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# Toll like receptor-2 regulates production of glial-derived neurotrophic factors in murine intestinal smooth muscle cells

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ABSTRACT

Gut microbiota-innate immunity axis is emerging as a key player to guarantee the structural and functional integrity of the enteric nervous system (ENS). Alterations in the composition of the gut microbiota, derangement in signaling of innate immune receptors such as Toll-like receptors (TLRs), and modifications in the neurochemical coding of the ENS have been associated with a variety of gastrointestinal disorders. Indeed, TLR2 activation by microbial products controls the ENS structure and regulates intestinal neuromuscular function. However, the cellular populations and the molecular mechanisms shaping the plasticity of enteric neurons in response to gut microbes are largely unexplored. In this study, smooth muscle cells (SMCs), enteric glial cells (EGCs) and macrophages/dendritic cells (MΦ/DCs) were isolated and cultured from the ileal longitudinal muscle layer of wild-type (WT) and Toll-like receptor-2 deficient ($TLR2^{-/-}$) mice. Quantification of mRNA levels of neurotrophins at baseline and following stimulation with TLR ligands was performed by RT-PCR. To determine the role of neurotrophins in supporting the neuronal phenotype, we performed co-culture experiments of enteric neurons with the conditioned media of cells isolated from the longitudinal muscle layer of WT or $TLR2^{-/-}$ mice. The neuronal phenotype was investigated evaluating the expression of βIII-tubulin, HuC/D, and nNOS by immunocytochemistry. As detected by semi-quantitative RT-PCR, SMCs expressed mRNA coding TLR1-9. Among the tested cell populations, un-stimulated SMCs were the most prominent sources of neurotrophins. Stimulation with TLR2, TLR4, TLR5 and TLR9 ligands further increased Gdnf, Ngf, Bdnf and Lif mRNA levels in SMCs. Enteric neurons isolated from $TLR2^{-/-}$ mice exhibited smaller ganglia, fewer $HuC/D^{+ve}$ and $nNOS^{+ve}$ neurons and shorter βIII-tubulin axonal networks as compared to neurons cultured from WT mice. The co-culture with the conditioned media from WT-SMCs but not with those from WT-EGCs or WT-MΦ/DCs corrected the altered neuronal phenotype of $TLR2^{-/-}$ mice. Supplementation of $TLR2^{-/-}$ neuronal cultures with GDNF recapitulated the WT-SMC co-culture effect whereas the knockdown of GDNF expression in WT-SMCs using shRNA interference abolished the effect on $TLR2^{-/-}$ neurons. These data revealed that by exploiting the repertoire of TLRs to decode gut-microbial signals, intestinal SMCs elaborate a cocktail of neurotrophic factors that in turn supports neuronal phenotype. In this view, the SMCs represent an attractive target for novel therapeutic strategies.



## 1. Introduction

In the enteric nervous system (ENS) distinct neuronal subpopulations encode excitatory and inhibitory neurotransmitters building up neural circuitries that regulate intestinal secretion and blood flow and finely control intestinal motility (Grundy and Schemann, 2007). Subtle alterations in transmitter production and release have been linked to morphological changes in the ENS and have been reported in functional and inflammatory diseases of the gastrointestinal (GI) tract (Delvaux, 2004; Geboes and Collins, 1998; Vasina et al., 2006; Villanacci et al., 2008). Noteworthy, specific trophic factors of the intestinal microenvironment control the commitment of enteric neuronal stem cells during development and also guide neural plasticity and regeneration following postnatal inflammatory, nutritional and microbiological challenges (Gershon et al., 1993; Schäfer et al., 2009). Thus, the mixture of soluble factors present in the gut such as neurotrophins and cytokines ensures the ENS to review its connections and to determine the neuronal phenotype and plasticity.

*Abbreviations:* Bdnf, Brain Derived Neurotrophic Factor; CM, Complete Medium; Cntf, Ciliary Neurotrophic Factor; EGCs, Enteric Glial Cells; ENS, Enteric Nervous System; FLA, Flagellin; FSL-1, Pam2CGDPKHPKSF; GDNF, Glial-Derived Neurotrophic Factor; Lif, Leukemia Inhibitory Factor; LMMP, Longitudinal Muscle Myenteric Plexus; LPS, Lipopolysaccharide; MΦ/DCs, Macrophages/Dendritic Cells; NGF, Nerve Growth Factor; Ntf, Neurotrophin; Pam3-CSK4, Pam3CysSerLys4; PAMPs, Pathogen Associated Molecular Patterns; Poly(I:C), Polyinosinic:polycytidylic acid; PRRs, Pattern Recognition Receptors; shRNA, short hairpin RNA; SMCs, Smooth Muscle Cells; TLRs, Tool-like Receptors.

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The gut microbiota, the complex ecosystem containing trillions of microbial cells is emerging as a chief regulator of gut homeostasis. Upon interacting with pattern recognition receptors (PRRs), including Toll-like receptors (TLRs), gut commensal microbes maintain mucosal integrity, ensure neuronal survival, and control the proper development of the immune system and the proliferation and differentiation of stem cell (Anitha et al., 2012; Sekirov et al., 2010). Among all TLRs, TLR2 enhances epithelial barrier function, regulates intestinal inflammation and neuromuscular function and controls ENS structure and neurochemical coding (Brun et al., 2013; Cario et al., 2007). In addition, the role of gut microbiota in GI neuromuscular activity has lately emerged. Experiments in germ-free mice suggested that gut microbes promote myoelectric activity of small bowel and influence the chemical coding and structure of the myenteric plexus (Collins et al., 2014; El Aidy et al., 2012; Husebye et al., 2001). Recent research papers further strengthened the relationship between gut microbiota and the ENS revealing that the gut microbes directly modify colonic neuropeptide content and receptor distribution (Nasser et al., 2007; Rousseaux et al., 2007).

Derangement in the composition of the gut microbial community is a central or a contributing cause of many GI diseases possibly by altering the intestinal neurotransmission pathways (Collins et al., 2014). However, the cell populations and the molecular mechanisms responsible for decoding of gut microbiota signals and for generation of the mixture of neurotrophins modulating integrity and function of the ENS are still largely unexplored. In this study we assumed that in the gut wall intestinal smooth muscle cells use TLRs to sense the gut microbiota-derived signals. The TLR engagement modulates in SMC the production of specific neurotrophins which in turn guide the plasticity of enteric neurons to acquire and maintain the proper phenotype.

## 2. Material and methods

### 2.1. Mice

Male TLR2$^{-/-}$ (B6.129-Tlr2$^{tm1Kir}$/J; postnatal day 30) and age-matched wild-type (WT) C57BL/6J mice (Harlan Laboratories, Oderzo, Italy) were housed in a temperature- and humidity-controlled animal facility under a 12-hour light–dark cycle. To normalize gut microbiota, mice colonies from both groups were housed 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. All experimental protocols were performed in compliance with the National and European directives for animal experiments and were approved by the Animal Care and Use Committee of the University of Padova.

### 2.2. Primary cell isolation and culture

Mice were sacrificed by cervical dislocation. The small intestine was aseptically removed, washed in Hanks balanced salt solution (HBSS; Gibco, Monza, Italy) and cut into pieces of 1 cm length. Intestinal segments were placed on a sterile glass rod and a small incision was created in the longitudinal muscle by gently rub the edge of the forceps. The longitudinal muscle layer with the adherent myenteric plexus (LMMP) was peeled off under a dissecting microscope (Leica, Milan, Italy). LMMP samples were immediately frozen for RNA extraction. For cell isolation, LMMP preparations were rinsed twice in HBSS and processed as elsewhere reported (Voss et al., 2013). Briefly, LMMP was dissociated by incubation for 10 min at 37 °C in HBSS containing collagenase type II from *Clostridium histolyticum* (14 mg mL$^{-1}$, Gibco), dispase (62.5 μg mL$^{-1}$, Sigma, Milan, Italy) and DNase (6.25 μg mL$^{-1}$, Calbiochem, Milan, Italy). Following enzymatic inactivation, tissues were further vigorously triturated with a Pasteur pipette.

To obtain mixed enteric cultures (including neurons, enteric glia, muscle cells and macrophages) LMMP-derived cell suspensions were seeded ($2 \times 10^3$ cells cm$^{-2}$) onto collagen type I (Sigma) coated cell culture plates (Corning, Milan, Italy) and incubated at 37 °C in 5% $CO_2$. Cells were cultured in Euromed-N (Euroclone, Milan, Italy) supplemented with 10% heat inactivated fetal bovine serum (FBS), penicillin (100 U mL$^{-1}$), streptomycin (50 μg mL$^{-1}$), 1% glutamine, N-2 supplement, and forskolin (1 μM), all purchased from Gibco (thereafter referred as complete medium, CM). CM was supplemented with nerve growth factor (NGF, 10 ng mL$^{-1}$, Gibco). Medium was renewed 18 h after cell isolation and replaced every 3 days. Cells were used after 7–10 days of culture.

To obtain cultures of primary smooth muscle cells (SMCs), LMMP-derived cell suspensions were seeded in 6-well plates and cultured in DMEM supplemented with 10% FBS, 1% glutamine, 1 mM sodium pyruvate, 100 U mL$^{-1}$ of penicillin and 50 μg mL$^{-1}$ of streptomycin. Cultures forming colonies were maintained in culture growth medium until confluence. Purity of SMCs was assessed by positive immunostaining for α-smooth muscle actin (α-SMA) and desmin and lack of von Willebrand factor and Thy1.1 expression (all antibodies were purchased from Sigma). SMC cultures that resulted in >95% αSMA$^{+ve}$ cells were used through passages 2 and 4.

To isolate enteric glial cells (EGCs), LMMP-derived cell suspensions were seeded onto 6-well culture plates and cultured in DMEM supplemented with 20% FBS, sodium pyruvate (1 mM), forskolin (1 μM), bovine pituitary extract (BPE 10 μM, Gibco), penicillin (100 U mL$^{-1}$) and streptomycin (50 μg mL$^{-1}$), as previously published (Rühl et al., 2001). Contaminating fibroblasts were removed by incubating primary cultures with anti-mouse Ty1.1 monoclonal antibody in the presence of complement (Sigma). Purity of EGCs was assessed by positive immunoreactivity for glial fibrillar protein (GFAP) and absence of von Willebrand factor, Thy1.1 and αSMA expression. EGC cultures reporting >95% GFAP positive cells were used through passages 2 and 4.

Purification of macrophages/dendritic cells (MΦ/DCs) from LMMP was performed using magnetic cell sorting (MACS; Miltenyi Biotec, Bologna, Italy). Briefly, cell suspensions from LMMP were incubated with mouse anti-CD11b MicroBeads (Miltenyi Biotec), according to the manufacturer's protocol. The resulting cell population was composed of >95% CD11b positive cells as assessed by flow cytometer analysis. Cells were cultured in DMEM supplemented with 10% FBS, 100 U mL$^{-1}$ of penicillin and 50 μg mL$^{-1}$ of streptomycin.

### 2.3. Exposure to bacterial PAMPs

Primary cultured cells isolated from WT or TLR2$^{-/-}$ were stimulated with TLR4 ligand lipopolysaccharide (LPS, 100 ng mL$^{-1}$, Calbiochem), TLR2/TLR1 heterodimer agonist Pam3CysSerLys4 (Pam3-CSK4, 10 μg mL$^{-1}$, InvivoGen, Milan, Italy), TLR2/TLR6 heterodimer agonist Pam2CGDPKHPKSF (FSL-1, 100 ng mL$^{-1}$, InvivoGen), TLR9 agonist CpG oligodeoxynucleotides class A (100 μg mL$^{-1}$, InvivoGen), TLR5 ligand flagellin (FLA, 1 μg mL$^{-1}$, InvivoGen), or TLR3 agonist polyinosinic:polycytidylic acid (Poly(I:C), 100 μg mL$^{-1}$, InvivoGen). After 6 h of incubation, cell cultures were washed in ice-cold HBSS and total RNA was extracted. All experimental conditions were previously established in our laboratories.

### 2.4. RNA isolation and quantitative PCR

Total RNA was isolated from LMMP or cultured cells using RNA purification kit (Promega, Milan, Italy) as previously described (Brun et al., 2013). Contaminating DNA was removed by DNase I treatment (Promega) and first-strand cDNA was synthesized. Five micrograms of total RNA was reverse transcribed using random primers and MuIV RT (Applied Biosystems, Monza, Italy). Five microliters of the RT reaction was subjected to semi-quantitative PCR to determine the presence of mRNA coding TLR. Amplification products were separated on 1.5% agarose gel and visualized by Nancy-520 DNA gel stain (Sigma) using UV illuminator. Quantitative PCR was performed using the ABI PRISM 7700