Toll-Like Receptor 2 Regulates Intestinal Inflammation by Controlling Integrity of the Enteric Nervous System

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BACKGROUND & AIMS: In the intestines, Toll-like receptor 2 (TLR2) mediates immune responses to pathogens and regulates epithelial barrier function; polymorphisms in TLR2 have been associated with inflammatory bowel disease phenotype. We assessed the effects of TLR2 signaling on the enteric nervous system (ENS) in mice. **METHODS:** TLR2 distribution and function in the ileal neuromuscular layer of mice were determined by immunofluorescence, cytofluorimetric analysis, immunoprecipitation, and immunoblot analyses. We assessed morphology and function of the ENS in *Tlr2*$^{-/-}$ mice and in mice with wild-type *Tlr2* (wild-type mice) depleted of intestinal microbiota, using immunofluorescence, immunoblot, and gastrointestinal motility assays. Levels and signaling of glial cell line–derived neurotrophic factor (GDNF) were determined using quantitative reverse transcriptase polymerase chain reaction, immunohistochemistry, and immunoprecipitation analyses. Colitis was induced by administration of dextran sulfate sodium or 2,4 dinitrobenzensulfonic acid to *Tlr2*$^{-/-}$ mice after termination of GDNF administration. **RESULTS:** TLR2 was expressed in enteric neurons, glia, and smooth muscle cells of the intestinal wall. *Tlr2*$^{-/-}$ mice had alterations in ENS architecture and neurochemical profile, intestinal dysmotility, abnormal mucosal secretion, reduced levels of GDNF in smooth muscle cells, and impaired signaling via Ret–GFRα1. ENS structural and functional anomalies were completely corrected by administration of GDNF to *Tlr2*$^{-/-}$ mice. Wild-type mice depleted of intestinal microbiota had ENS defects and GDNF deficiency, similar to *Tlr2*$^{-/-}$ mice; these defects were partially restored by administration of a TLR2 agonist. *Tlr2*$^{-/-}$ mice developed more severe colitis than wild-type mice after administration of dextran sulfate sodium or 2,4 dinitrobenzensulfonic acid; colitis was not more severe if *Tlr2*$^{-/-}$ mice were given GDNF before dextran sulfate sodium or 2,4 dinitrobenzensulfonic acid. **CONCLUSIONS: In mice, TLR2 signaling regulates intestinal inflammation by controlling ENS structure and neurochemical coding, along with intestinal neuromuscular function. These findings provide information as to how defective TLR2 signaling in the ENS affects inflammatory bowel disease phenotype in humans.**

Keywords: Innate Immunity; Immune Regulation; Ulcerative Colitis; Microbe.

Toll-like receptors (TLRs), a family of evolutionarily conserved proteins, play a key role in sensing microbial structures (eg, peptidoglycan, lipopolysaccharide, bacterial and viral nucleic acids), thereby triggering innate and adaptive immune responses against invading pathogens.[1] However, the finding of TLRs in tissues normally not exposed to microbes (eg, nervous system) and their ability to recognize endogenous molecules (danger-associated molecular patterns) produced by injured tissues clearly points to TLRs having an influence well beyond the induction of host defense responses.[2,3] The multifaceted activity of TLRs is well recognized in the intestinal mucosa, where they mediate inflammatory responses toward pathogens and activate beneficial signals for ensuring tissue integrity under physiological conditions.[4–6] Among all TLRs,[2] TLR2 appears to be a major player in gut homeostasis by exerting cytoprotective effects in intestinal epithelial cells.[7] The absence of TLR2 increases susceptibility to intestinal injury and inflammation, and TLR2 stimulation efficiently enhances epithelial barrier function.[7] Although polymorphisms in *TLRs* genes, including *TLR2,* have been associated with distinct disease phenotypes in patients with chronic inflammatory bowel diseases (IBD), their pathophysiological relevance is still unclear.[8]

Morphological and functional abnormalities of the enteric nervous system (ENS), the complex neuronal network that autonomously regulates most gastrointestinal functions, have been consistently reported in several

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Abbreviations used in this paper: DNBS, 2,4 dinitrobenzensulfonic acid; EGC, enteric glial cells; ENS, enteric nervous system; GDNF, glial cell line-derived neurotrophic factor; GFAP, glial fibrillary acidic protein; IBD, inflammatory bowel diseases; LMMP, longitudinal smooth muscle-myenteric plexus; mRNA, messenger RNA; nNOS, neuronal nitric oxide synthase; rGDNF, recombinant histidine-tagged GDNF; TLR2, Toll-like receptor 2; WT, wild type.

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bowel disorders.[9,10] However, it is unknown whether these ENS defects, ranging from subtle changes to severe structural modifications, are secondary to inflammatory processes or rather have a role in the pathogenesis of gut disorders.[9,10] Because TLRs are expressed in the central nervous system and their signaling is involved in nervous system development,[11,12] we hypothesized that TLR2 signaling is critical for ENS homeostasis. Here, we show that TLR2 is expressed in the ENS and intestinal smooth muscle layers. Its absence induces architectural and neurochemical coding changes in the ENS, leading to gut dysmotility and to higher IBD susceptibility. The finding that ENS anomalies resulting from inadequate TLR2-driven glial cell line–derived neurotrophic factor (GDNF) availability were completely corrected by GDNF treatment reveals the prominence of the TLR2-GDNF axis in ensuring ENS integrity and in the resolution of IBD outcomes.

Methods

Mice

All experimental protocols were approved by the Animal Care and Use Ethics Committee of the University of Padova under license from the Italian Ministry of Health and were in compliance with national and European guidelines for the handling and use of experimental animals. Male TLR2$^{-/-}$ (B6.129-Tlr2^{tm1Kir}/J; postnatal days 21) and age-matched wild-type (WT) C57BL/6J mice (Charles River Laboratories, Stone Ridge, NY) were housed in a temperature- and humidity-controlled room under a 12-hour light–dark cycle. To normalize gut microbiota, mice colonies from both groups were housed in the same room and generally in the same cages and maintained by the same personnel. All animals were specific pathogen-free and given standard chow diet and tap water ad libitum.

Mice Treatments

Recombinant histidine-tagged GDNF (rGDNF) was expressed in *Escherichia coli* and purified as described previously.[13] After endotoxin removal by gel chromatography (Pierce, Rockford, IL), endotoxin contamination was <0.1 pg/dose (Lymulus Amoebocyte Assay; BioWhittaker, Walkersville, MD). rGDNF (2 μg/g subcutaneously) or endotoxin-free saline were daily administered to P14 TLR2$^{-/-}$ and WT mice for 7 days, respectively.[14] For depletion of intestinal microbiota, mice were subjected to antibiotic treatment and 16S ribosomal RNA gene quantification in stool, as described previously[15] and indicated in the Supplementary Material. Pam3CysSerLys4 (Pam3-CSK4; (2 mg/kg intraperitoneally) was administered daily to P14 WT mice during antibiotic treatment for 7 days. No signs of illness were evident during treatment.

Organ Culture

Freshly isolated longitudinal smooth muscle-myenteric plexus (LMMP) cultures were prepared as reported previously, with minor modifications.[16] After a 30-minute equilibration period of LMMP in oxygenated sterile Kreb's solution at 37°C under gentle shaking, experiments were started by replacing incubation medium with fresh Kreb's solution in the presence or absence of Pam3-CSK4 (10 μg/mL; InvivoGen, San Diego, CA), Pam2CGDPKHPKSF (FSL-1, 10 μg/mL; InvivoGen), lipopolysaccharide (100 ng/mL; Calbiochem, Billerica, MA), or unmethylated CpG dinucleotides (CpG, 5 μg/mL; InvivoGen). After 2 or 6 hours, tissues were processed for RNA or protein extraction, respectively.

Additional Methods

Detailed methodology is described in the Supplementary Material.

Statistical Analysis

All results are given as mean ± SEM, except for gastric emptying and geometric center, which are presented as median ± SEM. Statistical significance was calculated with unpaired Student's *t* test or by 1-way analysis of variance with Newman-Keuls post-hoc test, using GraphPad Prism 3.03. $P < .05$ was considered statistically significant.

Results

Distinct Cell Populations of the Small Intestine Express Functional TLR2

Along with the reported presence of TLRs in tissues not directly exposed to microbial products (ie, central nervous system),[11] TLR2 was also expressed in smooth muscle layers of mouse ileum (Figure 1*A* and *B*). By performing multiparameter flow cytometry staining of cells dissociated from LMMP, we observed expression of TLR2 in neurons, glia, smooth muscle and endothelial cells, and macrophages (Figure 1*C*). Upon stimulation of LMMP organ cultures with the specific TLR2 ligand Pam3-CSK4,[7] TLR2 co-precipitated with the adapter MyD88 and p38 mitogen-activated protein kinase and phosphorylated nuclear factor (NF)-κB (Figure 1*D* and *E*), demonstrating the existence of functional TLR2-dependent signaling pathways in LMMP. The finding of MyD88 co-precipitation with TLR2 in the muscle layers, as shown in the mucosa (Figure 1*F*), suggests a potential role for TLR2 in the homeostasis of intestinal neuromuscular tissue, in addition to its well-known effects in gut epithelium.[7]

Absence of TLR2 Results in Altered Architecture and Neurochemical Coding of Myenteric Plexus

Because TLR signaling regulates postnatal neuronal plasticity,[11] we assessed the impact of TLR2 absence on ENS integrity. Although histological appearance and inflammatory mediator levels were comparable between TLR2$^{-/-}$ and WT mice (Supplementary Figure 1), a significant reduction of myenteric ganglia areas due to a lower number of HuC/D^{+} neurons and S100β^{+} enteric glial cells (EGC) in TLR2$^{-/-}$ mice was found (Figure 2*A* and *E* and Supplementary Figure 2). The distribution and expression of the neurofilament protein peripherin, but not βIII-tubulin, were altered in myenteric ganglia of TLR2$^{-/-}$ mice (Figure 2*B* and *E* and Supplementary Figure 3). The concomitant reduced expression of glial structural (ie, glial fibrillary acidic protein [GFAP])[17] and