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Toll-Like Receptor 2 Expression on Ovine Ileum throughout Fetal Development

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Abstract

Toll-like receptors (TLRs) family activated by endogenous host molecules or microbial components are pattern recognition receptors. Among all TLRs, TLR2 regulates intestinal inflammation, neuromuscular function, enteric nervous system structure, and neurochemical coding. In the present study, we aimed to investigate TLR2 expression on ovine ileum throughout prenatal development by using immunohistochemistry. We examined the fetal period in three different stages (fetal 60-100 days, fetal 100-125 days, and fetal 125-150 days). Moderate labeling was observed in most intestinal epithelial cells and follicle associated epithelium of Peyer's patches while some intestinal epithelial cells showed intense immunostaining. We also detected TLR2 expression in the majority of follicular immune cells, ganglion cells of submucosal and myenteric plexus, smooth muscles of the tunica and lamina muscularis, and blood vessels. In conclusion, this is the first study to describe expression profile of TLR2 in ovine ileum during prenatal development. TLR2 expression in the ileal ontogenesis has been shown to begin in the prenatal period. Almost all the intestinal epithelial cells and enteric neurons showed TLR2 expression. The presence of TLR2 expression in smooth muscles suggests that smooth muscles may be associated with immune system other than contracting task.

Key Words: Ileum, M cell, Peyer's patches, TLR2, sheep

Fötal gelişim Boyunca Koyun İleumundaki Toll like Reseptör 2 Ekspresyonu

Öz

Endojen veya mikrobiyal komponentlerle aktive olan Toll-like reseptörler (TLR) patern tanıyan reseptörlerdir. Bu reseptörler arasında yer alan TLR2 intestinal inflamasyonu, nöromüsküler fonksiyonu, enterik sinir sistemi yapısını ve nörokimyasal kodlamayı düzenler. Bu çalışmada, prenatal dönemde koyun ileumunda TLR2 ekspresyonunun immunohistokimyasal yöntemle araştırılması amaçlandı. Prenatal dönem 3 ayrı bölümde incelendi (60-100, 100-125, 125-150). İntestinal ve foliküle ilişkili epitelde orta derecede boyanma gözlenirken, bazı intestinal epitel hücrelerinde yoğun intrasitoplazmik immun reaksiyon tespit edildi. Ayrıca, foliküllerdeki hücrelerin büyük çoğunluğunda, submukozal ve myenterik pleksuslardaki gangliyon hücrelerinde, tunika muskularis, lamina muskularis ve damar duvarlarındaki düz kaslarda da TLR2 ekspresyonuna rastlandı. Sonuç olarak, bu çalışma prenatal gelişim süresince koyun ileumunda TLR2 ekspresyon profilini tanımlayan ilk araştırmadır ve ileumdaki TLR2 ontogenezisin doğum öncesi dönemde başladığı gösterilmiştir. Hemen hemen tüm intestinal epitel hücreleri ve enterik nöronlar TLR2 ekspresyonu gösterdi. Düz kaslarda TLR2 ekspresyonunun varlığı, düz kasların kasılma görevi dışında bağışıklık sistemi ile ilişkili olabileceğini göstermektedir.

Anahtar Kelimeler: İleum, M hücresi, Peyer plakları, TLR2, koyun

INTRODUCTION

Toll-like receptors (TLRs) are mammalian homologues of the Drosophila Toll protein playing a significant role in anti-fungal responses. TLRs were first detected in *Drosophila melanogaste*r and, thereafter, its one homolog known as TLR4 was found in humans (1, 2). This discovery of TLR4 inspired studies in the field of innate immune system. To date, researchers have discovered 10 functional TLRs in humans and 13 active TLRs in mouse (3). TLRs expression may be seen in extra- or intracellular compartments. While some TLRs (TLR1, -2, -4, -5, and -6) are found on the cell surface, others (TLR3, -7, -8, and -9) are expressed mainly in intracellular space including nucleic acids, endosomes, and their ligands (4). TLRs recognize pathogen-associated molecular patterns (PAMPs) and trigger innate immune system. These conserved molecular patterns are associated with both gram positive and gram-negative bacteria, the components of which include lipoteichoic acid, lipopeptides/lipoproteins, components of peptidoglycan, and zymosan (5). Furthermore, TLRs can be detected and triggered

by endogenous ligands released from necrotic cells and damaged tissues (6) such as high-mobility group box proteins 1, heat shock proteins, and saturated fatty acids (7, 8).

The multifunctional activity of TLRs is properly determined in the gastrointestinal mucosa. Among all the TLRs, TLR2 seems to be a key player in gastrointestinal tract homeostasis by performing protective effects in gastrointestinal epithelium (3, 9). While TLR2 deficiency predisposes intestines to inflammation and injury, TLR2 activation improves epithelial barrier integrity. Moreover, TLR2 regulates neuromuscular function and manages enteric nervous system structure and neurochemical coding (9, 10). However, we do not know the TLR2 expression of sheep intestine during fetal period. In prenatal period, the changes of TLR2 expression and cellular localization in ileum may shed light on intestinal immunity. For this purpose, we aimed to explore TLR2 expression on ovine ileum throughout prenatal development.

MATERIALS AND METHODS

Animal samples and tissue processing

We investigated 20 fetal sheep ileum at varying gestational ages ranging from 63 to 147 days. The ileal samples were collected from Akkaraman breed in slaughterhouses. To estimate the age of fetuses, we used Richardson's x=2.1(17+y) formula (11), where x indicates the age of the fetus in days, and y is the space between the anus and forehead of the fetus in cm. All experimental applications were conducted in accordance with the guidelines of the Animal Care and Use Committee of Ankara University (07 July 2014, No: 22724).

We employed Bouin's solution to fix ileal samples. Later, the samples were treated with 70% alcohol at overnight and passed through grade alcohols (80%, 90% and 100%). After this step, the tissues were passed through the methyl benzoate and benzol series for clearing and embedded in the paraplast.

Immunohistochemistry

We applied Streptavidin Biotin immunoperoxidase method to sections as previously determined (12). Briefly, the sections were deparaffinized and rehydrated using routine histological methods. Then, the slides were transferred into phosphate buffer saline (PBS). For antigen retrieval, the sections were immersed in 0.1 M, pH 6.0 citrate buffer solution in a microwave oven. To enhance membrane permeability, the samples were treated with 0.2 % Triton X-100 in PBS. In order to quench endogen peroxidase, the sections were transferred into 3% hydrogen peroxide. After this step, the slides were immersed in PBS and encircled with a hydrophobic PAP pen. In order to block nonspecific antigenic reactions, the sections were incubated with Ultra V Block solution (#TP-125-HL, Thermo Fisher Scientific). Later, the samples were incubated for 16 h at 4 °C with 1:300 diluted anti-TLR2 primary antibody (#bs-1019R, Bioss Antibodies). This step was followed by washing in PBS for 15 min. The slides were incubated with biotinylated secondary antibody for 30 min, followed by washing in PBS. Then, the sections were incubated with horseradish peroxidase - conjugated streptavidin (TP-125-HL, Thermo Fisher Scientific) for 30 min, followed by washing in PBS for 15 min. In order to visualize the resulting signal, we used 3-Amino-9-Ethylcarbazole (AEC) as a chromogen. We applied Gill's II hematoxylin for nuclear staining and the slides then were coverslipped with a hydrophilic mounting medium.

In order to validate immunostaining, the sections were treated with PBS instead of anti-TLR2 antibody. Moreover, the samples were incubated with nonimmune rabbit IgG (sc-2027, Santa Cruz Bio Inc.) for isotype control. We applied rat testis sections for the positive control (Figure 1). Immunostaining was assessed under a light microscope (BX51, Olympus, Japan) with digital camera (DP74, Olympus, Japan) and photographed with the aid of Olympus Cellsens software.

RESULTS

In order to validate the antibody specificity, we used rat testis sections. We detected TLR2 immunostaining in some cells of the spermatogenic series in rat testis (Figure 1a). We used fetal sheep ileum for negative control. We did not detect any nonspecific immunostaining in the sheep ileum (Figure 1b). We examined the fetal period in three different stages (fetal 60-100 days, fetal 100-125 days, fetal 125-150 days) considering the data on development of Peyer's patches as reported in our previous study (13). The semi-quantitative analysis of the immunostaining results is summarized in Table 1.

Table 1. A semiquantitative summary of the results obtained from present study

		Prenatal 60-100 days	Prenatal 100-125 days	Prenatal 125-150 days
		n: 6	n: 8	n:6
Intestinal epithelium	Villus intestinalis	++	++	++
	Crypts	+	+	+
Smooth muscles	Lamina muscularis	+++	+++	+++
	Tunica muscularis	+++	+++	+++
	Vessel wall	+++	+++	+++
Enteric ganglions	Plexus submucosus	+++	+++	+++
	Plexus myentericus	+++	+++	+++
Peyer's Patches	FAE	++	++	++
	Dome region	+	+	+
	Interfollicular region	-	+	+
	Follicle	+	+	+

Proportion of TLR2 staining cells was scored on a scale of - to +++ (- = no cells with staining; + = weak; ++ = medium; +++= intense). The number of staining cells was evaluated subjectively

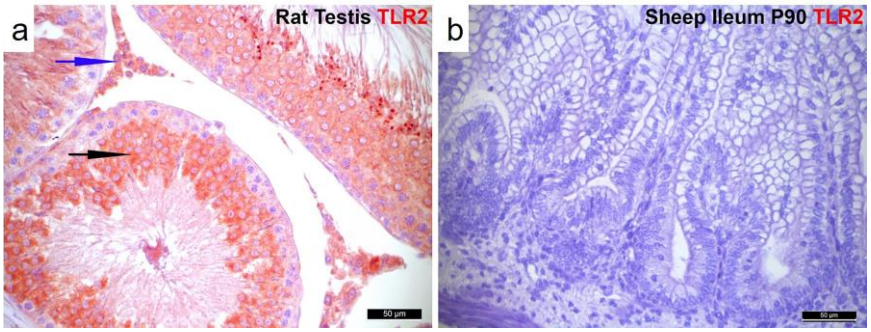

Figure 1. Representative figures demonstrating rat testis samples as positive control and fetal sheep ileum samples as negative control for immunohistochemical staining. TLR2 immunostaining in some cells of the spermatogenic series (black arrows) and interstitial cells (blue arrows) in rat testis. No immunostaining was observed in fetal ovine ileal samples that were treated without the primary antibody. P90; day of 90 prenatal. Bars: 50 µm (a, b).

Fetal 60-100 days

We observed moderate staining in the majority of the intestinal epithelial cells. Immunostaining was particularly on the apical faces of these cells. Moreover, we detected strong positive labelling in smooth muscles of the lamina muscularis, tunica muscularis and vessel wall. Ganglion cells of submucosal and myenteric plexus also showed positive immunostaining for TLR2 (Figure 2a and b).

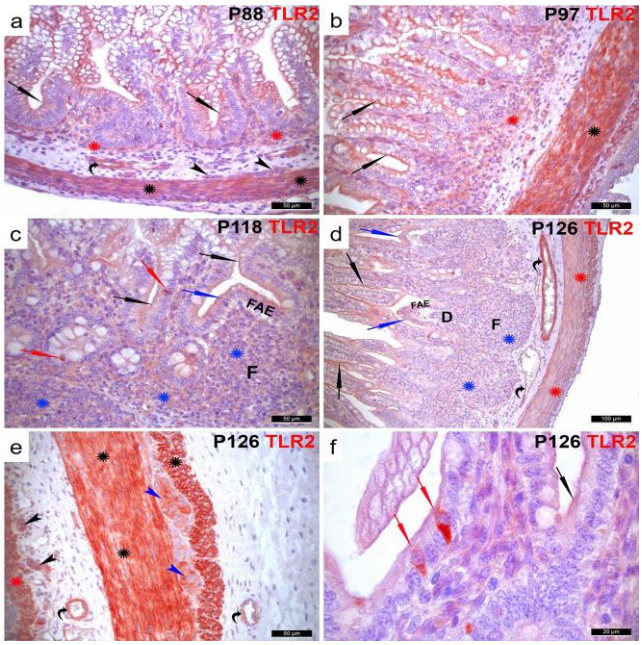

Figure 2. Representative figures demonstrating TLR2 in ileum of day 88 prenatal (a; P88), day 97 prenatal (b; P97), day 118 prenatal (c; P118) and day 126 (d, e, f; P126). Immunostaining was detected in apical surface of the intestinal epithelial cells (black arrows). Some intestinal epithelial cells showed strongly intracytoplasmic immunostaining (red arrows). Immunostaining in smooth muscles of the lamina muscularis (red asterisk), tunica muscularis (black asterisk), and vessel walls (black curve arrows). Enteric neurons in submucosal (black arrowheads) and myenteric plexus (blue arrowheads) showed positive immunostaining for TLR2. A positive reaction in FAE (blue arrows) and follicle (F, blue asterisk) of Peyer's patches. Bars: 20 µm (f), 50 µm (a, b, c, e), 100 µm (d).

Fetal 100-125 days

The apical site of intestinal epithelial cells showed a moderate staining for TLR2. Interestingly, we detected intense immunostaining in some epithelial cells of intestinal villi and crypts. Ganglion cells in myenteric and submucosal plexus also showed positive immunostaining for TLR2. Primordial Peyer's patches consisting of primordial follicles and domes were also seen in this period. The apical site of follicle associated epithelium (FAE) showed positive immunostaining for TLR2. Furthermore, we detected a positive reaction in the majority of immune cells in the follicle and dome region (Figure 2c, d, e and f).

Fetal 125-150 days

We observed histologically mature Peyer's patches in this period. Weak immunoreaction was determined in the majority of cells forming the dome and follicle region. TLR2 staining was also seen in the apical face of FAE. We generally observed a weak immunostaining in apical face of intestinal epithelium but an intense immune reaction in some intestinal epithelial cells. While the intensively stained cells were detected in intestinal crypts and villi, we did not observe these cells in FAE. In addition, we observed positive immunostaining in smooth muscle cells of the lamina muscularis, tunica muscularis and vessel wall, and in ganglion cells of myenteric and submucosal plexus (Figure 3a, b, c, and d).

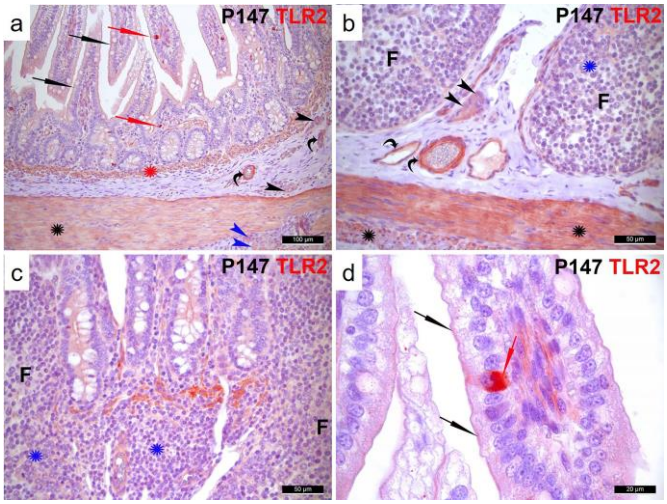

Figure 3. Representative figures demonstrating TLR2 in ileum of day 147 prenatal (a; P147). Immunoreaction was found in apical surface of the intestinal epithelial cells (black arrows). Some intestinal epithelial cells showed strongly intracytoplasmic immunostaining (red arrows). Immunoreaction in smooth muscles of the lamina muscularis (red asterisk), tunica muscularis (black asterisk), and vessel walls (black curve arrows). Enteric neurons in submucosal (black arrowheads) and myenteric plexus (blue arrowheads) showed positive labelling for TLR2. A positive labelling in follicle (F, blue asterisk) of Peyer's patches. Bars: 20 µm (d), 50 µm (b, c), 100 µm (a).

DISCUSSION AND CONCLUSION

Here, we investigated TLR2 expression on sheep ileum during prenatal development. TLR2 expression was detected in the epithelial, muscle and nerve cells of the ileal tissue at all stages of fetal period.

TLRs are involved in the regulation of intestinal epithelial barrier against a variety of enteric pathogens (14). Cario and Podolsky (2000) showed that the absence of TLR2 increases susceptibility to stress-induced intestinal barrier dysfunction (15). In the healthy gastrointestinal tract, TLR2 expression is seen in many different cell types including intestinal epithelial cells and some mononuclear cells of lamina propria (16). In healthy rats, TLR2 expression was located in only a few absorptive cells at the basal part in the jejunum, while it was found in absorptive and goblet cells in colon (17). However, the same researcher found that the immunostaining of TLR2 was observed across the whole height of some absorptive cells after 4 days post parasitic infection. In mouse, TLR2 expression was mostly observed in the proximal segment of colon (18). Moreover, the mRNA expression levels of TLR2 and TLR4 in jejunum and ileum increased gradually after the birth (19). In bovine, mRNA expression of all the TLRs was detected in cultured intestinal epithelial cells (20). In the present study, we found TLR2 expression in all the intestinal epithelial cells. TLR2 expression was more intense on the apical faces of intestinal epithelial cells. Since fetus swallows the amniotic fluid during prenatal period, the intestinal epithelium is subjected to some bioactive substances (21, 22). TLR2 expression in the intestinal epithelium may form a biological barrier to these substances. In addition, we also observed very intense staining in some intestinal epithelial cells. Considering the histomorphology of these cells, we think that they may be enteroendocrine cells. Besides, researchers reported that FAE and follicle of Peyer's patches showed TLR2 expression (23, 24) in concordance with our findings. In the previous study, we observed that most cells in the FAE consisted of M cells in the prenatal period (13). Therefore, M cells were also positive for TLR2 in the present study.

Researchers showed that the presence of TLR2 and TLR4 may play a significant role in the modulation of gastrointestinal motility. The expression of TLR2 and TLR4 has been observed in smooth muscle cells and enteric neurons of human and mouse intestines (10, 25, 26). Moreover, isolated murine smooth muscle cell expressed all TLRs mRNAs (TLR1, -2, -3, -4, -5, -6, -7, -8 and -9) at detectable levels (27). Some researchers indicated that enteric glial cells showed low/intermediate expressions of all the TLRs in small intestine from human patients with colorectal carcinoma (28). However, Brun et al. (2015) did not observe mRNA specific transcripts encoding TLR1, 3, and 7 in enteric glial cells. In our study, we observed TLR2 expression in smooth muscle cells and enteric neurons in accordance with previous findings. We speculated that TLR2 may be necessary for the functional integrity of smooth muscle cells and enteric neurons of sheep small intestine in the fetal period.

In conclusion, this is the first study to describe expression profile of TLR2 in ovine ileum during prenatal development. TLR2 expression in the ileal ontogenesis has been shown to begin in the prenatal period. Almost all the intestinal epithelial cells and enteric neurons showed TLR2 expression. The presence of TLR2 expression in smooth muscles indicates that smooth muscles may be associated with immune system other than contraction task.

REFERENCES

1. Hashimoto C, Hudson KL, Anderson KV. (1988). The Toll Gene of Drosophila, Required for Dorsal-Ventral Embryonic Polarity, Appears to Encode a Transmembrane Protein. Cell. 52: 269-279.
2. Medzhitov R, Preston-Hurlburt P, Janeway CA Jr. (1997). A Human Homologue of the Drosophila Toll Protein Signals Activation of Adaptive Immunity. Nature. 388(6640): 394-397.
3. Kawai T, Akira S. (2011). Toll-Like Receptors and Their Crosstalk with Other Innate Receptors in Infection and Immunity. Immunity. 34(5): 637-650.
4. Akira S, Uematsu S, Takeuchi O. (2006). Pathogen Recognition and Innate Immunity. Cell. 124(4):783-801.
5. Cario E. (2008). Barrier-Protective Function of Intestinal Epithelial Toll-Like Receptor 2. Mucosal Immunol Suppl 1: S62–66.
6. Seong SY, Matzinger P. (2004). Hydrophobicity: An Ancient Damage-Associated Molecular Pattern that Initiates Innate Immune Responses. Nat Rev Immunol. 4(6): 469-478.
7. Arslan F, Keogh B, Mcguirk P, Parker AE. (2010). TLR2 and TLR4 in Ischemia Reperfusion Injury. Mediators Inflamm. 2010: 704202.
8. Piccinini AM, Midwood KS. (2010). Dampening Inflammation by Modulating TLR Signalling. Mediators Inflamm. 2010: 672395.
9. Cario E, Gerken G, Podolsky DK. (2007). Toll-Like Receptor 2 Controls Mucosal Inflammation by Regulating Epithelial Barrier Function. Gastroenterology. 132: 1359-1374.
10. Brun P, Giron MC, Qesari M, et al. (2013). Toll-Like Receptor 2 Regulates Intestinal Inflammation by Controlling Integrity of the Enteric Nervous System. Gastroenterology. 145(6): 1323-1333.
11. Richardson C, Hebert CN, Terlecki S. (1976). Estimation of the Developmental Age of the Ovine Fetus and Lamb. Vet Rec. 99: 22-26.
12. Öztop M, Özbek M, Liman N, Beyaz F, Ergün E, Ergün L. (2019). Localization Profiles of Natriuretic Peptides in Hearts of Pre-Hibernating and Hibernating Anatolian Ground Squirrels (Spermophilus Xanthoprymnus). Vet Res Commun. 43(2): 45-65.
13. Özbek M, Bayraktaroğlu AG. (2019). Developmental Study on the Ileal Peyer's Patches of Sheep, and Cytokeratin-18 as a Possible Marker for M Cells in Follicle Associated Epithelium. Acta Histochem. 121(3): 311-322.
14. Moncada DM, Kammanadiminti SJ, Chadee K. (2003). Mucin and Toll-Like Receptors in Host Defense against Intestinal Parasites. Trends Parasitol. 19(7): 305-311.
15. Cario E, Podolsky DK. (2000). Differential Alteration in Intestinal Epithelial Cell Expression of Toll-Like Receptor 3 (TLR3) and TLR4 in Inflammatory Bowel Disease. Infect Immun. 68(12): 7010-7017.

16. Cario E, Rosenberg IM, Brandwein SL, Beck PL, Reinecker HC, Podolsky DK. (2000). Lipopolysaccharide Activates Distinct Signaling Pathways in Intestinal Epithelial Cell Lines Expressing Toll-Like Receptors. J Immunol. 164(2): 966-972.
17. Kosik-Bogacka DI, Wojtkowiak-Giera A, Kolasa A, Salamatin R, Jagodzinski PP, Wandurska-Nowak E. (2012). Hymenolepis Diminuta: Analysis of the Expression of Toll-Like Receptor Genes (TLR2 and TLR4) in the Small and Large Intestines of Rats. Exp Parasitol. 130(3): 261-266.
18. Ortega-Cava CF, Ishihara S, Rumi MA, et al. (2003). Strategic Compartmentalization of Toll-Like Receptor 4 in the Mouse Gut. J Immunol. 170(8): 3977-3985.
19. Inoue R, Yajima T, Tsukahara T. (2017). Expression of TLR2 and TLR4 in Murine Small Intestine during Postnatal Development. Biosci Biotechnol Biochem. 81(2): 350-358.
20. Yang Z, Fu Y, Gong P, et al. (2015). Bovine TLR2 and TLR4 Mediate Cryptosporidium Parvum Recognition in Bovine Intestinal Epithelial Cells. Microb Pathog. 85:29-34.
21. Kanai M, Rosenberg I, Podolsky DK. (1997). Cytokine Regulation of Fibroblast Growth Factor Receptor 3 IIIb in Intestinal Epithelial Cells. Am J Physiol. 272(4): G885-G893.
22. Ng AY, Waring P, Ristevski S, et al. (2002). Inactivation of the Transcription Factor Elf3 in Mice Results in Dysmorphogenesis and Altered Differentiation of Intestinal Epithelium. Gastroenterology. 122(5): 1455-66.
23. Hausmann M, Kiessling S, Mestermann S, et al. (2002). Toll-Like Receptors 2 and 4 Are Up-Regulated during Intestinal Inflammation. Gastroenterology. 122(7): 1987-2000.
24. Yuasa H, Mantani Y, Masuda N, et al. (2017). Differential Expression of Toll-Like Receptor-2, -4 and -9 in Follicle-Associated Epithelium from Epithelia of Both Follicle-Associated Intestinal Villi and Ordinary Intestinal Villi in Rat Peyer's Patches. J Vet Med Sci. 78(12): 1797-1804.
25. Filippova LV, Malyshev FS, Bykova AA, Nozdrachev AD. (2012). Expression of Toll-Like Receptors 4 in Nerve Plexuses of the Rat Duodenum, Jejunum, and Colon. Dokl Biol Sci. 445: 215-217.
26. Ammoscato F, Scirocco A, Altomare A, et al. (2013). Lactobacillus Rhamnosus Protects Human Colonic Muscle from Pathogen Lipopolysaccharide-Induced Damage. Neurogastroenterol Motil. 25(12): 984-E777.
27. Brun P, Gobbo S, Caputi V, et al. (2015). Toll Like Receptor-2 Regulates Production of Glial-Derived Neurotrophic Factors in Murine Intestinal Smooth Muscle Cells. Mol Cell Neurosci. 68 : 24-35.
28. Turco F, Sarnelli G, Cirillo C, et al. (2014). Enteroglial-Derived S100B Protein Integrates Bacteria-Induced Toll-Like Receptor Signalling in Human Enteric Glial Cells. Gut. 63(1): 105-115.

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