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Original Paper

NOD2 Modulates Serotonin Transporter and Interacts with TLR2 and TLR4 in Intestinal Epithelial Cells

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Key Words
PRRs • 5-HT • SERT • RIP2/RICK • TLR2 • TLR4 • Intestine

Abstract
Background/Aims: Serotonin (5-HT) is a chief modulator of intestinal activity. The effects of 5-HT depend on its extracellular availability, which is mainly controlled by serotonin transporter (SERT), expressed in enterocytes. On the other hand, innate immunity, mediated by Toll-like receptors (TLRs) and nucleotide oligomerization domain (NOD)-like receptors (NLRs), is known to control intestinal microbiota and maintain intestinal homeostasis. The dysregulation of the intestinal serotonergic system and innate immunity has been observed in inflammatory bowel diseases (IBD), the incidence of which has severely increased all over the world. The aim of the present study, therefore, was to analyze the effect of NOD2 on intestinal SERT activity and expression, as well as to study the crosstalk of NOD2 with TLR2 and TLR4. ***Methods:*** Intestinal epithelial cell line Caco-2/TC7 was used to analyze SERT activity and SERT, NOD2, TLR2 and TLR4 molecular expression by real-time PCR and western blotting. Moreover, intestinal tract (ileum and colon) from mice deficient in TLR2, TLR4 or TLR2/4 receptors was used to test the interdependence of NOD2 with these TLR receptors. ***Results:*** NOD2 activation inhibits SERT activity in Caco-2/TC7 cells, mainly due to the decrement of SERT molecular expression, with RIP2/RICK being the intracellular pathway involved in this effect. This inhibitory effect on SERT would yield an increment of extracellular 5-HT availability. In this sense, 5-HT strongly inhibits NOD2 expression. In addition, NOD2 showed greater interdependence with TLR2 than with TLR4. Indeed, NOD2 expression significantly increased in both cells treated with TLR2 agonists and the intestinal tract of *Tlr2*$^{-/-}$ mice. ***Conclusions:*** It may be inferred from our data that NOD2 could play a role in intestinal pathophysiology not only through its inherent innate immune role but also due to its interaction with other receptors as TLR2 and the modulation of the intestinal serotonergic system decreasing SERT activity and expression.

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Introduction

Serotonin (5-hydroxytryptamine, 5-HT) is an essential intestinal neuromodulator, mainly synthesized by enterochromaffin cells from the intestinal epithelium, where it is released to apical and basolateral cell surfaces, activating specific 5-HT receptors and triggering different responses [1]. 5-HT is critical in the control of the whole intestinal physiology because it mediates several functions, such as motility, secretion or absorption [2], and has also been involved in several intestinal inflammatory processes [3]. 5-HT activity principally depends on its extracellular availability, mostly regulated by the serotonin transporter (SERT) located in the apical and basolateral membranes of enterocytes. 5-HT is taken up by SERT from the interstitial space into enterocytes, ending its effects. Serotonin extracellular levels are subsequently controlled by the equilibrium between serotonin synthesis (by enterochromaffin cells) and uptake (by SERT from the enterocyte membrane), which is vital for the triggering of 5-HT effects. Apart from the essential importance of 5-HT in the maintenance of intestinal balance, serotonin also plays a critical role at the systemic level [4].

Several studies have described the important implications of intestinal serotonergic alterations in many chronic gastrointestinal diseases, including inflammatory bowel diseases (IBD). In this context, recent works have shown that 5-HT [5], 5-HT receptors [6] and SERT [7] would play a chief role in the development of IBD. In fact, recent studies have suggested that modifications of the activity of selective serotonin receptors and reuptake transporter in the gut could be effective for controlling IBD activity and associated symptoms [8, 9].

In addition to the serotonergic system, modifications in intestinal innate immunity have also been described in IBD [10]. Pattern recognition receptors (PRRs) are innate immune components, which mainly involves Toll-like receptors (TLRs) and nucleotide oligomerization domain (NOD)-like receptors (NLRs). PRRs are expressed in intestinal epithelial cells [11, 12] and are able to recognize microbial-associated molecular patterns (MAMPs) to develop either inflammatory or tolerance responses [13]. In this context, several studies have reported that numerous PRRs, as TLR2, TLR4 [14] and NOD2 [15], seem to be implicated in IBD due to the dysfunctional recognition of commensal microbiota [16]. TLR2 is expressed in the cell surface and can detect molecular patterns associated with Gram-positive bacteria [17] through their heterodimers (TLR2/1 and TLR2/6) [18] while TLR4, also located on the cell surface, detects lipopolysaccharide (LPS), the major cell wall component of Gram-negative bacteria [19].

Regarding NLRs, NOD2 is a cytoplasmic receptor expressed in intestinal epithelial cells, which recognizes a specific peptidoglycan shared by both Gram-positive and Gram-negative bacteria: muramyl dipeptide (MDP) [20]. NOD2 plays a key role in intestinal homeostasis, developing a tolerance response to the commensal microbiota and protecting the host against pathogenic microorganisms through pro-inflammatory intracellular pathways, such as NF-κB [11].

Recent works have shown that both TLR2 and TLR4 activation downregulates intestinal serotonin transporter activity and expression [21, 22]. However, the implication of other PRRs, such as NOD2, in the intestinal serotonergic system remains unknown. Therefore, the aim of this work was to analyze the effects of NOD2 activation on SERT activity and molecular expression. Moreover, we have also aimed to study the possible interaction between NOD2 with TLR2 and TLR4.

Materials and Methods

Reagents and antibodies

The following drugs and substances were used (abbreviations and respective suppliers in parentheses): serotonin (5-hydroxytryptamine, 5-HT) from Sigma–Aldrich (St. Louis, MO, USA) and [^{3}H]-5-HT (specific activity 28 Ci/mmol) was from Perkin-Elmer (Boston, MA, USA). N-glycolytated muramyldipeptide-NOD2 ligand (MDP), lipopolysaccharide (LPS; *Escherichia coli* O111:B4), N114

Palmitoyl-S-[2, 3-bis(palmitoyloxy)-(2RS)-propyl]-[R]-cysteinyl-[S]-seryl-[S]-lysyl-[S]-lysyl-[S]-lysyl-[S]-lysine (Pam3CSK4), S-[2, 3-bis(palmitoyloxy)-(2RS)-propyl]-[R]-cysteinyl-[S]-seryl-[S]-lysyl-[S]-lysyl-[S]-lysyl-[S]-lysine x 3 CF3COOH (Pam2CSK4) and Gefitinib RIP2 Tyrosine Kinase Inhibitor (gentamicin, receptor interacting protein-2 [RIP2] inhibitor) from InvivoGen (San Diego, CA, USA) were also used. The specific primary antibodies used are indicated in Table 1. The secondary antibodies coupled to horseradish peroxidase were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). All generic reagents were purchased from Sigma-Aldrich and Roche Applied Sciences (Sant Cugat del Vallés, Barcelona, Spain).

Cell culture

This study was carried out with the human enterocyte-like cell line Caco-2/TC7 [23]. We have used these human epithelial cells as *in vitro* model, since previous works have demonstrated that this cell line expresses SERT and innate immunity receptors, thus being an optimal intestinal model for analyzing SERT [24] and PRRs [12, 25]. Caco2/TC7 cells were cultured at 37°C in an atmosphere of 5% CO_2 with high-glucose DMEM, supplemented with 2 mM glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 1% non-essential amino acids and 20% heat-inactivated fetal bovine serum (FBS) from Life Technologies (Carlsbad, CA, USA). The medium was changed two days after seeding and daily afterwards. The experiments were carried out nine days after reaching confluence (14 days after seeding), as SERT activity is maximal at this point [24]. The cell medium was free of FBS 24 hours before the experiment to avoid interference. NOD2 agonist (N-glycolytated muramyldipeptide, MDP) and the different modifiers were added to the culture medium at different concentrations and periods. Prior to the experiments, the cell monolayer was analyzed, and none of the different conditions seemed to affect the morphology, proliferation or monolayer integrity of the Caco-2/TC7 cells (data not shown).

Animals

Wild-type C57BL/10ScSn mice (WT) as well as TLR2-deficient C57BL/10ScSn (*Tlr2*$^{-/-}$), TLR4-deficient C57BL/10ScN (*Tlr4*$^{-/-}$) and TLR2/TLR4 double-deficient C57BL/10ScN mice (*Tlr2, 4*$^{-/-}$) [26] were bred at the Centro de Investigación y Tecnología Agroalimentaria (CITA, Zaragoza, Spain). All of the mice genotypes were periodically tested. Mice from 10 to 12 weeks of age were used in the experiments and were maintained under pathogen-free conditions on a 12-hour light/dark cycle with food and water *ad libitum*. The Ethic Committee for Animal Experiments of the Zaragoza University (P136/12) approved the experiments carried out in this study. The care and use of animals were performed in accordance with the Spanish Policy for Animal Protection RD53/2013, which meets the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (Council of Europe Nº 123, Strasbourg 1985) and the European Union Directive 2010/63/EU on the protection of animals used for scientific purposes. Mice euthanasia was performed by using cervical dislocation, and immediately after that, the intestinal tract (ileum and colon) was removed and cleaned with an ice solution of NaCl 0.9%. Intestinal samples for RNA studies were collected in RNAlater from Qiagen (Hilden, Germany), stored for one day at 4°C and then subsequently frozen at -80°C. The samples for protein analyses were immediately frozen in ice-cold isopropyl alcohol and stored at -80°C.

5-HT uptake studies

Uptake measurements were performed in cell cultures in 24-well plates, as previously described [24], either under the control condition or after treatment with a specific NOD2 ligand. The transport medium composition in mM was as follows: 137 NaCl, 4.7 KCl, 1.2 KH_2PO_4, 1.2 $MgSO_4$, 2.5 $CaCl_2$, 10 HEPES pH 7.4, 4 glutamine, 1 ascorbic acid, 0.1% BSA, and both 0.2 µM 5-HT and [^{3}H]-5-HT

Table 1. Primary antibodies used for western blot analysis. Primary antibodies were purchased from (a) Abcam (Cambridge, UK) and (b) Santa Cruz Biotechnology (Santa Cruz, CA, USA). Capital letters indicate animal species: M: mouse; R: rat; Rb: rabbit; H: human; G: goat

Antibody	reference	Description	Species reactivity	Dilution
Anti-SERT	ab130130 [a]	G polyclonal	M, R, Rb, H	1:1,000
Anti-NOD2	ab197030 [a]	Rb monoclonal	M, R, H	1:2,500
Anti-TLR2	ab24192 [a]	Rb polyclonal	M, H	1:1,000
Anti-TLR4	ab13556 [a]	Rb polyclonal	M, H	1:1,000
Anti-Actin	SC-1615 [b]	G polyclonal	M, R, H	1:10,000

(1.5 µCi/ml) as the substrate. Before uptake studies, cells were pre-incubated at 37°C in an atmosphere of 5% CO_2 with a substrate-free transport medium for 30 minutes. The cells were washed with the substrate free transport medium at 37°C and then, incubated with the transport medium at 37°C for 6 minutes.

The uptake was stopped by removing the transport medium and washing the cells twice with an ice-cold transport medium containing 20 µM 5-HT. Finally, the cells were solubilized in 0.5 ml of 0.1 N NaOH and a sample of 200 µl taken for radioactivity counting (Wallac Liquid Scintillation Counter, Perkin-Elmer). The protein concentration was calculated using the Bradford method from Bio-Rad (Hercules, CA) with BSA as the standard. All results were calculated in pmol 5-HT/mg protein being and expressed as a percentage of the control value (100%).

RNA extraction, reverse transcription and real-time PCR

Caco-2/TC7 cells were cultured in 6-well plates and total RNA was extracted using the RNeasy mini kit and the RNase-free DNase set from Qiagen, following the manufacturer's protocol. For intestinal RNA extraction, prior the use of the RNeasy mini kit, the samples were thawed in an ice-cold lysis RTL buffer (Qiagen) and homogenized using the Ultra Turrax T25 from IKA (Staufen, Germany). The extracted RNA (1 µg) was used as a template for first-strand cDNA synthesis using oligo(dT) primers and a reverse transcriptase (Lucigen, Middleton, USA). A negative amplification was done without reverse transcriptase. The cDNAs obtained by reverse transcription (RT) were used to determine the mRNA level by real-time PCR using SYBR Green.

The quantification of the SERT, NOD2, TLR2 and TLR4 mRNA in Caco-2/TC7 cells and NOD2 mRNA in mice was carried out by using the StepOne Plus Real-Time PCR System (Applied Biosystems, Foster City, USA), with GAPDH and HPRT1 as housekeeping. The specific primers used are detailed in Table 2. All samples analyzed were determined in triplicate, and the fluorescence raw data were analyzed by the Applied Biosystems StepOne Software v2.3 (Applied Biosystems). Thus, the mRNA relative expression was calculated as $\Delta\Delta Ct = \Delta Ct_{control} - \Delta Ct_{treatment}$, being $\Delta Ct = Ct_{gene} - Ct_{calibrator}$. Finally, the levels of the relative gene expression were transformed and expressed as the fold difference ($=2^{-\Delta\Delta Ct}$).

Cell homogenates and brush border-enriched fraction for western blot analysis

Caco-2/TC7 cells were cultured in 75 cm^2 flasks and the cell brush border membrane-enriched fraction was obtained following the method described in a previous paper [27]. The cells were washed twice with PBS and re-suspended with an ice-cold Tris-mannitol buffer (2 mM Tris, 50 mM Mannitol, pH 7.1) containing protease inhibitors and 0.02% sodium azide. The samples were disrupted using a Potter-Elvehjem homogenizer with a PTFE pestle and the obtained suspension was homogenized and disrupted by sonication (fifteen 1-s bursts, 60W). The brush border membrane-enriched fraction was obtained through the addition of 20 mM $CaCl_2$ to the cell lysate and was maintained for 10 minutes in ice. After this time, samples were centrifuged for 10 minutes at 950 g. The supernatant was centrifuged (30 minutes, 40, 000 g) and the pellet was re-suspended in a phosphate buffer (10 mM KH_2PO_4/K_2HPO_4 pH 6.8) to obtain the brush border membrane-enriched fraction. Mice intestinal samples (ileum and colon) were cleaned and homogenized using ultra-turrax in Tris-mannitol buffer pH 7.1 [21]. Then, all of the samples were disrupted using a Potter-Elvehjem homogenizer with a PTFE pestle and the obtained suspension was homogenized and disrupted by sonication (fifteen 1-s bursts, 60W). All protein samples were measured using the Bradford method (Bio-Rad).

Cell lysate and brush border-enriched fraction from

Table 2. Primer sequences used for real-time PCR analysis of human (h) cells or intestinal mice (m) samples

Name	Forward primer (5'-3')	Reverse primer (5'-3')
hSERT	GGCCTGGAAGGTGTGATCA	GCGCTTGGCCCAGATGT
hNOD2	CTGGCAAAGAACGTCATGCTA	CCTGGGATTGAATCTTGGGAA
hTLR2	GAAAGCTCCCAGCAGGAACATC	GAATGAAGTCCCGCTTATGAAGACA
hTLR4	TTGAGCAGGTCTAGGGTGATTGAAC	ATGCGGGACACACACACTTTCAAATA
hGADPH	CATGACCACAGTCCATGCCATCACT	TGAGGTCCACCACCCTGTTGCTGTA
hHPRT1	CTGACCTGCTGGATTACA	GCGACCTTGACCATCTTT
mNOD2	CTTCATTTGGCTCATCCGTAG	CTGGAGATGTTGCAGTACAAAG
mGADPH	AACGACCCCTTCATTGAC	TCCACGACATACTCAGCAC
mHPRT1	CTGGTGAAAAGGACCTCTCGAA	CTGAAGTACTCATTATAGTCAAGGGCAT

Caco-2/TC7 cells (60 µg of total protein) as well as ileum and colon homogenates (30 µg) of WT, *Tlr2*$^{-/-}$, *Tlr4*$^{-/-}$ and *Tlr2, 4*$^{-/-}$ mice were electrophoresed in 8% SDS-PAGE gels and later transferred to PVDF membranes by electroblotting. Membranes were blocked with 4% non-fat dried milk plus 1% BSA and were probed with the primary antibodies and their correspondent concentration indicated in Table 1. Primary antibodies were detected using specific secondary antibodies coupled with horseradish peroxidase and the WesternBright Sirius HRP substrate from Advansta (Menlo Park, CA, USA). In addition, the signal was visualized used VersaDoc™ from Imaging System Bio-Rad. After stripping, membranes were re-probed with anti β-actin antibody (Table 1) for the determination of differences in the sample loading. The SERT, NOD2, TLR2 and TLR4/β-actin protein ratio was calculated in densitometry units using Quantity One 1-D Analyses Software from Bio-Rad, and the results were expressed as a percentage of the control values (100%).

Statistical analyses

All results were expressed as the mean ± the standard error of the mean (SEM). Statistical comparisons were performed using Student's unpaired t-test or one-way ANOVA. One-way ANOVA was followed by the Bonferroni post-test with a confidence interval of 95% ($p < 0.05$). Previously, normal distribution was confirmed with the D'Agostino-Pearson test. The statistical analysis is indicated in each figure and was developed with the computer-assisted Prism GraphPad Program (Prism version 5.03, GraphPad Software, San Diego, CA).

Results

SERT inhibition by NOD2 activation in Caco-2/TC7 cells

Several studies have already described NOD2 expression in myeloid and lymphoid cells, but also in non-hematopoietic cells, such as Paneth cells, stem cells, goblet cells and enterocytes [28]. Nevertheless, we considered it essential to detect NOD2 expression in our enterocyte-like model. For this reason, we determined NOD2 mRNA and protein expression in Caco-2/TC7 cells by RT-PCR and western blot respectively (data not shown). NOD2 mRNA was amplified by PCR (317 bp amplicon size) and the NOD2 protein was immunodetected in cell homogenates, with both agreeing with the expected NOD2 mRNA and protein size.

After NOD2 expression was demonstrated, we analyzed the effect of NOD2 activation by muramyl dipeptide (MDP) on SERT activity in Caco-2/TC7 cells. Thus, cells were treated with different MDP concentrations (1, 10 and 50 µg/ml) for a short (30 minutes), medium (6 hours) or long (1 day) term, and afterwards, 5-HT uptake was measured. The results showed that NOD2 activation with MDP significantly diminished 5-HT uptake with short and medium-term treatment. However, with long-term MDP treatment, 5-HT uptake was significantly diminished only with the higher MDP concentration (Fig. 1A).

Because NOD2 activation decreases SERT activity, we measured the effect of NOD2 activation in SERT molecular expression in Caco-2/TC7 cells. Thus, cells were treated for 1 day with MDP 50 µg/ml and the SERT mRNA and protein levels were analyzed. The results showed that activation of NOD2 over a period of 1 day strongly decreased the SERT mRNA level (85%) (Fig. 1B), and similarly, SERT protein expression was notably reduced in cells treated with MDP in both the cell homogenate (42%) and apical membrane (30%) (Fig. 1C). The lowest inhibition observed in SERT protein expression would be due to the SERT protein turnover in these cells. Therefore, NOD2 activation inhibits SERT activity in Caco-2/TC7 cells and induces a significant decrease of SERT molecular expression.

RIP2/RICK intracellular signaling pathway is involved in NOD2 inhibitory effect on SERT

To gain in depth knowledge about the NOD2 inhibitory effect on SERT activity and expression, the intracellular signaling pathway involved was studied. To do so, 1 hour before the cell treatment with MDP (50 µg/ml) for 1 day, the cells were pre-treated with a specific RIP2 inhibitor (gentamicin 1 µM) and then the 5-HT uptake was measured.

Results showed that the specific RIP2 inhibitor (gentamicin) reversed the NOD2 inhibitory effect on SERT activity in the Caco-2/TC7 cell line (Fig. 2A). Moreover, gentamicin alone did not appear to affect 5-HT transport. To confirm the role of the RIP2/RICK pathway

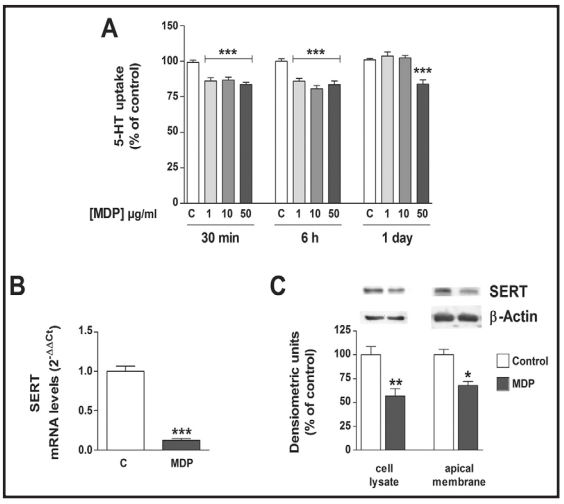

Fig. 1. SERT function and expression after NOD2 activation. A. 5-HT uptake was measured after 6 minutes of incubation of 5-HT 0.2 µM. MDP concentrations assayed were 1, 10 and 50 µg/ml. The treatment periods were 30 minutes (short term), 6 hours (medium term) and 1 day (long term). The results are expressed as the percentage of the uptake control (100%) and are the mean ± SEM of seven independent experiments. Absolute control values were 7.71±0.61, 5.77±0.7 and 7.0±1.1 pmol 5-HT/mg protein at 30 minutes, 6 hours and 1 day, respectively. B: Real-time PCR analysis of the SERT mRNA expression level in cells treated for 1 day with MDP 50 µg/ml. Relative quantification was performed using comparative Ct method ($2^{-\Delta\Delta Ct}$). Results are expressed as arbitrary units (control = 1) and are the mean ± SEM of five independent experiments. C: Immunodetection of SERT by western blot in cell lysate and apical membrane from Caco-2/TC7 cells treated with NOD2 50 µg/ml for 1 day. The quantitation of SERT protein using β-actin as an internal control of the protein load (SERT/β-actin ratio) was achieved. The results are expressed as a percentage of the control value and are the mean ± SEM of four independent experiments. *P<0.05, **P<0.01 and ***P<0.001 compared with the corresponding control value.

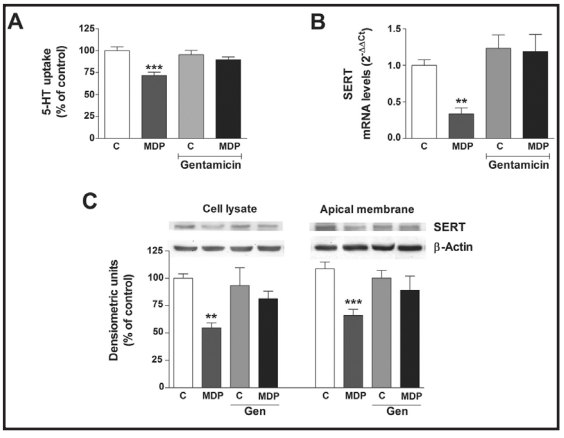

Fig. 2. RIP2/RICK intracellular pathway is involved in MDP effect on SERT. Caco-2/TC7 cells were treated for 1 day with MDP 50 µg/ml and/or gentamicin 1 µM. A. Uptake of 5-HT was measured after 6 minutes of incubation of 5-HT 0.2 µM. Absolute control value was 6.03±0.45 pmol 5-HT/mg protein. The results are expressed as the percentage of the uptake control (100%) and are the mean ± SEM of four independent experiments. B. Real-time PCR analysis of SERT mRNA expression. Relative quantification was performed using comparative Ct method ($2^{-\Delta\Delta Ct}$). Results are expressed as arbitrary units (control = 1) and are the mean ± SEM of three independent experiments. C. Immunodetection and quantification of SERT protein expression level by western blot in both cell lysate and apical membrane of Caco-2/TC7 cells. Results are expressed as SERT/β-actin ratio and are the mean ± SEM of four independent experiments. **P<0.01 and ***P<0.001 compared with the corresponding control value.

in the long-term effect of NOD2 activation, SERT mRNA and protein expression was determined in the same conditions mentioned above – 1 day of treatment with MDP 50 µg/ml with or without gentamicin. These data suggest that the specific RIP2 inhibitor rescued the SERT mRNA reduction that MDP induced (Fig. 2B). SERT protein expression was also studied in similar conditions and, in accordance with the previous results, the RIP2 inhibitor reversed the SERT protein level reduction that MDP induced in both the cell homogenate and apical membrane (Fig. 2C).

5-HT feedback regulation of NOD2 expression

From the results above, we can conclude that NOD2 activation by MDP inhibits SERT activity and expression in Caco-2/TC7 cells. As a result, SERT inhibition could lead to an increase of 5-HT extracellular availability in the intestinal epithelium, which has been

demonstrated to be a common factor in IBD [3]. Nonetheless, the effect of 5-HT in NOD2 molecular expression has not yet been determined. To do so, cells were treated for 1 day with low (physiological) or high (pathological) concentrations of 5-HT (10^{-8} M or 10^{-4} M respectively). The results showed that NOD2 mRNA expression was strongly decreased in the treated cells with both 5-HT concentrations, without a difference between them (Fig. 3A). NOD2 protein expression was also analyzed and a similar decrease in the NOD2 protein level was found (Fig. 3B and Fig. 3C).

Interdependence among TLR2, TLR4 and NOD2 in Caco-2/TC7 cells

The above results show that NOD2 activation decreases SERT expression and activity. Similarly, previous results have concluded that TLR2 (either TLR2/1 or TLR2/6) and TLR4 activation decrease significantly 5-HT uptake in Caco-2/TC7 cells [21, 22]. Moreover, the over-expression of both receptors has been implicated in IBD [14]. Accordingly, we have analyzed the possible interaction between TLR2 and TLR4 with NOD2. For this, Caco-2/TC7 cells were treated with TLR4 agonist (LPS 30 μg/ml), TLR2/1 agonist (Pam3CSK4 10 μg/ml) and TLR2/6 agonist (Pam2CSK4 100 ng/ml) for 1 day, after which the NOD2 mRNA and protein expression were assessed. The results showed that the NOD2 mRNA level was significantly decreased in cells treated with LPS (TLR4 agonist). Moreover, the NOD2 mRNA level increased after TLR2/6 activation, whereas TLR2/1 activation did not induce an alteration in NOD2 mRNA expression (Fig. 4A). In line with the mRNA results, NOD2 protein expression was significantly increased by TLR2/6 activation and non-changes after TLR4 activation; however, TLR4 activation decreased NOD2 protein expression but not significantly (Fig. 4B).

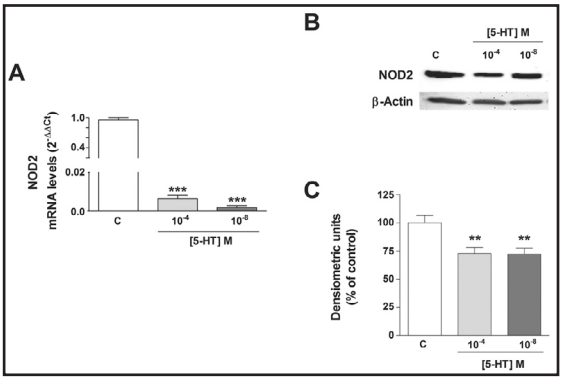

Fig. 3. Serotonin inhibits NOD2 expression. Caco-2/TC7 cells were treated with 5-HT 10^{-4} and 10^{-8} M for 1 day. A. Real-time PCR analysis of NOD2 mRNA expression level. Relative quantification was performed using comparative Ct method ($2^{-\Delta\Delta Ct}$). Results are expressed as arbitrary units (control = 1) and are the mean ± SEM of three independent experiments. B. Immunodetection of NOD2 by western blot in cell lysate of treated cells. C. Quantification of NOD2 protein relative expression in treated cells using β-actin as an internal control of the protein load (NOD2/β actin ratio). The results are expressed as a percentage of the control value (100%) and are the mean ± SEM of three independent experiments. **P<0.01 and ***P<0.001 compared with the control value.

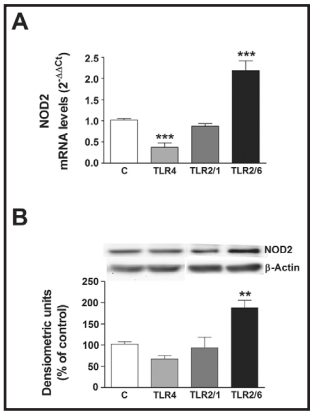

Fig. 4. Activation of TLR2 and TLR4 modulates NOD2 expression. Cells were treated with LPS 30 μg/ml (TLR4 ligand), Pam3CSK4 10 μg/ml (TLR2/1 ligand) or Pam2CSK4 100 ng/ml (TLR2/6 ligand) for 1 day. A. Real-time PCR analysis of NOD2 mRNA expression in treated cells. Relative quantification was performed using comparative Ct method ($2^{-\Delta\Delta Ct}$). Results are expressed as arbitrary units (control = 1) and are the mean ± SEM of four independent experiments. B. Immunodetection and quantification of NOD2 protein expression in cell lysate using β-actin as an internal control of the protein load (NOD2/β actin ratio). The results are expressed as a percentage of the control value (100%) and are the mean ± SEM of six independent experiments. **P<0.01 and ***P<0.001 compared with the control value.

We also analyzed the effect of NOD2 activation on TLR2 and TLR4 expression. Here, Caco-2/TC7 cells were treated with NOD2 ligand (MDP 50 μg/ml) for 1 day, and then, TLR2 and TLR4 mRNA and protein expression was assessed. These data showed that the TLR2 and TLR4 mRNA expression level was decreased after NOD2 activation (Fig. 5A); however, TLR2 and TLR4 protein expression was unchanged (Fig. 5B).

Analysis of NOD2 expression in ileum and colon of Tlr2$^{-/-}$, Tlr4$^{-/-}$ and Tlr2, 4$^{-/-}$ mice

To corroborate the previous results, we analyzed NOD2 expression in the ileum and colon of *Tlr2*$^{-/-}$, *Tlr4*$^{-/-}$ and *Tlr2, 4*$^{-/-}$ mice. In the ileum, NOD2 mRNA (Fig. 6A) and protein (Fig. 6B) expression strongly increased in *Tlr2*$^{-/-}$ mice, whereas NOD2 expression did not seem to be modified in *Tlr4*$^{-/-}$ and *Tlr2, 4*$^{-/-}$ mice (Fig. 6A and Fig. 6B). Regarding the colon, NOD2 mRNA and protein expression followed a similar pattern to the ileum, being significantly increased only in *Tlr2*$^{-/-}$ mice (Fig. 6C and Fig. 6D). Surprisingly, NOD2 expression seemed not to be modified in both ileum and colon from double knockout *Tlr2, 4*$^{-/-}$ compared with WT mice, suggesting a possible collaborative action between TLR2 and TLR4.

Discussion

It is well known that most of the body's serotonin (5-HT) is synthesized and stored in the intestine (approximately 95%). 5-HT is recognized as a signaling molecule implicated in a wide number of physiological (gastrointestinal and non-gastrointestinal) functions [2], being also implicated in the development and maintenance of gut inflammatory pathologies, such as IBD [8]. These pathologies also involve altered intestinal innate immunity [29], which plays an important role in intestinal homeostasis.

In this paper, we have described how NOD2 activation decreases intestinal SERT activity and molecular expression, through the RIP2/RICK intracellular signaling pathway. The

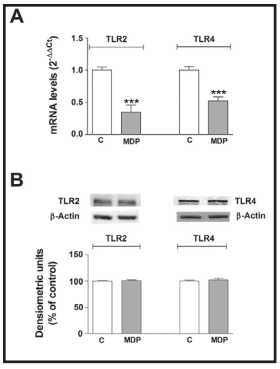

Fig. 5. NOD2 modulates TLR2 and TLR4 expression. Cells were treated with MDP 50 μg/ml for 1 day. A. Real-time PCR analysis of TLR2 and TLR4 mRNA expression in treated cells. Relative quantification was performed using comparative Ct method ($2^{-\Delta\Delta Ct}$). Results are expressed as arbitrary units (control = 1) and are the mean ± SEM of four independent experiments. B. Immunodetection and quantification of TLR2 and TLR4 protein expression in cell lysate using β-actin as an internal control of the protein load (TLR2 and TLR4/β actin ratio). The results are expressed as a percentage of the control value (100%) and are the mean ± SEM of five independent experiments. ***P<0.001 compared with the control value.

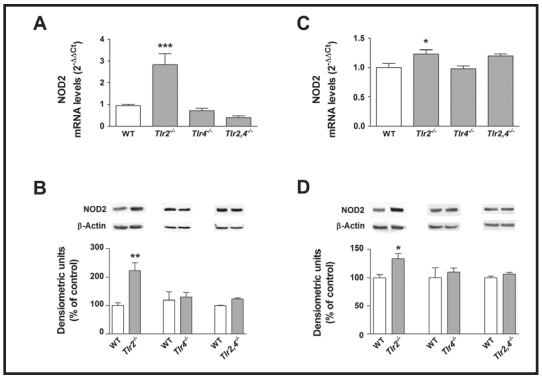

Fig. 6. NOD2 expression in ileum (A, B) and colon (C, D) of Tlr2$^{-/-}$, Tlr4$^{-/-}$, and Tlr2,4$^{-/-}$ mice. Real-time PCR analysis of NOD2 mRNA expression level in ileum (A) and colon (C) of knockout mice. Relative quantification was performed using comparative Ct method ($2^{-\Delta\Delta Ct}$). Results are expressed as arbitrary units (WT = 1) and are the mean ± SEM of five animals. Immunodetection and quantification of NOD2 by western blot in ileum (B) and colon (D) of knockout mice, using β-actin as an internal control of the protein load (NOD2/β-actin ratio). The results are expressed as a percentage of the control value (100%) and are the mean ± SEM of four animals. *P<0.05, **P<0.01 and ***P<0.001 compared with the control value.

inhibitory effect on SERT activity yields an increment of extracellular 5-HT, which seems not to affect NOD2 expression. Analysis of SERT activity showed that NOD2 activation, in the short and medium terms, reduces SERT activity at all of the concentrations of MDP tested. However, NOD2 long-term activation showed that only the highest concentration of MDP used was able to reduce SERT activity, with a reduction of SERT molecular expression. This suggests that long-term NOD2 activation may decrease SERT expression via transcriptional and/or post-transcriptional mechanisms, offering an explanation for the 5-HT uptake reduction. In agreement with our results, other PRRs have also demonstrated inhibition of SERT activity with [21, 22] or without [30] alteration of SERT expression.

The activation of NOD2, with low concentrations of MPD, induces an inhibitory effect observed only in the short (30 minutes) and medium terms (6 hours), suggesting that a brief stimulation of NOD2 induces a rapid inhibition of SERT activity, increasing 5-HT extracellular availability to promote a fast inflammatory response. The observed effect in the long term may be due to the activation of mechanisms that could regulate NOD2 over-activation after a long-term treatment [31]. In this case, only a high concentration of MDP in the long term would maintain NOD2 activation [32].

The inhibition of SERT expression and activity by NOD2 activation would lead to an increase of the 5-HT extracellular availability, which could induce the effect on NOD2 expression via feedback regulation. Our results have shown that 5-HT strongly diminished NOD2 mRNA and protein expression, suggesting that 5-HT could act as a repressor of NOD2 expression. However, when we compared high 5-HT concentration (10^{-4} M), which occurs in inflammatory pathologies [2] with lower (5-HT, 10^{-8} M) physiological conditions no significant differences were observed in NOD2 expression, suggesting that a NOD2 induced extracellular 5-HT increase would not modify the observed effect. Previous studies, however, have demonstrated feedback regulation between TLR2 and 5-HT [21], where a high 5-HT concentration decreases TLR2 expression, modulating TLR2 responses. Our results demonstrate that 5-HT, an intestinal non-immunological modulator, could regulate the expression of innate immune receptors in different ways and may contribute to modulation of the intestinal response to inflammation.

This study has demonstrated that NOD2 activation could inhibit SERT activity and expression through the RIP2/RICK intracellular pathway. Other pro-inflammatory pathways have been pointed out as being implicated in SERT activity [33, 34], but the implication of RIP2/RICK in SERT activity has not been studied until now, although several studies have shown that MDP could act throughout RIP2/RICK to develop pro-inflammatory responses [35]. In fact, recent results have demonstrated that MDP could increase the infiltration of inflammatory-related cells and intestinal damage with a transcript expression increment of NOD2 and RIP2/RICK [36]. Moreover, several works have shown that pro-inflammatory factors inhibit SERT [37, 38].In addition, serotonin has been also reported as a pro-inflammatory molecule in the intestine because SERT knockout mice showed exacerbated intestinal inflammation due to an uncontrolled increase of mucosal 5-HT levels [39, 40]. In this context, experimental colitis was also more intense in SERT knockout mice compared with WT, suggesting the critical implication of serotonin and SERT in intestinal inflammation [39, 40]. In this sense, gut microbiota have also been linked to immune responses and to the pathophysiology of intestinal inflammatory disease. Several members of the microbiota produce metabolites, including serotonin. These metabolites induce intestinal epithelial cells to release molecules that modulate cell signaling [41]. Indeed, spore-forming bacteria, primarily *Clostridium* spp., modulated the colonic luminal metabolome, including short-chain fatty acids, thereby inducing serotonin biosynthesis by enterochromaffin cells [42].

The innate immune system needs both TLRs and NLRs for developing an effective response against potential pathogens in the gut. Numerous studies have shown that crosstalk takes place between different TLRs, especially between TLR2 and TLR4 in different cell types [43, 44]. However, these results seem to be controversial. Some investigations showed synergy between PRRs to improve the intestinal defense [45] and to promote the inflammatory situation [46], whereas other authors pointed out that TLRs and NLRs are

inversely related, with the activation of one PRR inhibiting the other [47]. In this regard, and considering that TLR2 and TLR4 also inhibit SERT expression and activity [21, 22], we have analyzed the possible relation between TLR2 and TLR4 with NOD2 to clarify the role of NOD2 in homeostasis and inflammation. Our results showed that only TLR2/6 activation increased significantly NOD2 expression (mRNA and protein) in Caco-2/TC7 cells. In addition, NOD2 activation decreased TLR2 mRNA in Caco-2/TC7 cells. To confirm the interdependence between TLR2 and NOD2 in the intestinal tract, NOD2 expression in the ileum and colon of *Tlr2*$^{-/-}$ mice was analyzed. Surprisingly, *Tlr2*$^{-/-}$ mice also showed increased NOD2 expression (mRNA and protein) in both the ileum and colon, although TLR2 activation upregulated NOD2 expression. However, recently, we observed that *Tlr2*$^{-/-}$ mice had increased TLR4 expression and vice versa [25], suggesting a compensatory mechanism between TLR2 and TLR4 expression that might explain our results. Similarly, we have recently demonstrated that *Tlr2*$^{-/-}$ mice also exhibit increased expression of SERT [21].

In agreement with our results, recent studies showed a synergic effect between NOD2 and TLR2 [48], where specific NOD2 activation with MDP increased the induction of TNFα, IL-1 and IL-10 upon co-stimulation with TLR2 agonists. Other studies showed that the activation of NOD2 could modify the expression of TLR2 negatively, although this seems to be determined by the different kinds of studied cells [49]. However, it seems that NOD2 could also recognize the specific bacterial fragments recognized by TLR2 heterodimers (TLR2/1 and TLR2/6) [50]. Therefore, TLR2 could act synergistically with NOD2 in the MAMP's recognition, and this recognition would increase NOD2 expression, reinforcing the inflammatory process. Moreover, increased NOD2 expression in both ileum and colon of *Tlr2*$^{-/-}$ mice may mean that the lack of TLR2 could be compensated by the increment of the NOD2 receptor to maintain the intestinal homeostasis.

In contrast to TLR2, it seems that TLR4 activation by LPS treatment in Caco-2/TC7 cells decreases the NOD2 mRNA level, without causing changes in protein expression, suggesting transcriptional NOD2 modifications that apparently are not translated into NOD2 protein modifications. It may be possible that LPS treatment for one day was not enough to trigger a decrease in NOD2 protein expression but would be sufficient for decreasing significantly NOD2 mRNA expression. These results are not in agreement with recent studies in which macrophages treated with LPS increased NOD2 mRNA expression, although it could be due to the different cell model used [51]. Moreover, NOD2 activation seemed to decrease TLR4 mRNA level. In relation to NOD2 expression in the intestinal tract of *Tlr4*$^{-/-}$ mice, it seems that there was no compensation response by NOD2 as a result of missing TLR4 activity. NOD2 molecular expression (mRNA and protein) was modified neither in the ileum nor in the colon of *Tlr4*$^{-/-}$ and *Tlr2, 4*$^{-/-}$ mice. Other factors could also be implicated in NOD2 regulation, where the lack of receptors TLR2 and TLR4 leads to a non-modification of the NOD2 level. Thus, as the intestinal tract results showed, regulation of NOD2 may be different with the absence of TLR2 or/and TLR4 in the ileum and colon, producing a different response to microorganisms. In this context, several studies have indicated a possible TLR2-TLR4 interaction, which could explain the different results from single and double knockout [52, 53].

In summary, our work shows that NOD2 activation inhibits SERT activity and expression in intestinal epithelial cells and proposes RIP2/RICK as the signaling pathway involved. Therefore, the intracellular detection of bacteria by NOD2 would induce an increase in intestinal 5-HT levels through the inhibition of SERT, contributing in this way to the inflammatory response against the pathogenic bacteria but also by interacting with other PRR as TLR2 and TLR4 to trigger several defense pathways (Fig. 7). Moreover, elevated levels of 5-HT could affect the intestinal physiology, contributing to the symptoms of a gastrointestinal disorder as diarrhea, increase motility, and abdominal pain. In this context, the activation of others PRRs, such as TLR2 and TLR4, would affect NOD2, and in turn, NOD2 activation could influence both TLRs, which also seems to modify SERT. Thus, intestinal serotonin is widely modulated by innate immunity to maintain intestinal homeostasis and inflammation.

Acknowledgements

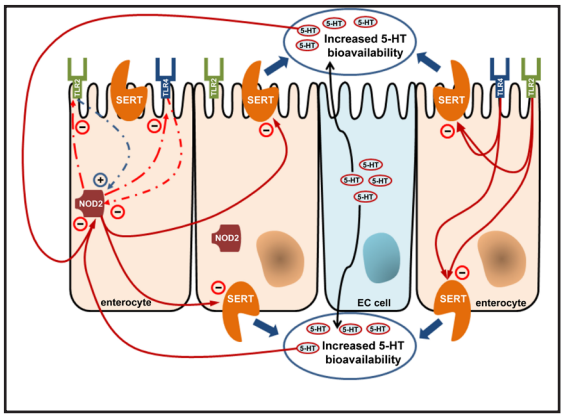

Fig. 7. Schematic drawing of NOD2 effect on SERT and crosstalk between NOD2, SERT, TLR2 and TLR4. NOD2 activation as well as TLR2 or TLR4 activation induces a decrease of SERT activity and expression, which would induce an increase in intestinal 5-HT levels, contributing to the inflammatory response and gastrointestinal disorders (diarrhea, increase motility and abdominal pain). In a feedback interaction to avoid an exacerbated response, 5-HT could act as a repressor of NOD2 expression. In this context of regulation, NOD2 also inhibits TLR2 and TLR4 expression, like TLR4 inhibits NOD2, while TLR2 stimulates NOD2 expression.

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In memoriam of Ana I. Alcalde.

Disclosure Statement

The authors declare to have no competing interests.

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