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$^{-/-}$, and TLR2$^{-/-}$ 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$^{-/-}$ or TLR2$^{-/-}$ 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.**

The intestinal epithelium is covered by a protective mucus layer that is in continuous intimate contact with myriad commensal bacteria. The mucus layer is composed predominantly of mucin glycoproteins and trefoil factor (TFF) 3 that are synthesized and secreted by goblet cells (GC) throughout the small and large intestines. TFF3 plays a major role in wound healing and repair of the intestinal mucosa.[1] TFF3$^{-/-}$ mice are highly susceptible to chemical, hypoxia, or radiation stress-induced colonic injuries and fail to mount an effective repair response.[2–4] TFF3 does not exhibit intrinsic activity in regulating cell proliferation but promotes essential migration during epithelial restitution.[5] Commensals may drive GC functions by modulating synthesis of mucus layer components, thus maintaining mucosal homeostasis in the intestine.[6] Alterations of the intestinal mucus composition may contribute to imbalanced activation of immune responses in inflammatory bowel diseases (IBD).[7] However, the innate immune mechanisms of beneficial commensal-host interactions[8] that specifically affect GC dynamics have not been elucidated yet.

Toll-like receptor 2 (TLR2), one member of the TLR family, recognizes conserved molecular patterns associated with both gram-negative and gram-positive bacteria, including lipopeptides, such as synthetic $Pam_3CysSK4$ (PCSK).[9] TLR2 has been shown to be functionally expressed in 3 out of the 4 intestinal epithelial cell lineages: enterocytes,[10] Paneth cells, and enteroendocrine cells[11] but not in GC so far. Recently, progress has been made in defining TLR2-dependent defense mechanisms that help maintain functional tight junction (TJ) barrier integrity of the intestinal epithelial layer. TLR2 directly enhances transepithelial resistance via protein kinase C α/δ of the enterocyte barrier in vitro. Treatment with the TLR2 ligand PCSK protects TJ-associated integrity and decreases intestinal permeability, leading to significant amelioration of acute dextran sulfate sodium (DSS)-induced colonic inflammation during the recovery phase.[12,13] Mice deficient in TLR2 exhibit delayed or diminished tissue repair responses.[13,14] Absence of TLR2 leads to deficient antiapoptotic protection of the intestinal mucosa against toxic stress-induced injury, which further compromises TJ-associated barrier integrity and perpetuates intestinal inflammation.[13] However, the molecular and cellular mechanisms of TLR2-mediated antiapoptosis in mucosal inflammation of the intestine have not yet been further defined.

Abbreviations used in this paper: DSS, dextran sulfate sodium; GC, goblet cell; IBD, inflammatory bowel diseases; PCSK, $Pam_3CysSK4$; TFF3, trefoil factor 3 (intestinal); TJ, tight junction; UC, ulcerative colitis; WT, wild type.

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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,[15] 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 Pam_3Cys-SKKKKx3HCl (PCSK; lot No. L08/02) was obtained from EMC Microcollections GmbH (Tübingen, Germany).[12] 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.[16] 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[12] or as recommended by the manufacturer, respectively.

Mice

TLR2$^{-/-}$ (*Tlr2*^{tm1Kir};>F10 [C57BL6/J]) with wild-type (WT) (TLR2$^{+/+}$) controls (C57BL6/J) and TFF3$^{-/-}$ (*Tff3*^{tm1Dkpy};>F7 [129S2/SvPaf]) with WT (TFF3$^{+/+}$) controls (129S2/SvPaf) have previously been described.[2,17] 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.[13]

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-