR2 Ligand in Colitis Depends on TFF3 Induction**

Induction of antiapoptosis has been reported to contribute to TLR2-induced accelerated mucosal recovery after acute inflammatory injury, yet the underlying cellular mechanism has not been resolved so far. TFF3 has been shown to block apoptosis, thus accelerat-

![Figure 2. Oral treatment with TLR2 agonist induces GC regeneration and increases TFF3 peptide expression in acute DSS colitis. DSS-colitis was induced in WT mice (C57BL6/J) that received 2.7% DSS for 6 days, followed by oral treatment with 150 \(\mu\)g/mL PCSK or water for 6 subsequent days. Mice were killed on day 12. Representative histology of distal DSS-colon with or without PCSK treatment is shown: left: alcian blue (AB)/periodic acid-schiff (PAS); right: TFF3 (CY5: white) immunofluorescence assessed by confocal laser microscopy (original magnification, 40×/1.3, oil, scan zoom 0.7).](image)
ing wound healing and repair of the intestinal mucosa.\textsuperscript{2–4,16,21,22} Given these properties, we investigated whether TLR2-mediated therapeutic effects in acute colonic inflammation may depend functionally on induction of antiapoptotic TFF3 in GC. Consistent with previous findings,\textsuperscript{2} TFF3\textsuperscript{−/−} mice were highly susceptible to DSS-colitis with an overall mortality rate of 40% (vs 0% in the DSS-TFF3\textsuperscript{+/+} groups). Whereas PCSK treatment ameliorated all colitis-associated signs in TFF3\textsuperscript{+/+}/mice, colitis in TFF3\textsuperscript{−/−}/mice was not influenced during the early phase by the TLR2 ligand (Figure 3A–C). Treatment with PCSK abolished mucosal apoptosis in DSS-TFF3\textsuperscript{+/+} during the acute phase of colitis by day 8. However, absence of TFF3 led to complete inhibition of TLR2-mediated antiapoptosis in acute mucosal inflammation (Figure 3A), resulting in significantly delayed mucosal healing of inflammatory stress-induced injury, as assessed by any of the several parameters (including body weight, histology [Figure 3B and C], and colon length [Supplementary Figure 4A]). Increased cellular apoptosis was found throughout the lamina propria and submucosa as well as surface intestinal epithelium, leading to recruitment of prominent leukocyte infiltrations with transmural involvement, which persisted in DSS-TFF3\textsuperscript{−/−} up to day 12, irrespective of PCSK therapy. These findings indicate that TLR2 critically suppresses mucosal apoptosis in acute colitis via TFF3 in GC. However, compared with untreated DSS-TFF3\textsuperscript{−/−}, PCSK-treated DSS-TFF3\textsuperscript{−/−}/mice showed significantly less rectal bleeding and demonstrated improved restoration of ZO-1-associated barrier integrity in differentiated enterocytes during the recovery phase—even in close proximity to inflammatory infiltrates (Figure 3D; Supplementary Figure 4B and C), suggesting that TLR2 stabilizes colitis-induced, T\textsuperscript{J}-associated barrier disassembly in the intestinal epithelial cell layer independent of TFF3.

**TLR2\textsuperscript{−/−} Mice Exhibit a Selective Defect in TFF3 During Intestinal GC Maturation**

To substantiate further the direct molecular link between TLR2 and TFF3 in the intestine, we next examined GC morphology and related product expression in the absence of TLR2. Intestinal GC in healthy TLR2\textsuperscript{−/−} mice were hypotrophic along the upper half of the crypt (Figure 4A), apparently lacking the ability to transform from pre-GC to mature GC during migration from mid-crypt to villus surface. Quantitative morphometry revealed almost a one-third decrease in GC size in TLR2\textsuperscript{−/−} colons, but no reduction in total GC number, when compared with TLR2\textsuperscript{+/+} colons (Supplementary Table 1A). Colonic TFF3 mRNA levels were decreased by almost 50% in healthy TLR2\textsuperscript{−/−} mice (Supplementary Figure 5A), and TFF3 peptide expression was significantly diminished in the upper portions of the crypt epithelium of healthy TLR2\textsuperscript{−/−} mice (Figure 4B). Furthermore, during the acute phase of DSS-induced inflammation, expression of TFF3 was almost completely lost on mRNA and peptide levels in the absence, but not in the presence, of TLR2 (Figure 4B; Supplementary Figure 5A). In contrast, MUC2 mRNA expression in TLR2\textsuperscript{−/−} mice was comparable with control WT and MUC2 protein was abundantly expressed in GC along the crypt-villus axis in both healthy and inflamed TLR2\textsuperscript{−/−} and WT colons (Supplementary Figure 5A and B). There were no microscopic signs of inflammation and no evidence of infection, essentially excluding secondary causes of GC hypoplasia and TFF3 deficiency.

We considered whether perturbation of stem cell transcriptional factors or associated stromal mediators known to be involved in terminal GC differentiation may have contributed to the observed GC phenotype in TLR2\textsuperscript{−/−}. We observed only a statistically significant increase in G6-1 mRNA levels in healthy TLR2\textsuperscript{−/−} compared with WT colons, whereas the mRNA levels of other progenitor/postmitotic GC regulators remained unchanged (Supplementary Table 2). However, baseline keratinocyte growth factor (KGF) was decreased, potentially contributing secondarily to aberrant GC-specific lineage differentiation in the absence of TLR2.

**TFF3 Supplementation Rescues TLR2–Deficient Mice From Tissue Injury-Induced Lethality**

We and others have recently demonstrated that mice deficient in TLR2 exhibit severe morbidity and mortality during the acute phase of DSS-colitis.\textsuperscript{13,14} We hypothesized that increased tissue injury-induced lethality may be caused by lack of TFF3 in the absence of TLR2. We therefore assessed the effect of oral supplementation of recombinant TFF3 (rTFF3 [0.4 mg/mL]) on the survival rate of TLR2\textsuperscript{−/−} mice after DSS exposure (4%; 5 days). As shown in Figure 5, the overall mortality rate was 100% in DSS-TLR2\textsuperscript{−/−}/control mice vs only 40% in DSS-TLR2\textsuperscript{−/−}/mice that received rTFF3, implying that TFF3 supplementation can rescue TLR2\textsuperscript{−/−} mice from early colitis-induced wasting disease and subsequent death. Consistent with previous findings,\textsuperscript{13} the mortality rate of TLR2\textsuperscript{+/+} controls that did not receive rTFF3 was less than 10% (data not shown).

**rTFF3 Ameliorates Colitis in TLR2-Deficient Mice by Suppressing Mucosal Apoptosis**

We hypothesized that the selective lack in TFF3 production because of impaired GC maturation in TLR2\textsuperscript{−/−} is an important contributing factor for impaired healing of the intestinal mucosa after inflammatory stress-induced damage. To investigate further the disease course during mucosal restitution, we lowered the concentration of DSS (2.0% for 6 days) to reduce its toxicity in TLR2\textsuperscript{−/−} mice. DSS-alone TLR2\textsuperscript{−/−} mice still showed marked colitis-associated signs, whereas administration of rTFF3 significantly ameliorated all signs of DSS-induced colonic inflammation (Figure 6A–C; Supplementary Figure 6A–C). Mucosal cells in DSS-TLR2\textsuperscript{−/−}
mice underwent mitochondrial-dependent apoptosis via initiation of the apoptosome-catalyzed caspase-9 → 7-cascade (intrinsic cell death pathway) (Figure 6C and D). Excessive apoptosis in DSS-TLR2−/− mice coincided with increased chemokine expression, peak migration, and delayed clearance of leukocytes at the site of mucosal inflammation (Supplementary Table 3; Figure 6B and C). Remarkably, TFF3 supplementation completely abolished ongoing caspase-dependent apoptosis in DSS-TLR2−/− mice and consequently reduced apoptosis-associated inflammatory recruitment of leukocytes.

**UC-Associated R753Q Variant of the TLR2 Gene Impairs Wound Healing Because of TFF3 Deficiency**

Up to 40% of UC patients develop extensive colonic disease, called pancolitis,7 which still represents a therapeutic challenge for the clinician. This more severe phenotype has recently been associated with the heterozygous TLR2-R753Q polymorphism,8,9 but the underlying pathogenesis remained so far unresolved. TFF3 promotes essential migration during epithelial restitution.5 We therefore hypothesized that this UC-associated TLR2 variant delays wound repair by failing to induce TFF3 synthesis, thus leading to disease exacerbation. To address this, we stably transfected the intestinal epithelial cell line Caco-2 (which inducibly express TFF323) with self-active24 HA-tagged plasmids of TLR2 full-length (TLR2-FL) or TLR2 mutant (TLR2-R753Q) and assessed basal production of TFF3 and its functional effects on epithelial cell migration in an in vitro wound healing model of IEC-6 monolayers. Overexpression of Caco-2 cells with TLR2-FL led to significant induction of cellular synthesis (Figure 7A) and constitutive secretion of TFF3 peptide into the culture supernatant (Figure 7B), which markedly promoted restitution (Figure 7C). Other factors present in the conditioned media of Caco-2-TLR2-FL may have secondarily contributed to increased IEC-6 restitution, but the degree of migration was comparable with that observed of rTFF35 using the same protein concentration. In contrast, Caco-2 cells overexpressing the TLR2-R753Q mutant did not lead to intracellular production (Figure 7A) or secretion of TFF3 into the supernatants (Figure 7B), which impaired epithelial cell migration (Figure 7C), comparable with untransfected Caco-2 cells (mock). Although stable protein expression levels of HA-tagged full-length were lower than mutant TLR2, transfection efficiency was sufficient to initiate robust TFF3 production in Caco-2-TLR2-FL cells, which was neither evident in Caco-2-mock nor in Caco-2-TLR2-R753Q cells.

**Discussion**

Commensals serve as an important stimulus for diverse innate immune functions that protect intestinal mucosal homeostasis. TLRs are involved in host recognition and response to commensals and play a key role in innate immunity of the gastrointestinal tract. Here, we identify a previously unappreciated TLR2-regulated pathway necessary for induction of TFF3 synthesis during terminal GC maturation, thus critically balancing mucosal homeostasis against proinflammatory apoptosis. These studies provide a molecular link between the innate immune system and commensal-mediated modulation of GC-derived TFF3 in the intestine.

Our data from in vitro and ex vivo studies indicate that stimulation of TLR2 with PCSK promptly induces sustained TFF3 synthesis in murine and human GC. TFF3 transcriptional activation occurs through Ras/MEK/MAPK and PI3K/Akt pathways,19,25 which are both shared by TLR2 signaling.10,13 Downstream, TFF3 transcription is regulated through induction of the goblet cell–specific transcription factor GCSI-BP.26 Future studies will need to determine whether TLR2-induced signaling modules may recruit GCSI-BP or other regulatory elements that may distinctly drive the expression of this key GC product. TLR2 did not modulate MUC2 gene transcription in vitro and in vivo, suggesting a selective and direct GC-regulatory effect on TFF3 synthesis by TLR2 in the intestine.

Furthermore, our findings demonstrate that TLR2 activation distinctly modulates GC function in vivo. A significant increase in TFF3-producing GC via TLR2 was detected in PCSK-treated intestine. The specific and anti-inflammatory effects of PCSK via TLR2 have previously been shown,13 yet the full array of cell-specific protective mechanisms of TLR2 remains to be resolved. We now demonstrate that PCSK-mediated inhibition of inflammatory destruction of the intestinal mucosa correlated...
with rapid induction of GC regeneration and increased TFF3 expression. TLR2-induced suppression of mucosal apoptosis in acute DSS-colitis was essentially regulated through TFF3 in GC. Lack of TFF3 led to abrogation of TLR2-mediated antiapoptosis and significantly delayed mucosal healing of acute stress-induced injury of the intestine. However, independent of TFF3, the TLR2 ligand PCSK efficiently maintained TJ-associated barrier integrity in differentiated enterocytes and thus accelerated wound repair in DSS-TFF3−/− mice during the late recovery phase. These findings imply that TLR2 exerts diverse mucosa-protective properties in different epithelial cell types, critically suppressing mucosal apoptosis in acute colitis via TFF3 in GC. However, because TLR2 is expressed by many cell types within intestinal mucosa, the contribution of additional anti-inflammatory responses mediated by TLR2 on lamina propria mononuclear cells cannot be excluded.

TFF3 expression correlates with terminal GC differentiation. TLR2−/− GC seen in the small intestine and colon were immature because of impairment of commensal-mediated TFF3 production. These cells most likely represent pre goblet cells because the total GC number was similar and MUC2 expression remained unchanged in TLR2−/− when compared with WT controls, suggesting that the GC differentiation program is not completely ablated, rather the terminal part of the GC differentiation pathway essential for TFF3 synthesis is disturbed because of lack of TLR2 stimulation. Degree of intestinal TFF3 expression may be programmed in pluripotent stem cells under the direct or indirect influence of TLR2. However, only Gfi1 was significantly elevated in TLR2−/− colons. Transcription and other factors with well-defined roles in intestinal epithelial stem cell control, such as KLF4 or Math1, were largely unaffected by loss of TLR2. Gfi1 is essential for normal lineage allocation in the intestine and controls TFF3 expression during GC migration. Therefore, increase of Gfi1 may reflect an innate compensatory mechanism of secretory lineage progenitor cells in the attempt to enforce TFF3+ GC maturation in the absence of TLR2 stimulation. Aberrant mesenchymal-epithelial interactions have been shown to impair colonic epithelial progenitor responses in TLR/MyD88−/− mice. Of note, we observed a decrease in KGF in TLR2−/− colons, reflecting proliferative

Figure 4. Intestinal TLR2-deficient GC are hypotrophic because of TFF3-deficiency. (A) Representative PAS histology of the distal colon of healthy WT (C57BL6/J) or TLR2−/− mice (original magnification, 10× or 40× for insert). Black arrow indicates example of hypotrophic GC. (B) Representative TFF3 (white)/propidium iodide (PI) (red) immunofluorescence of the distal WT or TLR2-deficient colon with or without DSS exposure (4% for 5 days), as assessed by confocal laser microscopy (original magnification, 40×/1.3, oil, scan zoom 0.7). White arrows indicate examples of TFF3-deficient GC.

Figure 5. TFF3 supplementation rescues TLR2-deficient mice from colitis-induced lethality. Survival rate of TLR2−/− mice (n = 5 per group) that first received 4.0% DSS for 5 days followed by rescue therapy with recombinant TFF3 (0.4 mg/mL per os) or water for 6 subsequent days. Mice were then followed up to day 21 after DSS start.
alteration in the pericryptal mesenchyme. KGF is an endogenous paracrine effector synthesized by stromal fibroblasts in the colonic stem cell niche. KGF regulates mouse TFF3 transcription through the GCSI element, which is essential for goblet cell–specific expression of TFF3. Reduction of homeostatic KGF in the TLR2-deficient intestinal mucosa may have contributed secondarily to the defect in GC differentiation. The IL-6/Gp130/STAT-pathway has also been linked to TFF3 production, but we were also not able to detect any modulation of baseline interleukin-6 in healthy TLR2−/− intestines.

Figure 6. Administration of rTFF3 ameliorates acute DSS colitis in TLR2-deficient mice by abolishing mucosal apoptosis and associated leukocyte recruitment. TLR2−/− mice (n = 8 per group) received 2.0% DSS for 6 days followed by treatment with rTFF3 (0.4 mg/mL p.o.) or water for 6 subsequent days. Mice were killed on days 8 or 12. Data are presented as means ± SEM. (+rTFF3) vs (−rTFF3): *P < .05; **P < .01; ***P < .001; P > .05. (A) Evolution of body weight during DSS colitis and subsequent supplementation of rTFF3. (B) Representative histology of the distal DSS colon with or without rTFF3 treatment on day 12 (H&E staining on frozen sections [original magnification, 2.5×]). Arrows indicate leukocyte infiltrates; star indicates ulcer. (C) TUNEL (FITC: green) assay of distal colonic tissues using confocal immunofluorescence on days 8 or 12 after DSS exposure with or without subsequent rTFF3 therapy in TLR2−/− mice. Cells were counterstained with propidium iodide (PI) for nuclei (rhodamine: red) and anti-pan-cytokeratin (CY5: white) specific for IEC (original magnification, 40×/1.3, oil, scan zoom 0.7). Representative immunofluorescent images (day 12) are shown; arrows indicate apoptotic cells. (D) Assessment of cleavage of caspases 9, 8, and 7 in whole distal colonic tissues of DSS-TLR2−/− with or without rTFF3 treatment on day 12 by Western blotting.
Our findings indicate that GC-dependent injury responses require functional TLR2 to abolish excessive mucosal apoptosis through TFF3 induction. Lack of TFF3 contributed to increased morbidity and mortality in TLR2−/− mice in stress-induced damage. This defect was reversed by rescue with topical TFF3. The proapoptotic phenotype of DSS-exposed TLR2−/− was highly similar to the intestinal mucosal phenotype exhibited by mice deficient in TFF3 after DSS challenge. TFF3 has been shown to block the induction of both p53-dependent and p53-independent apoptosis.21,22 Remarkably, TFF3 supplementation in DSS-TLR2−/− mice completely abolished ongoing caspase-dependent apoptosis, which rapidly limited the inflammatory recruitment of leukocytes to the inflamed mucosa. The precise mechanisms responsible for (direct or indirect) attenuation of apoptosis remain to be resolved in future studies. TFF3 may induce x-linked inhibitor of apoptosis protein (XIAP), which binds tightly to caspase-9 in the apoptosome complex, resulting in abrogation of caspase-7 processing.35 TFF3 also exerts antiapoptotic effects through an EGFR-dependent mechanism.21 Collectively, these studies reveal that TFF3 represents an important antiapoptotic checkpoint in protective innate immune signalling in intestinal GC via TLR2.

We have previously shown that human TLR2 is not up-regulated in inflamed epithelium from UC patients.36 Novel risk variants in the TLR2 gene have been associated with a more severe disease phenotype in UC patients.15 Our study demonstrates that the UC-associated variant TLR2-R753Q fails to induce TFF3 synthesis, which impairs restitution during wound healing. Dysfunction in GC-TLR2 by the R753Q mutant could be responsible for the typical features seen in UC, including reduction of expression of TFF3 and enhanced apoptosis,7 thus leading to more extensive disease because of impaired innate immune host defense in a subgroup of IBD patients.
In summary, these studies provide first evidence that a specific TLR, namely TLR2, acts to control terminal GC differentiation by selectively regulating TFF3 expression in the intestine, thus conferring antiapoptotic protection of the intestinal mucosa. They suggest that TLR2 deficiency results in an innate immune defect of GC-derived TFF3, contributing to exacerbation of mucosal apoptosis and associated leukocyte influx during acute inflammatory stress-induced damage of the intestine, which can be reversed by supplementation with recombinant TFF3 peptide. They also demonstrate that cell type–specific functional differences in mucosa-protective effects via TLR2 exist within the intestinal epithelial lineages. Finally, the more severe disease phenotype seen in UC patients with the TLR2-mutant haplotype is pathogenetically linked to intestinal TFF3 reduction. As recognition grows for TLRs to play a major role in IBD pathogenesis,7 significant efforts have begun to find a cellular approach of therapeutic TLR manipulation in the gastrointestinal tract.37 Our results suggest that specifically targeting TLR2 in intestinal GC could help in the design of an adjuvant therapeutic means by enhancing cell survival through TFF3 induction, thus protecting the inflamed mucosa during acute gastrointestinal injuries, such as IBD, but potentially also other causes of damage, including, eg, radiation- or chemotherapy-induced mucositis. In conclusion, these data demonstrate a novel function of TLR2 in intestinal GC that links products of commensal bacteria to innate immune protection of the host.

Supplementary Data

Note: To access the supplementary material accompanying this article, visit the online version of Gastroenterology at www.gastrojournal.org, and at doi:10.1053/j.gastro.2009.03.007.

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Received January 13, 2009. Accepted March 10, 2009.

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Acknowledgments
The authors thank The GI Company (Framingham, MA) for the gift of recombinant TFF3, Dr Michael Rünzi (Kliniken Essen-Süd) for kindly providing human colonial biopsy specimens, Kathryn L. Devaney (MGH-GI Unit) for breeding and genotyping the TFF3-knockout mice, and Yvonne Schwafertz (University of Hospital Essen) for technical assistance.

Conflicts of interest
The authors disclose the following: D.K.P. is a founder and holds equity in The GI Company, which has licensed rights for commercial development of TFF3. The remaining authors disclose no conflicts.

Funding
Supported by CCFA grant SRA-1790, DFG grant Ca226/4-2, IFORES program (to E.C.), and NIH grants DK60049 and DK43351 (to D.K.P.).
Supplemental figures and tables to

Colitis-associated variant of TLR2 causes impaired mucosal repair due to TFF3 deficiency

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Suppl. Fig. 1

*Genotyping of TLR2-/- and TFF3-/- mice by PCR.*

Allele-specific genotyping by PCR of mice deficient in TLR2 (A) or TFF3 (B) genes. Tail-extracted genomic DNA was amplified as described in *Supplemental Materials and Methods*. Representative results (n=5 mice per group) are shown.
Suppl. Fig. 2

**Dose response curve of PCSK-mediated TFF3 mRNA increase.**

Dose-dependent increase of TFF3 mRNA expression in GC-like Ls174T cells by PCSK stimulation (30 mins), as determined by real-time RT-PCR analysis. The amount of PCSK (200ng/ml, 2000ng/ml, 20000ng/ml) is plotted as the log of ligand concentration. Each data point represents the mean (± SEM (n=3 independent experiments)) increase of TFF3 mRNA in relation to mRNA expression for the housekeeping gene Gapdh and normalized to unstimulated Ls174T cells. The solid line is the sigmoidal dose-response curve computer-fitted to the data points (GraphPad Prism vers 4.03, GraphPad Software). EC_{50} value was calculated using the log dose-response function and non-linear regression analysis of the software-standardized sigmoidal curve-fitting algorithm.
Suppl. Fig. 3

**The GC-specific product MUC2 is not regulated by TLR2.**

(A) Increase of TFF3 mRNA, but not MUC2 mRNA, in distal DSS-colon with or without PCSK treatment during the recovery phase of colitis (day 12), as determined by real-time RT-PCR analysis. Results are shown in relation to mRNA expression for the housekeeping gene Gapdh and normalized to control H2O-colon (set as 1 arbitrary unit). Data are presented as means ± SEM (n=5-6 mice per group): **, p<0.01; +, p>0.05.

(B) Representative MUC2 (white) -immunofluorescence of distal DSS-colon with or without PCSK treatment on day 12, as assessed by confocal laser microscopy (40x/1.3, oil, scan zoom 0.7).
Suppl. Fig. 4

**Additional parameters for assessment of the therapeutic effects of TLR2 ligand therapy in TFF3-deficient mice.**

TFF3+/+ or TFF3-/- mice [129S2/SvPaf] received 2.5% DSS for 5 days followed by treatment with PCSK 150µg/ml or water for 7 subsequent days. Mice were sacrificed on days 8 or 12. Assessment of total colon length (A). Evolution of blood scores (B) during DSS colitis and subsequent treatment with or without PCSK. Data are presented as means ± SEM. (+PCSK) vs. (-PCSK): *p<0.05; **p<0.01; ***p<0.001; +p>0.05. Representative histology (C) on day 12 of DSS-TFF3-/- colon with or without PCSK treatment (hematoxylin-eosin staining; original magnification: x10 or x40 as indicated). White arrows indicate immature epithelial cells over a large wound defect, black arrows indicate improved epithelization over damaged mucosa.
Suppl. Fig. 5

**TFF3 expression is distinctly reduced in TLR2-deficient GCs.**

(A) Reduction of TFF3 mRNA, but not MUC2 mRNA, in distal colon of TLR2-/− mice with or without DSS exposure (4% for 5 days) during the acute phase of colitis (day 5), as determined by real time RT-PCR analysis. Results are shown in relation to mRNA expression for the housekeeping gene Gapdh and normalized to healthy WT-colon [C57BL6/J]. Data are presented as means ± SEM (n=5-6 mice per group): **, p<0.01; *, p<0.05; +, p>0.05. (B) Representative MUC2 – immunofluorescence of the distal WT or TLR2-deficient colon with or without DSS exposure (4% for 5 days), as assessed by confocal laser microscopy (40x/1.3, oil, scan zoom 0.7).
Suppl. Fig. 6

Additional parameters for assessment of the effects of recombinant TFF3 supplementation in TLR2-deficient mice.

TLR2-/− mice received 2.0% DSS for 6 days followed by treatment with rTFF3 (0.4mg/ml p.o.) or water for 6 subsequent days. Mice were sacrificed on day 12. Evolution of rectal bleeding (A) during DSS colitis and subsequent supplementation of rTFF3. Assessment of (B) total colon length and (C) histology score after DSS exposure with or without rTFF3 on day 12.
### Suppl. Table 1

#### A

<table>
<thead>
<tr>
<th>PAS+ cells in distal colons of healthy mice</th>
<th>total cell number (per vision field)</th>
<th>individual cell size (in square pixels)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>- PCSK</td>
<td>+ PCSK</td>
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<tr>
<td><strong>WT</strong></td>
<td>114.3 ± 6.9</td>
<td>152.1 ± 6.1</td>
</tr>
<tr>
<td><strong>TLR2/-</strong></td>
<td>105.6 ± 5.3</td>
<td>115.8 ± 3.7</td>
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</tbody>
</table>

**Suppl. Table 1A**

**Oral supplementation with PCSK increases number and size of colonic GC via TLR2.**

WT [C57BL6/J] or TLR2/- mice were treated with or without 150µg/ml PCSK in drinking water for 5 subsequent days. GC number and size in distal colons from healthy WT or TLR2/- mice were analyzed as indicated in *Methods*. Data are presented as means ± SEM (n (mice per group): (-PCSK)=4-5; (+PCSK)=2). ***p<0.001; +p>0.05: 1) WT (-PCSK) vs. WT (+PCSK); 2) TLR2/- (-PCSK) vs. WT (-PCSK); 3) TLR2/- (-PCSK) vs. TLR2/- (+PCSK).

#### B

<table>
<thead>
<tr>
<th>PAS+ cells in distal colons of DSS mice</th>
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<tr>
<td></td>
<td>- PCSK</td>
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<tr>
<td><strong>WT</strong></td>
<td>144.5 ± 12.1</td>
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</tbody>
</table>

**Suppl. Table 1B**

**Treatment with PCSK increases GC number in acute colonic inflammation.**

WT [C57BL6/J] mice received 2.7% DSS for 6 days and were treated with or without 150µg/ml PCSK in drinking water for 6 additional days. GC number in distal colons from DSS-WT on day 12 was quantified as described in *Methods*. Data are presented as means ± SEM (n (mice per group)=4 mice). ***p<0.001 (-PCSK) vs. (+PCSK).
Suppl. Table 2

<table>
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<td>Gfi1</td>
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<tr>
<td>Hes5</td>
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<tr>
<td>Klf4</td>
<td>0.93+</td>
</tr>
<tr>
<td>Math1</td>
<td>0.95+</td>
</tr>
<tr>
<td>Notch1</td>
<td>0.84+</td>
</tr>
<tr>
<td>IL-6</td>
<td>1.24+</td>
</tr>
<tr>
<td>KGF</td>
<td>0.63*</td>
</tr>
</tbody>
</table>

# mean ratio (TLR2-/ / WT) of normalized colonic expression levels

Suppl. Table 2

**Gene expression changed in healthy TLR2-/ colons.**

Results are shown as ratios of mRNA expression levels of healthy distal colons between TLR2-/ and WT mice [C57BL6/J], normalized for the housekeeping gene Gapdh. Values are presented as average fold-change, as determined by real-time RT-PCR analysis (n=5-6 mice per group): * p<0.05; ** p<0.01; + p>0.05.
Supp. Table 3

*Treatment with rTFF3 of TLR2-/-- mice reduces colonic levels of chemokines in acute inflammation.*

TLR2-/-- mice were exposed to 2.0% DSS for 6 days and treated with or without 0.4mg/ml rTFF3 in drinking water for additional 6 days. Total protein of distal colons was subjected to cyto-/chemokine array analysis as described in *Methods*. Table lists only proteins downregulated at least 1.5-fold relative to untreated DSS-TLR2-/- mice (n=2 mice per group).