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

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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, 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 TLR2 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

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.

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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; LMP, 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.
bowel disorders. 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. Because TLRs are expressed in the central nervous system and their signaling is involved in nervous system development, 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-Tlr2tm1Ker/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. 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. For depletion of intestinal microbiota, mice were subjected to antibiotic treatment and 16S ribosomal RNA gene quantification in stool, as described previously 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. 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), TLR2 was also expressed in smooth muscle layers of mouse ileum (Figure 1A 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 1C). Upon stimulation of LMMP organ cultures with the specific TLR2 ligand Pam3-CSK4, TLR2 co-precipitated with the adapter MyD88 and p38 mitogen-activated protein kinase and phosphorylated nuclear factor (NF)-κB (Figure 1D 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 1F), suggests a potential role for TLR2 in the homeostasis of intestinal neuromuscular tissue, in addition to its well-known effects in gut epithelium.

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

Because TLR signaling regulates postnatal neuronal plasticity, 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 2A 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 2B and E and Supplementary Figure 3). The concomitant reduced expression of glial structural (ie, glial fibrillary acidic protein [GFAP]) and
regulatory (ie, S100\(\beta\))\(^{18}\) proteins suggests that TLR2 signaling in
fluences development of both enteric neurons and EGC (Figure 2A, C, and E and Supplementary Figures 2 and 3).

We next asked whether the absence of TLR2 modified the distribution of excitatory (ie, cholinergic and tachykinergic) and inhibitory (ie, nitricergic and VIPergic) neurons in myenteric ganglia. In TLR2\(^{-/-}\) mice, neuronal nitric oxide synthase positive (nNOS\(^{+}\)) neurons were significantly reduced compared with WT mice, and vasoactive intestinal polypeptide\(^{+}\) (VIP\(^{+}\)), substance P\(^{+}\), and cholinergic (choline acetyltransferase\(^{+}\)) neurons were not affected in terms of expression and numbers (Figure 2D and Supplementary Figure 4). In addition, acetylcholine esterase\(^{+}\) fibers were significantly decreased in TLR2\(^{-/-}\) mice as compared with WT (Figure 2F), reflecting a dysfunction of the cholinergic system, possibly dependent on reduced neuronal HuC/D levels (Figure 2A and E).\(^{19}\)

Overall, these data support the view that intestinal TLR2 influences neuronal network integrity and neurochemical coding controlling gastrointestinal functions.

**TLR2\(^{-/-}\) Mice Experience Intestinal Neuromuscular Dysfunctions**

Abnormalities of the ENS have been described in various gastrointestinal motor disorders.\(^{20}\) Isolated ileal segments of TLR2\(^{-/-}\) mice displayed a higher contraction frequency and amplitude in spontaneous rhythmic activity (Figure 3A). Altered excitatory neurotransmission in TLR2\(^{-/-}\) mice was shown by enhanced electric field stimulation-elicited contractions (Figure 3B). The electric field stimulation\(-\)induced contractions up to 20 Hz were of neuronal cholinergic origin as confirmed by their sensitivity to tetrodotoxin, and to the muscarinic receptor

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**Figure 1.** Expression of functional TLR2 in the ileal smooth muscle layers of WT mice. (A) Western blots of TLR2 in protein extracts from full-thickness ileum, ileal mucosa and LMMP of WT mice (n = 5 per group). \(\beta\)-actin was used as loading control. (B) Dual-label immunohistochemistry showing expression of TLR2 and neural marker peripherin in ileal cryosections from WT and TLR2\(^{-/-}\) mice. Nuclei were stained with TOTO-3. Scale bar = 50 \(\mu\)m. Enlarged view of peripherin\(^{+}\) and TLR2\(^{+}\) cells in LMMP from WT mice from boxed area in overlay (scale bar = 12.5 \(\mu\)m). (C) Expression of TLR2 in HuC/D\(^{+}\), GFAP\(^{+}\), \(\alpha\)-smooth muscle actin\(^{+}\), CD31\(^{+}\), F4/80\(^{+}\), CD3\(^{+}\) cells harvested from LMMP of WT mice and analyzed by flow cytometry. For each LMMP, 10\(^5\) cells were collected. Representative dot plots for each experiment are reported. The histogram shows the percentage of double-positive cells for each population subset (n = 6 per group). *\(P < .05\) vs WT. (D) LMMP of WT mice (n = 5 per group) were incubated in the presence or absence of TLR2 ligand Pam3-CSK4 and protein lysates were immunoprecipitated with anti-TLR2. MyD88 in the immunoprecipitates was determined by Western blot. IP, immunoprecipitation. (E) Western blot analysis of phosphorylated nuclear factor-\(\kappa\)B p65, I\(\kappa\)B\(\alpha\), total p38 mitogen-activated protein kinase on proteins extracted from LMMP of WT mice (n = 5) after incubation in presence or absence of TLR2 ligand Pam3-CSK4, \(\beta\)-actin was used as loading control. (F) Protein lysates were obtained from full-thickness ileum, ileal mucosa, and LMMP of WT mice (n = 5 per group) and were immunoprecipitated with anti-TLR2. MyD88 in the immunoprecipitates was determined by Western blot.
blocker atropine (data not shown). In addition, muscle responsiveness after stimulation with the cholinergic agonist carbachol (30 μM) was comparable between genotypes (Supplementary Figure 5), demonstrating that the observed changes in contractility are generated by erroneous neurochemical coding.

Figure 2. Absence of TLR2 signaling perturbs myenteric plexus architecture. (A–D) Immunofluorescence analysis of LMMP from WT and TLR2<sup>−/−</sup> mice, labeled for pan-neuronal marker HuC/D, glial marker S-100β, neurofilaments βIII-tubulin and peripherin, glial filament GFAP, and nNOS. Nuclei were stained with TOTO-3. Scale bars = 37.5 μm in (A) and 75 μm in (B–D). nNOS<sup>+</sup> HuC/D<sup>+</sup> neurons/mm<sup>2</sup> in LMMP of WT and TLR2<sup>−/−</sup> mice. *P < .05 vs WT. (E) Western blot analysis of HuC/D, S-100β, βIII-tubulin, peripherin, GFAP, and nNOS in protein extracts from LMMP of WT and TLR2<sup>−/−</sup> mice (n = 5 per group). β-actin was used as loading control. Protein signals, quantified using densitometry analysis, are reported in Supplementary Figure 3. (F) Acetylcholine esterase (AChE)–stained neurons and fibers within the myenteric plexus of WT and TLR2<sup>−/−</sup> mice. Scale bar = 150 μm. Percentage change in the number of AChE<sup>+</sup> fibers. *P < .05 vs WT.
Considering the strong impact of the main inhibitory neurotransmitter nitric oxide on intestine, we verified the functional consequences of altered nNOS expression in TLR2−/− myenteric plexus by evaluating intestinal contractility induced by nonadrenergic, noncholinergic nerve stimulation. In agreement with previous reports,21 nonadrenergic, noncholinergic-evoked relaxation in both WT and TLR2−/− mice was abolished by the NOS inhibitor N^G^-nitro-L-arginine methyl ester, indicating its nitrergic origin (data not shown). The reduced number of nNOS+ neurons in myenteric ganglia of TLR2−/− mice (Figure 2D) was accompanied by a loss of nitrergic modulation in intestinal contractility (Figure 3C). Together with enhanced contractility in TLR2−/− mice, there was an altered distribution of fluorescein isothiocyanate–labeled dextran in the gastrointestinal tract caused by the significant increase of both gastric emptying and gastrointestinal transit (Figure 3D–F).

**TLR2 Influences the Architecture and Functional Integrity of Submucosal Plexus**

For a more comprehensive analysis of the impact of TLR2 on ENS we evaluated structural and functional integrity of the submucosal plexus. In TLR2−/− mice...
submucosal βIII-tubulin+ neurons and fibers were significantly reduced with no apparent changes in peripherin+ fibers and GFAP+ glial bundles (Figure 4A–D). Because neurons of submucosal plexus exert an integrated control of mucosal functions,22 we evaluated neuromediated secretion of ileal explants from TLR2+/−/− and WT mice. Carbachol induced a strong tetrodotoxin-dependent chloride secretion in WT mice, shown by short-circuit current increment,23 but not in TLR2+/−/− mice (Figure 4E). Overall, these data highlight that structural and functional anomalies are present in both myenteric and submucosal plexuses and are TLR2-dependent.

To verify that TLR2-dependent functional anomalies are nonhematopoietic cell-dependent, we generated bone marrow chimeric mice from WT. As shown in Supplementary Figure 6, the structure and function of ENS were similar in WT mice given either WT or TLR2+/−/− bone marrow, demonstrating that TLR2 signaling in nonhematopoietic cells is a main contributor to ENS homeostasis.

**Figure 4.** Loss of TLR2 signaling causes morphofunctional abnormalities in submucosal plexus. (A–C) Immunofluorescence analysis of submucosal plexus (SMP) from WT and TLR2+/−/− mice. SMP were labeled for the neurofilaments βIII-tubulin, peripherin and the glial filament GFAP. Nuclei were stained with TOTO-3. Scale bar = 75 μm. (D) Western blot analysis of βIII-tubulin, peripherin, and GFAP in protein extracts from SMP of WT and TLR2+/−/− mice (n = 5 per group). β-actin was used as loading control. Protein signals were quantified using densitometry analysis. (E) Measure of short-circuit current (Isc) in basal condition and after stimulation with carbachol (Cch) in ileal segments from WT and TLR2+/−/− mice mounted in Ussing’s chambers (n = 6 per condition). Experiments were done with or without the neuronal blocker tetrodotoxin (TTX). *P < .05 vs WT without TTX.

**TLR2 Signaling Affects Intestinal Expression of Neurotrophic Factors**

In accordance with the recognized role of GDNF for the proper development and maintenance of ENS structural and functional integrity,14,24,25 TLR2+/−/− mice showed a decrement in messenger RNA (mRNA) transcripts and immunoreactivity of GDNF in muscle layers (Figure 5A and B). These findings were associated with a reduced GDNF signaling via RET/GFRA1 complex in freshly isolated LMMP of TLR2+/−/− mice (Figure 5C). To demonstrate the direct effect of TLR2 signaling on GDNF expression we exposed LMMP organ cultures to different TLRs agonists. TLR2 engagement by Pam3-CSK4 (TLR2/TLR1 agonist) or FSL1 (TLR2/TLR6 agonist), but not TLR4 or TLR9 activation with lipopolysaccharide or CpG, respectively, significantly increased GDNF mRNA transcripts (Figure 5D). In addition, TLR2-driven GDNF upregulation was dependent on nuclear factor-κB and p38 mitogen-activated protein kinase signaling (Figure 5E).
Considering that GDNF influences the abundance of ENS subpopulations by controlling cell proliferation and maturation rather than inducing apoptosis, we found no differences in apoptotic cell death and caspase-7 activation in the ileum of WT and TLR2−/− mice (Figure 5F).

**GDNF Administration Ameliorates ENS Defects in TLR2−/− Mice**

To confirm that ENS abnormalities in TLR2−/− mice are secondary to inadequate GDNF levels, the effect of GDNF treatment in vivo was evaluated. In TLR2−/− mice, administration of rGDNF for 7 days restored RET/GFRα1 complex signaling in LMMP (Figure 6A). Consistent with the crucial role of RET-mediated signaling, we observed increased expression of HuC/D and S100β protein in myenteric plexus (Figure 6B). No changes in myenteric ganglia area and HuC/D+ cell number were detected, and S100β+ EGC were almost normalized (Figure 6B and Supplementary Figure 7). In addition, rGDNF supplementation to TLR2−/− mice prevented loss of nNOS+ neurons in myenteric ganglia (Supplementary Figure 8), correcting the contractile abnormalities in ileum segments (Figure 6C) and gastrointestinal transit (Supplementary Figure 9). The rGDNF-mediated therapeutic effect was also observed in submucosal plexus, as shown by restored βIII-tubulin expression and secretory response to carbachol in TLR2−/− mice (Figure 6D and E). Overall, these data confirm that ENS anomalies in TLR2−/− mice are mostly GDNF-dependent.

To investigate the gut microbiota-mediated effects on ENS integrity/function, WT mice were treated with broad-spectrum antibiotics for 2 weeks. Microbiota-depletion efficacy was confirmed by the marked reduction in bacterial loads in mouse feces (Supplementary Figure 10). WT mice treated with antibiotics showed reduced expression of neuronal peripherin and nNOS, and glial S100β proteins in LMMP (Supplementary Figure 10). These ENS anomalies were associated with a marked reduction of electric field...
stimulation-induced excitatory response and to a decreased level of GDNF (mRNA and immunoreactivity) and signaling in LMMP (Supplementary Figure 10). Supplementation with GDNF or Pam3CSK4 partially corrected these anomalies in ENS structure and intestinal contraction, confirming that ENS integrity relies on gut microbiota–TLR2–GDNF axis (Supplementary Figure 10).

Correction of ENS Defects in TLR2^−/− Mice Reduces Colitis Severity

Because TLR2^−/− mice present increased morbidity and mortality during acute experimental colitis,7 we hypothesized that enhanced colitis severity depends on the inadequate regulatory role of the altered ENS. ENS architecture and function were restored by daily

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Figure 6. GDNF administration prevents ENS abnormalities in TLR2^−/− mice and regulates mucosal inflammatory responses. (A) Protein lysates from LMMP of WT and TLR2^−/− mice treated with GDNF or vehicle were immunoprecipitated with anti-Ret. GFRα1, p38 mitogen-activated protein kinase (MAPK), RET phosphorylated tyrosine residues, extracellular signal-regulated kinase (ERK)1/2 were determined by immunoblot. IP, immunoprecipitation. (B) Immunofluorescence of LMMP from WT and TLR2^−/− mice treated with GDNF or vehicle were labeled for HuC/D and S-100β. Nuclei were stained with TOTO-3. Scale bar = 75 μm. Western blot of HuC/D and S-100β in protein extracts from LMMP of WT and TLR2^−/− mice treated with GDNF or vehicle. β-actin was used as loading control. Protein signals were quantified using densitometry. (C) Electric field stimulation (EFS)–elicited contractions in ileum segments of WT and TLR2^−/− mice treated with GDNF or vehicle (n = 6 per group). **P < .01 vs WT; ***P < .01 vs TLR2^−/− treated with GDNF. (D) Immunofluorescence of SMP from TLR2^−/− mice treated with GDNF or vehicle were labeled for βIII-tubulin. Nuclei were stained with TOTO-3. Scale bar = 75 μm. Western blot of βIII-tubulin in protein extracts from SMP of WT and TLR2^−/− mice treated with GDNF or vehicle, β-actin was used as loading control. Protein signals were quantified using densitometry. (E) Basal and carbachol-induced short-circuit current (Isc) in ileal segments from TLR2^−/− mice treated with GDNF mounted in Ussing’s chambers (n = 6 per condition). Experiments were done with or without tetrodotoxin (TTX). *P < .05 vs stimulation with TTX. (F) Body-weight changes and H&E-stained ileal sections during dextran sulfate sodium (DSS) colitis. WT and TLR2^−/− control (CTR) received tap water; WT and TLR2^−/− DSS received 3% DSS; TLR2^−/− DSS+GDNF were treated with GDNF from P14 to P21; GDNF treatment was then discontinued and 3% DSS administration started (n = 6 per group). Scale bar = 90 μm. *P < .05 vs CTR, ^P < .05 vs TLR2^−/− DSS, §P < .05 vs WT DSS.
administration of GDNF in TLR2−/− mice for 7 days. To avoid a direct protective effect of administered GDNF, the activity of which vanished within 24 hours (Supplementary Figure 11), GDNF treatment was stopped 24 hours before colitis induction with dextran sulfate sodium exposure for 5 days or DNBS clisma. Correction of ENS defects in TLR2−/− mice reduced inflammation severity to levels comparable with WT animals in both experimental colitis models (Figure 6F and Supplementary Figures 12 and 13).

Discussion

TLRs are expressed in diverse cell populations throughout the body. They not only sample microbial products, but also recognize endogenous ligands to regulate tissue development, repair, and regeneration. TLR2 that is primarily located into the gut mucosal layer maintains barrier integrity and modulates immune responses. Here, we now show that TLR2 is not merely a host immune sentinel, but a dynamic guardian of ENS integrity as well as indispensable for ensuring gut function in physiological and pathological conditions. The novel findings here are the following: functional TLR2 is expressed on neuronal, glial, and smooth muscle cells; genetic deficiency of TLR2 provokes complex anomalies in the structure, neurochemical coding, and function of ENS, leading to intestinal dysmotility and altered neuro-mediated mucosal secretion; TLR2 signaling influences GDNF availability; in vivo GDNF treatment of TLR2−/− mice largely corrects ENS defects and consequently gut function; and once ENS integrity and function is re-established, TLR2+/− mice experience a response to mucosal inflammatory stimuli comparable with that found in WT animals.

Although TLR2 was first documented in immune and epithelial cells exposed to environmental pathogens, there is increasing evidence that other cell populations, such as endothelial cells, airway smooth muscle cells, and cardiomyocytes, respond to TLR2 ligands. This report is the first description of functional TLR2 expression on distinct cell types in the intestinal smooth muscle layers, namely smooth muscle cells, enteric neurons, and glia. In the brain, TLR2 directly regulates neuronal progenitor cell proliferation and fate; however, only minimal architectural abnormalities have been described in TLR2−/− mice. Here, the absence of TLR2 signaling is shown to determine architectural anomalies in the ENS.

On the basis of the premise that gut–brain disease paradigms can exist, the structural defects of regulatory proteins and neurofilaments expressed in ENS appear to be phenotypic characteristics resembling those found in neurodegenerative disorders of the central and peripheral nervous system. Reduced expression of HuC/D, a RNA-binding protein stabilizing several key transcripts (eg, acetylcholine esterase, neurofilament M, etc) has been described in diabetic neuropathy. Anomalies of type III intermediate filament peripherin and the microtubule element βIII-tubulin have been reported in several pathological conditions characterized by neuronal suffering. In addition, expression of S100β and GFAP, specific markers for EGC, is significantly affected in gut neuropathies associated with metabolic and inflammatory diseases. Although the pathophysologic relevance of the observed alterations in these regulatory and structural proteins is still unknown in the ENS, they clearly indicate the presence of an underlying gut neuropathy in the absence of proper TLR2 signaling. The morphological abnormalities of ENS in TLR2−/− mice were associated with impaired small bowel function, evidenced by altered neuromuscular contractility and mucosal secretion. The balanced excitatory and inhibitory innervation of the intestinal muscle is a key requirement for coordinated propulsion. We suggest that the observed gut dysmotility in TLR2−/− mice can be ascribed to an unbalanced inhibitory/excitatory neuromuscular activity. Changes in intestinal innervation are either considered secondary to genetic diseases or interpreted as neuroplastic responses during inflammatory or metabolic disorders. Strikingly, in our study, neuronal dysfunction in the absence of histological and biochemical signs of inflammation identifies TLR2 signaling as a key ingredient in the development and maintenance of ENS. Functional or organic abnormalities of ENS can arise after disturbances in neurotrophic factor signaling. For example, complete deficit in GDNF signaling determines aganglionosis in children, and its reduction contributes to changes in ENS architecture and function during diabetic neuropathy. In agreement with the newly discovered ability of TLRs to mediate central nervous system neurotrophic factor production, we observed that TLR2 signaling is required for adequate GDNF expression in the gut. In line with previous reports, intestinal smooth muscle cells during postnatal development appeared to be the primary source of GDNF, which is required for neuronal cell survival, growth, and innervation of target tissues. The reduced density of HuC/D+ neurons in the myenteric plexus of TLR2−/− mice and ENS anomalies might be attributed to defective GDNF signaling caused by either lack of the neurotrophic factor or absence of its receptor GFRα2. The importance of the TLR2-GDNF axis in ENS integrity and activity was shown by the ability of recombinant GDNF administration to rescue most of the observed defects in ENS architecture and function. TLR2−/− mice are more susceptible to colitis after exposure to chemical mucosal insults, which has been attributed to compromised barrier integrity. However, the fact that the ENS in TLR2−/− mice could be stabilized to the level of WT mice after GDNF administration clearly demonstrates for the first time that a primary dysfunction of ENS results in development of more severe IBD. In contrast to previous studies showing that administration of recombinant adenoviral vector encoding GDNF during colitis ameliorates inflammatory damage by reducing epithelial cell apoptosis, our protective effects on colitis...
in TLR2−/− mice were evident in the absence of concurrent GDNF administration, but were induced by earlier GDNF-mediated ENS restoration. These findings support the novel view that structural changes in the ENS, observed in healthy gut segments of Crohn’s disease patients, might precede the onset of mucosal inflammation and eventually contribute to spreading and exacerbation of the disease.25

Genetic defects or inadequate ligand availability could be responsible for the deficit in TLR2 signaling contributing to ENS dysfunction. Clinical studies have shown that TLR2 polymorphisms26 causing deficient signaling correlate with a more extensive disease localization in IBD, and the presence of ENS anomalies in noninfamed Crohn’s disease resection margins are indicative of early postoperative recurrence.27,28 On the other hand, because gut microbiota—derived pathogen-associated molecular pattern can interact with innate immunity receptors potentially in any sterile body district,29,30 perturbations of gut microbiota composition induced by infection or pharmacological treatment might indirectly affect ENS structure and function, paving the way for functional or inflammatory gastrointestinal disorders.31 The different pattern of functional and neurochemical alterations observed in the ENS in the presence of defective TLR2 and/or TLR4 signaling or potentially of others TLRs strengthens the importance of an harmonized TLRs activity in preserving ENS homeostasis. This concurrent action of multiple TLRs in the regulation of the same pathway is not a novelty, but has been extensively demonstrated in metabolic disorders.32

In conclusion, our data demonstrate that TLR2 is not merely a host immune sensor, but a dynamic guardian of ENS integrity instrumental to preserve gut homeostasis. Most recently, several reports have expanded the role of gut microbiota to directly influencing gut—brain axis function, in view of the fact that alterations in microbiota are linked not just to gut inflammation, but also to changes in host behavior.33 Because stimuli delivered through TLR2 can directly affect immunological responses and ENS integrity, it is evident that in the gut TLR2 function lies squarely at the crossroads of gut microbiota, epithelial barrier, and ENS, finely tuning beneficial and harmful insults. In the context of multifactorial pathologies, such as IBD, tuning innate immunity—ENS crosstalk might represent an attractive target for novel therapeutic strategies.

Supplementary Material

Note: To access the supplementary material accompanying this article, visit the online version of Gastroenterology at www.gastrojournal.org, and at http://dx.doi.org/10.1053/j.gastro.2013.08.047.

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Conflicts of interest
The authors disclose no conflicts.

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