Title:

TLR2 AND TLR4 INTERACT WITH SULFIDE SYSTEM IN THE MODULATION OF MOUSE COLONIC MOTILITY

Running title:

TLR2 and TLR4-sulfide system interaction

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Abstract

Background: H_2S is a neuromodulator that may inhibit intestinal motility. H_2S production in colon is yielded by cystathionine β-synthase (CBS) and cystathionine γ-lyase (CSE) enzymes and sulphate-reducting bacteria (SRB). Toll-like receptors (TLRs) recognize intestinal microbiota. The aim of this work was to evaluate the influence of TLR2 and TLR4 on the endogenous and SRB-mediated synthesis of H_2S and its consequences on the colonic motility of mouse.

Methods: Muscle contractility studies were performed in colon from WT, *Tlr2*$^{-/-}$ and *Tlr4*$^{-/-}$ mice. The mRNA levels of TLR2, TLR4, CBS, CSE and SRB were measured by real-time PCR. Free sulfide levels in colon and feces were determined by colorimetric assays.

Results: NaHS and GYY4137, donors of H_2S, reduced the contractility of colon. Aminooxyacetic acid (AOAA), inhibitor of CBS, and D-L propargylglycine (PAG), inhibitor of CSE, increased the contractility of colon. *In vivo* treatment with NaHS or GYY4137 inhibited the spontaneous contractions and upregulated TLR2 expression. The *in vivo* activation of TLR4 with lipopolysaccharide increased the contractile response to PAG, mRNA levels of CSE and the free sulfide levels of H_2S in colon. In *Tlr2*$^{-/-}$ and *Tlr4*$^{-/-}$, the contractions induced by AOAA and PAG and mRNA levels of CBS and CSE were lower with respect to WT mice. Deficiency of TLR2 or TLR4 provoke alterations in free sulfide levels and SRB of colon.

Conclusions & Inferences: Our study demonstrates interaction between TLR2 and TLR4 and the sulfide system in the regulation of colonic motility and contributes to the pathophysiology knowledge of intestinal motility disorders.

Keywords: microbiota, Toll-like receptors, H_2S, intestinal motility

Key points

- H_2S is synthesized by two enzymes of the colon and sulphate-reducting bacteria (SRB) of the intestinal lumen. Toll-like receptors (TLRs) recognize intestinal bacteria. We evaluate the influence of TLRs on the sulfide system in the modulation of mouse colonic motility.
- Our study demonstrates the interaction between TLR2 and TLR4 and the sulfide system in the regulation of colonic motility.
- These findings contribute to the pathophysiology knowledge of intestinal motility disorders such as Inflammatory Bowel Disease.

Introduction

Colonic microbiota acts as a barrier against pathogens, drives the maturation of the immune system and contributes to the maintenance of gut homeostasis[1]. Bacteria recognition is carried out by Toll-like receptors (TLRs), a family of transmembrane proteins localized in intestinal epithelial cells and immune cells that trigger innate immune responses [2,3]. Among TLRs, the most important bacteria-sensing receptors are TLR2 and TLR4, which recognize the bacterial fragments lipoteichoic acid (LTA) and lipopolysaccharide (LPS), respectively. Expression of TLR2 and TLR4 has been found in enteric neurons, glial cells and smooth muscle cells of mouse colon, suggesting that TLR2 and TLR4 may play a chief role in the regulation of intestinal motility [4-6]. In fact, our group and other authors have described that TLR4 but not TLR2 is involved in the regulation of the normal spontaneous colonic motility in mice [7,8].

Hydrogen sulfide (H_2S) has emerged as an important gaseous signaling molecule playing numerous roles in health and disease. Previous studies have suggested that H_2S is implicated in the regulation of several cellular processes in the gastrointestinal (GI) tract including inflammation, epithelial secretion, nociception and motility [9]. In fact, H_2S has been proposed as a third gaseous neuromodulator, after nitric oxide and carbon monoxide (CO), that may inhibit GI motility [10].

The endogenous production of H_2S in the smooth muscle layer of rat and mouse colon is mainly through two enzymes: the cystathionine γ-lyase (CSE) and the cystathionine β-synthase (CBS) [11,12]. In fact, both CBS and CSE enzymes have been identified in enteric neurons, smooth muscle layers and epithelial cells of colon [11-13]. Additionally, large amounts of H_2S can be produced by the sulphate-reducting bacteria (SRB) living in the colon [9]. SRB are anaerobic microorganisms that conduct dissimilatory sulphate

reduction to obtain energy, resulting in the release of a great quantity of sulfide in the intestine.

Understanding of H_2S interactions with other signaling mediators is still a topic of active research. In fact, the cross-talk between TLR and the H_2S system in the intestine has not yet been elucidated in detail. As TLR2 and TLR4 have been found in the enteric nerves and smooth muscle, the same type of cells in which CBS and CSE are expressed, we hypothesize that TLR may modulate the synthesis of H_2S and the motor responses induced by H_2S in the intestine. Thus, the aim of the present study was to evaluate the influence of TLR2 and TLR4 on the endogenous and SRB-mediated synthesis of H_2S and its consequences on the colonic motility of mouse.

Materials and methods

Animals

All procedures were carried out under Project Licenses PI03/16 and PI13/17, approved by the in-house Ethics Committee for Animal Experiments from the University of Zaragoza. Inbred C57BL/10 and mouse strains deficient for TLR2 (*Tlr2*$^{-/-}$) and TLR4 (*Tlr4*$^{-/-}$) were kindly provided by Ignacio Aguiló from the University of Zaragoza, and bred at the Centro de Investigación Biomédica de Aragón (CIBA), Zaragoza, Spain. Their genotypes were periodically analyzed as described [14]. Male *Tlr2*$^{-/-}$ and *Tlr4*$^{-/-}$ knockout and age-matched (8 to 12 weeks old) wild-type (WT) mice were used in the experiments. All mice were housed under pathogen-free conditions on a 12-hour light/dark cycle with food and water ad libitum.

The WT, *Tlr2*$^{-/-}$ and *Tlr4*$^{-/-}$ mice were treated with a single IP injection of NaHS or GYY4137 5 mg Kg^{-1} for 3 or 24 h, respectively. These treatments were previously shown to have effects on the digestive system [15-18].

Other WT mice were treated with a single IP injection of lipoteichoic acid (LTA, 20 mg Kg^{-1} for 3 h) to activate TLR2 or ultrapure lipopolysaccharide (LPS, 5 mg Kg^{-1} for 24 h) to activate TLR4. These treatments have been used by other authors previously [19,20].

Muscle contractility studies

Strips of proximal-mid colon with intact mucosa were suspended in the direction of circular smooth muscle fibers in an organ bath and muscle contractility studies were performed as previously described [8].

To study *in vitro* the effect of the H_2S donors in WT, *Tlr2*$^{-/-}$ and *Tlr4*$^{-/-}$ mice, NaHS (a rapid donor, 10-1000 µM) or GYY4137 (a slow donor, 0-1000 µM) was added every 15 min and the cumulative concentration-response curves were performed.

To study the inhibitory effect *in vitro* of the H_2S synthesis enzymes in WT, *Tlr2*$^{-/-}$ and *Tlr4*$^{-/-}$ mice, aminooxyacetic acid (AOAA, 0.01-10 mM), an inhibitor of CBS, or D-L Propargylglycine (PAG, 0.01-10 mM), an inhibitor of CSE, was added every 15 min and the cumulative concentration-response curves were performed.

In mice treated *in vivo* with NaHS or GYY4137, only the spontaneous activity of the colon was recorded.

In mice treated with LTA or LPS, we added a single concentration of NaHS (1 mM) or GYY4137 (1 mM) or AOAA (10 mM) or PAG (10 mM) in each colonic strip.

To estimate the responses to the drugs, the area under the curve (AUC) of spontaneous contractions from the minimum was measured before and after drug addition, and expressed as g min^{-1}. The inhibition or increase of the spontaneous contractions were quantified as the inhibition or increase in the AUC after the addition of the drugs over the baseline AUC, before the addition of the drugs.

mRNA expression by real-time PCR

The relative abundance of TLR2, TLR4, CBS and CSE mRNA in the colon from mice was measured by real-time PCR. Tissue samples for mRNA analysis were collected in RNAlater (Qiagen, Hilden, Germany), and RNA extractions were carried out with the RNeasy mini kit (Qiagen, Hilden, Germany) and the cDNA was synthesized using the NX M-MuLV Reverse Transcriptase kit (Lucigen, Middleton, WI, USA) according to the supplier's protocol. The cDNAs obtained were used to measure the mRNA levels by SYBR Green and using specific primers (Table 1). Reactions were run using the StepOne Plus Real-Time PCR System (Life Technologies, Carlsbad, California, USA). The reaction mixture (10 µL) was comprised of 4.5 µL FastStart Universal SYBR Green Master (Roche, Mannheim, Germany), 0.5 µL of each primer 30 µM, 2.5 µL of sterile distilled water, and 2 µL of cDNA template (200 ng). Each sample was run in triplicate, and the mean Ct was determined from the three runs. Thus, the relative mRNA expression was calculated as $\Delta\Delta Ct = \Delta Ct_{\text{treatment or type of mice}} - \Delta Ct_{\text{control or WT mice}}$ being $\Delta Ct = Ct_{\text{target gene}} - Ct_{\text{reference gene}}$. GAPDH and actin were used as reference genes. Finally, the relative gene expression levels were converted and expressed as fold difference ($= 2^{-\Delta\Delta Ct}$).

Determination of free sulfide levels in mouse colon

Sulfide levels in mouse colon were measured by using the zinc sulfide precipitation method with some modifications [21]. Two samples of colonic tissue (120 mg) were harvested from each animal. The fresh samples were weighted out and immediately immersed in 1 mL of cold phosphate 50 mM pH 6 or bicarbonate-carbonate buffer 50 mM pH 9.4. Samples were then homogenized on ice followed by centrifugation at 15000 g for 10 min at 4°C. 500 µL of the clear supernatant from each sample was added to 400 µL of a pre-mixed 1% w/v zinc acetate (350 µL) solution and 50 µL of NaOH

1.5 M and incubated for 1 h at RT. This was followed by centrifugation at 1200 g for 5 min to pellet the zinc sulfide formed. The supernatant was removed and the pellet washed with 1.5 mL of Milli-Q water by vortexing thoroughly, followed by a centrifugation at 1200 g for 5 min. The supernatant was then removed and the pellet reconstituted with 480 µL of Milli-Q water and mixed with 120 µL of pre-mixed dye (60 µL of N,N-dimethyl-p-phenylenediamine, NNDP, 20 mM in HCl 7.2 M and 60 µL of $FeCl_3$ 30 mM in HCl 1.2 M). The solution was then incubated for 10 min to allow the color to develop and the absorbance was read on a spectrophotometer at 670 nm.

To determine free sulfide levels, one sample was prepared in a sodium phosphate buffer 50 mM pH 6 and another sample in a bicarbonate-carbonate buffer 50 mM pH 9.4. At pH 6, >90% of free sulfides should exist as the neutral H_2S species which are highly volatile and easily lost by dissipation into the atmosphere. At pH 9, >90% of free sulfides should exist as the anionic HS^-, which is retained in solution. Free tissue sulfide was then calculated by deducting the value of sulfides measured at pH 6 from pH 9.

A standard curve of serially diluted NaHS was prepared in bicarbonate-carbonate buffer 50 mM pH 9.4. The concentrations of free sulfide were expressed as nmole per mg of tissue weight.

Determination of free H_2S levels in mouse feces

In order to evaluate the role of H_2S produced in the colonic lumen by bacteria, free H_2S levels were determined in mouse feces by a previously described method with some modifications [22,23].

A sample of fresh mouse feces was collected into a pre-weighed tube containing 500 µl of cold NaOH 1 M. The tube with the feces was weighed again and the weigh of the feces was calculated (30-60 mg). Free sulfide was measured using the supernatant fraction following centrifugation (14500 g, 5 min, 4ºC) of NaOH slurries. The sulfide

levels were determined using the methylene blue reaction by mixing 300 µl of faecal slurry with 150 µl of NNDP 0.17 M in HCl 6 M and adding immediately 150 µl of $FeCl_3$ 0.37 M in HCl 6 M. This was quickly sealed and allowed 20 min for full color development. Following centrifugation (12000 g, 5 min), 100 µl of supernatant was diluted in 0.9 ml maleic acid buffer 0.86 M pH 0.6, mixed and the absorbance was measured at 670 nm. A standard curve of NaHS was fresly prepared for each experiment and faecal sulfide was therefore calculated by extrapolation. The concentrations of sulfide were expressed as nmole per mg of feces.

Microbial analysis

Freeze-dried feces (20 mg) were homogenized in tubes containing zirconium and glass beads in a Precellys 24 tissue homogenizer (2 cycles of 5000g for 30 seconds). Total DNA was isolated using FavorPrep Stool DNA Isolation Mini Kit (Favorgen Biotech Corp). Extracted DNA was eluted in 50µl and quantified by NanoDrop-100 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Total bacteria gene copy number and four groups of bacteria were targeted by real time-PCR in QuantStudio 6 Flex Real-Time PCR system (Thermo Fisher Scientific, Waltham, MA, USA) with PerfeCTa SYBR Green SuperMix Low ROX (Quantabio, Beverly, MA, USA). The analysis was performed with QuantStudio RT PCR software v1.3. The primers used in this study are in Table 2. Relative quantification to 16S rDNA gene was calculated using ΔΔCt method. The total bacteria 16S rRNA gene copy numbers was determined by standard curves made using serial dilutions of plasmid (containing 16S rRNA gene fragment) of known concentrations on a tenfold basis.

Data analysis and statistics

Differences in the responses to the different concentrations of the drugs in mice, were compared by one or two-way analysis of variance (one or two-way ANOVA) followed

by Bonferroni's post-hoc test. IC_{50} values were calculated using a conventional concentration-response curve with variable slope. Differences in bacteria levels, gene expression or sulfide levels were compared by Student´s unpaired t-test.

The results were expressed as the mean ± SEM with *n* denoting the number of animals used. The data were analyzed using the software GraphPad Prims version 5.00 (GraphPad Software, San Diego, CA, USA), and the differences between P-values < 0.05 were considered to be statistically significant.

Drugs and solutions

Lipoteichoic acid from *Bacillus subtilis*-TLR2 ligand and ultrapure lipopolysaccharide from *E. coli* O111:B4 strain-TLR4 ligand were purchased from InvivoGen (San Diego, CA, USA). NaHS, GYY4137, AOAA and PAG were acquired from Sigma (Madrid, Spain). AOAA, PAG and NaHS solutions were prepared in milliQ water for *in vitro* studies. GYY4137 was dissolved in DMSO to obtain a stock solution of 3 mg ml^{-1} and the following solutions were made with milliQ water or saline for *in vitro* or *in vivo* studies, respectively. NaHS, LPS and LTA solutions were prepared in saline for *in vivo* studies.

Results

***In vitro* effect of H_2S on colonic spontaneous motility**

To study the effect of H_2S in colon motility, concentration-response curves to rapid (NaHS, 10-1000 µM) and slow (GYY4137, 10-1000 µM) donors of H_2S were performed in whole strips of mouse colon suspended in the circular direction in an organ bath. Both NaHS and GYY4137 reduced the contractility of the circular smooth muscle of the mouse colon in a concentration-dependent manner ($p < 0.001$) (Figure 1A-D). The IC_{50} values were 465 µM for NaHS (95% confidence interval log IC_{50} = -3.33 ±

0.44; n= 7) and 192 µM for GYY4137 (95% confidence interval log IC_{50} = -3.71 ± 0.32; n= 7).

To analyze the participation of H_2S synthesis enzymes on the spontaneous contractions, concentration-response curves to AOAA (0.01-10 mM), an inhibitor of CBS, and PAG (0.01-10 mM), an inhibitor of CSE, were performed on colonic strips in the same way in the organ bath. Both AOAA and PAG increased the contractility of the circular smooth muscle of the mouse colon in a concentration-dependent manner ($p < 0.01$ and $p < 0.001$, respectively) (Figure 1E-H). The IC_{50} values were 40 µM for AOAA (95% confidence interval log IC_{50} = -4.38 ± 1; n= 4) and 700 µM for PAG (95% confidence interval log IC_{50} = -3.15 ± 0.48; n= 10).

***In vivo* effect of H_2S donors**

To corroborate the *in vitro* inhibitory effect of the H_2S donors on colonic motility, we examined the spontaneous motility of muscle strips isolated from mice treated *in vivo* with a single IP injection of NaHS or GYY4137 5 mg Kg^{-1} for 3 or 24 h, respectively. Both the rapid release of H_2S through the donor NaHS and the slow release of H_2S through the donor GYY4137 induced an inhibition of the spontaneous contractions of colon (Figure 2A, B).

In order to establish a possible interaction between the H_2S and the receptors TLR2 and TLR4 in the modulation of the intestinal motor function, we first studied mRNA expression of TLR2 and TLR4 in the colon of WT mice treated with NaHS or GYY4137. TLR2 expression was upregulated after the treatment with NaHS or GYY4137, being the expression of this receptor much higher (17-fold) in the intestine of mice treated with GYY4137, the slow donor of H_2S (Figure 2C). However, TLR4 mRNA level was not significantly modified by any of the donors of H_2S (Figure 2D).

TLR2 and TLR4 activation modulates H_2S synthesis and H_2S induced motor responses

The above results suggest that H_2S could be a regulator of TLR expression. In the next step, we studied the effect of the TLR activation with specific ligands on the motor responses induced by H_2S donors in colon. The *in vitro* addition of both NaHS 1 mM and GYY4137 1 mM reduced the contractility of the circular smooth muscle of mice treated with LTA (20 mg Kg^{-1} for 3 h), a specific ligand of TLR2, similar to those observed in mice treated with saline for 3 h (Figure 3A, B). In the same way, NaHS and GYY4137 reduced the contractility in the mice treated with LPS (5 mg Kg^{-1} for 24 h), a specific ligand of TLR4, similar to those observed in mice treated with saline for 24 h (Figure 3A, B).

The effects of the inhibition of CBS and CSE enzymes on the spontaneous motility of mice treated with LTA or LPS was also studied. AOAA (10 mM) caused a contractile response in the circular smooth muscle of mice treated with LTA or LPS, similar to those observed in mice treated with saline (Figure 3C). PAG 10 mM caused a contractile response in mice injected with LTA, similar to those observed in mice treated with saline (Figure 3D). However, the contractile response to PAG was significantly increased in mice treated with LPS (Figure 3D), suggesting that TLR4 could alter H_2S synthesis mediated by CSE enzyme.

Thus, to establish a possible relation between the activation of TLR2 and TLR4 and the H_2S synthesis in the modulation of the motor responses mediated by H_2S, we studied mRNA expression of the H_2S synthesis enzymes CBS and CSE and the levels of free sulfide in colon from mice treated with LTA or LPS.

The activation of TLR2 or TLR4 with LTA or LPS, respectively, did not modify the mRNA expression levels of CBS in colon (Figure 3E). Although the mRNA expression

levels of CSE was not modified in the colon from mice treated with LTA, the mRNA levels of this enzyme were highly increased after the activation of TLR4 with LPS (Figure 3F).

In addition, regarding sulfide levels, we observed that the activation of TLR2 with LTA did not modify the free sulfide levels in colon. However, the activation of TLR4 with LPS increased the free sulfide levels in colon (Figure 3G), suggesting that TLR4 activation increases the H_2S synthesis mediated by CSE.

H_2S synthesis and H_2S induced motor responses in *Tlr2*$^{-/-}$ and *Tlr4*$^{-/-}$ mice.

To corroborate the previous results in which both TLR2 and TLR4 seem to show interaction with the H_2S synthesis and the motor responses induced by H_2S, experiments were performed on TLR2 or TLR4 deficient mice. NaHS (10-1000 µM) reduced the contractility in a concentration-dependent manner in *Tlr2*$^{-/-}$ ($p < 0.001$) and *Tlr4*$^{-/-}$ ($p < 0.05$) mice (Figure 4A). The IC_{50} values of NaHS were 137 µM for *Tlr2*$^{-/-}$ mice (95% confidence interval log IC_{50} = -3.86 ± 0.24; n= 9) and 509 µM for *Tlr4*$^{-/-}$ mice (95% confidence interval log IC_{50} = -3.29 ± 0.66; n= 6).

GYY4137 (10-1000 µM) reduced the contractility in a concentration-dependent manner in *Tlr2*$^{-/-}$ ($p < 0.001$) and *Tlr4*$^{-/-}$ ($p < 0.001$) mice (Figure 4B). The IC_{50} values of GYY4137 were 110 µM for *Tlr2*$^{-/-}$ mice (95% confidence interval log IC_{50} = -3.95 ± 0.27; n= 6) and 175 µM for *Tlr4*$^{-/-}$ mice (95% confidence interval log IC_{50} = -3.75 ± 0.40; n= 11).

No differences were found between the decrease in the contractility induced by NaHS or GYY4137 in *Tlr2*$^{-/-}$ or *Tlr4*$^{-/-}$ with respect to WT mice.

Regarding enzyme inhibition, AOAA (0.01-10 mM) increased the contractility in a concentration-dependent manner in *Tlr2*$^{-/-}$ ($p < 0.05$) and *Tlr4*$^{-/-}$ ($p < 0.01$) mice (Figure

4C). The IC_{50} values of AOAA were 20 µM for *Tlr2*$^{-/--}$ mice (95% confidence interval log IC_{50} = -4.57 ± 1.4; n= 9) and 580 µM for *Tlr4*$^{-/-}$ mice (95% confidence interval log IC_{50} = -2.23 ± 0.4; n= 7). PAG (0.01-10 mM) increased the contractility in a concentration-dependent manner in *Tlr2*$^{-/-}$ ($p < 0.001$) and *Tlr4*$^{-/-}$ ($p < 0.01$) mice (Figure 4D). The IC_{50} values of PAG were 170 µM for *Tlr2*$^{-/-}$ mice (95% confidence interval log IC_{50} = -3.75 ± 0.55; n= 5) and 140 µM for *Tlr4*$^{-/-}$ mice (95% confidence interval log IC_{50} = -3.83 ± 0.55; n= 5).

The contractions induced by AOAA or PAG were significantly lower in *Tlr2*$^{-/-}$ and *Tlr4*$^{-/-}$ with respect to WT mice ($p < 0.001$ and $p < 0.05$, respectively), which could be due to the presence of lower H_2S levels in these TLR deficient mice.

For this reason, and in order to investigate the effects of the deficiency of TLR2 and TLR4 on the H_2S synthesis, we studied the gene expression of CBS and CSE and the levels of free sulfide in colon from *Tlr2*$^{-/-}$ and *Tlr4*$^{-/-}$ mice.

The expressions of CBS and CSE were significantly reduced in both *Tlr2*$^{-/-}$ and *Tlr4*$^{-/-}$ mice (Figure 4 E, F). Surprisingly, the levels of free sulfide were incremented in *Tlr4*$^{-/-}$ but unchanged in *Tlr2*$^{-/-}$mice (Figure 4G).

Sulphate-reducing bacteria and H_2S levels in feces from *Tlr2*$^{-/-}$ and *Tlr4*$^{-/-}$ mice

The high levels of H_2S in *Tlr4*$^{-/-}$ mice could come from external sources to the intestinal tissue itself. Therefore, we decided to analyze by quantitative PCR some of the sulphate-reducing bacteria (SRB) contained in the feces of *Tlr2*$^{-/-}$ and *Tlr4*$^{-/-}$ mice, as these bacteria may synthesize large amounts of H_2S and influence in the total H_2S levels present in the GI tract.

The determination of total bacteria DNA in the feces showed that the number of 16S rRNA gene copies was similar in *Tlr2*$^{-/-}$ and *Tlr4*$^{-/-}$ with respect to WT mice (Figure 5A).

Dissimilatory sulfite reductase (*dsrA*) gene copy number was quantified to determine the abundance of SRB in feces [24]. As it is shown in Figure 5B, mice with a deficiency in TLR2 showed a high reduction, while mice with a deficiency in TLR4 showed an increase in the *dsrA* gene copy number.

When we studied the expression of specific SRB in *Tlr2*$^{-/-}$ mice, we found a decrease in *Bilophila wadsworthia*, a depletion of *Desulfovibrio desulfuricans*, but a high increase in *Desulfovibrio piger* bacteria (Figure 5C-E). On the other hand, mice with a deficiency in TLR4 showed an increase in *D. desulfuricans* and *D. piger*, and no modification of *B. wadsworthia* bacteria (Figure 5C-E).

Finally, we determined the free H_2S levels in the feces of *Tlr2*$^{-/-}$ and *Tlr4*$^{-/-}$ mice. The H_2S levels were reduced in *Tlr2*$^{-/-}$ and unchanged in *Tlr4*$^{-/-}$ with respect to WT mice (Figure 5F).

Discussion

H_2S has been considered as a gaseous signalling molecule regulating several functions in the GI tract, including the regulation of the intestinal motility [25] . Here, we have shown that H_2S and innate immune system are interconnected, showing that H_2S increases TLR2 expression, TLR4 increases H_2S synthesis mediated by CSE enzyme, and that TLR2 and TLR4 modify the populations of SRB in the colon.

Previous studies have described that NaHS relaxes the smooth muscle of guinea-pig and rabbit ileum [26,27] and human, rat and mouse colon [11,25] . Our experiments *in vitro* and *in vivo* clearly demonstrate that NaHS, a rapid donor of H_2S, and GYY4137, a slow donor of H_2S, at physiologically relevant concentrations, inhibit the spontaneous motility of the circular smooth muscle in mouse colon, corroborating the inhibitory effect of H_2S on the intestinal motility.

The endogenous production of H_2S in the smooth muscle layer of rat and mouse colon is mainly through CBS and CSE enzymes [11,12]. Both CBS and CSE enzymes have been identified in enteric neurons and smooth muscle layers of colon [11-13], which are the main tissues involved in the intestinal motility. Our results demonstrate that the inhibition of CBS with AOAA or CSE with PAG increased the colonic motility, indicating that H_2S might be a molecule that could regulate intestinal motility. Similarly, other authors have reported that AOAA and PAG are able to cause a smooth muscle depolarization and increase the spontaneous motility in rat and human colon [11,28].

As far as we know, the cross-talk between the H_2S system and the bacteria-sensing receptors (TLR) in the gastrointestinal tract has not yet been explored. In this work, we first demonstrate that the treatment with the H_2S donors NaHS and GYY4137 increase the expression of TLR2 but not TLR4 in mouse colon. Interestingly, the release of H_2S maintained in time through the administration of the slow donor GYY4137 induced a higher expression of TLR2 than in the case of the rapid release of H_2S by NaHS. However, TLR2 activation with LTA did not modify the gene expression levels of CBS or CSE nor the free sulfide levels present in colon. These facts may explain that the responses induced by the CBS inhibitor AOAA and the CSE inhibitor PAG in the mice treated with LTA are similar to mice treated with saline.

Previous studies have shown interactions between H_2S and TLR4 but not TLR2. In this sense, NaHS has an additive effect on osteoclast differentiation through activation of the TLR4 but not the TLR2 pathway, in rat periodontal tissue [29]. H_2S treatment decreased the expression of TLR4 and reduced inflammation in a rat model of renal ischemia-reperfusion injury [30]. In fact, H_2S donors are able to reduce the infiltration of neutrophils and lymphocytes in several models and reduce the expression of many pro-inflammatory cytokines, most likely related to its ability to suppress the activation of

NF-κB, a factor regulated by TLR2/TLR4 signaling [31] .Also, H_2S supplementation inhibited TLR4 expression in a mouse model of mesangial cell overproliferation induced by high glucose [32]. These evidences suggest that H_2S is able to regulate TLR expression, although this regulation seems to be specific for the different tissues, species or models used.

Interestingly, and in relation to previous studies showing a relationship between H_2S and TLR4, activation with LPS induces an increase in CSE expression and the free sulfide levels in colon, resulting in an increase of the motor response induced by PAG. Other studies have shown that the activation of TLR4 by LPS increases the biosynthesis of CSE and H_2S in mouse macrophages through p38/MAPKs and NF-κB signaling [33]. On the contrary, LPS decreased CSE expression in human endothelial cells and blocked H_2S production in mouse aorta tissues [34]. All these findings indicate that the activation of TLR4 by LPS may modulate the synthesis of H_2S through the enzyme CSE. However, while a list of evidence of cellular regulation of H_2S enzymes have been studied in other systems, the cellular source of the gene regulation could not be identified in the current study. In addition, TLR receptor activation of enzyme regulation may in fact take place in different cells types, like neurons and smooth muscle cells, but also in immune and epithelial cells of the mucosa and submucosa. In these sense, it is well-known that mucosa-submucosa-muscle interactions might affect the muscle contractility.

On the other hand, the responses induced by the H_2S donors NaHS and GYY4137 were not modified in mice with TLR2 or TLR4 activated or in mice with a deficiency in TLR2 or TLR4, indicating that TLR2 or TLR4 seem not to be involved in the direct relaxant effect of H_2S on the intestinal smooth muscle. In fact, other authors have demonstrated that NaHS induces a smooth muscle hyperpolarization mediated by K

channels, particularly apamin-sensitive SK channels and glybenclamide-sensitive K (ATP) channels in human and rat colon [25].

In our study, the deficiency of TLR2 or TLR4 downregulates the gene expression levels of CBS and CSE present in colon. These facts may explain that the responses induced by the CBS inhibitor AOAA and the CSE inhibitor PAG in the *Tlr2*$^{-/-}$ and *Tlr4*$^{-/-}$ are also reduced in comparison with WT mice. Thus, our results indicate that the presence of TLR2 and TLR4 is necessary for maintaining the endogenous levels of H_2S synthesized by CBS and CSE that modulate the intestinal motility. However, although TLR receptors may be required to maintain endogenous H_2S enzymes, we can not determine exactly whether this regulation occurs at the cellular level or through multi-cellular signaling pathways, where different cell types can interact to obtain the final biological effect desired.

Surprisingly, the free sulfide levels found in the colon from *Tlr2*$^{-/-}$ or *Tlr4*$^{-/-}$ mice are not reduced, as it could be expected because of the downregulation of the CBS and CSE enzymes found in these mice. Several putative origins of H_2S in the large intestine have been described: enteric neurons and smooth muscle layers expressing CBS and CSE enzymes [11-13], blood or vascular tissues [35] and finally, the luminal bacteria [36]. H_2S produced by luminal bacteria has the potential to modify GI function and participates in motility disorders when intestinal microbiota is altered [37].

In the colon, H_2S can be free or bound to luminal contents. Faecal components have a large capacity for binding and catabolizing H_2S, thereby rendering it inactive. Therefore, total concentrations are not representative of the active luminal sulfide; free levels of H_2S have been reported between 0.2-1.1 nanomol mg^{-1} in mouse feces [38]. Then, we measured the free H_2S levels and the sulphate-reducing bacteria present in the feces of *Tlr2*$^{-/-}$ or *Tlr4*$^{-/-}$ mice, detecting levels of free H_2S about 0.6 nanomol mg^{-1} in

feces of WT mice and these levels were reduced in *Tlr2*$^{-/-}$ but not modified in *Tlr4*$^{-/-}$ mice.

Sulphate-reducing bacteria (SRB) can perform anaerobic respiration utilizing sulphate (SO_4^{2-}) as terminal electron acceptor, reducing it to H_2S. In our study, the amount of total bacteria was not modified in *Tlr2*$^{-/-}$ or *Tlr4*$^{-/-}$ compared to WT mice. In fact, it has been described that the impact of TLR deficiency on the composition of the intestinal microbiota is minimal under homeostatic conditions [39]. However, SRB abundance was strongly reduced in *Tlr2*$^{-/-}$ and increased in *Tlr4*$^{-/-}$ mice, indicating that TLR may be able to modulate the populations of SRB.

SRB include a large amount of bacteria of different genera [40]. In our work, we have focused in studying the levels of the sulphate-reducing bacteria *Desulfovibrio desulfuricans* and *Desulfovibrio piger*, since they are considered the most abundant SRB in human feces, and the sulphite-reducing bacterium *Bilophila wadsworthia*, which has also been isolated in these samples [41-43]. In our study, we demonstrate that effectively TLR2 and TLR4 modulate the levels of *D. piger*, *B. wadsworthia* and *D. desulfuricans*. In this way, alterations in the amount of bacteria of genus *Desulfovibrio* present in the cecal contents of TLR2 deficient mice, but not in TLR4 deficient mice have been reported previously [39].

Several authors have described alterations in faecal sulfide concentrations and activity of SRB in chronic intestinal motility disorders as inflammatory bowel disease (IBD) [38]. Within this context, recent studies have described in IBD different alterations of TLRs showing the critical importance of these innate immunity components in sensing bacteria to maintain intestinal homeostasis [44]. Our study demonstrates the interaction between the TLR and the sulfide system in the regulation of the colonic motility, an intestinal function altered in IBD patients. Thus, our study contributes to the

understanding of the pathophysiology of the motility disorders and suggest the modulation of the sulfide system as a possible therapeutic target.

In conclusion, our study demonstrates the interaction between TLR2 and TLR4 and the sulfide system in the regulation of colonic motility and contributes to the pathophysiology knowledge of motility disorders such as IBD.

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Conflict of Interest

The authors declare no conflict of interest.

Author contributions

LG designed the research study; LG, RF, LA, AP and SF performed the research and analyzed the data; LG, JEM, EL and EL wrote the paper.

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Tables

Table 1. Primers used for quantification of TLRs and H_2S synthesis enzymes in mouse colon by real-time PCR.

Gene	Reference	GenBank accession number	Sense and antisense primers
TLR2	[45]	NM_011905	GCCACCATTTCCACGGACT GGCTTCCTCTTGGCCTGG
TLR4	[45]	NM_021297	AGAAATTCCTGCAGTGGGTCA TCTCTACAGGTGTTGCACATGTCA
CBS	[46]	NM_001271353.1	CCAGGACTGTCGGGATGAAG TGAACCAGACGGAGCAAACA
CSE	[47]	NM_145953.2	GGGCCAGTCCTCGGGTTTTGAATA TAATCGTAATGGTGGCAGCAAGAC

Table 2. Primers used for quantification of bacteria by real-time PCR.

Species	Reference	Sense and antisense primers	Annealing T (° C)
Total bacteria	[48]	ACTCCTACGGGAGGCAGCAG ATTACCGCGGCTGCTGG	60
dsrA	[24]	ACSCACTGGAAGCACGGCGG GTGGMRCCGTGCAKRTTGG	60
Desulfovibrio desulfuricans	[42]	GGTACCTTCAAAGGAAGCAC GGGATTTCACCCCTGACTTA	60
Desulfovibrio piger	[49]	GCGGCGTGCTTAACACAT CCTCACGGTATCGCTGC	60
Bilophila wadsworthia	[50]	ACCCTGGTAGTCCACGCTGT TGAGTTCAGCCTTGCGACCG	60

Figures

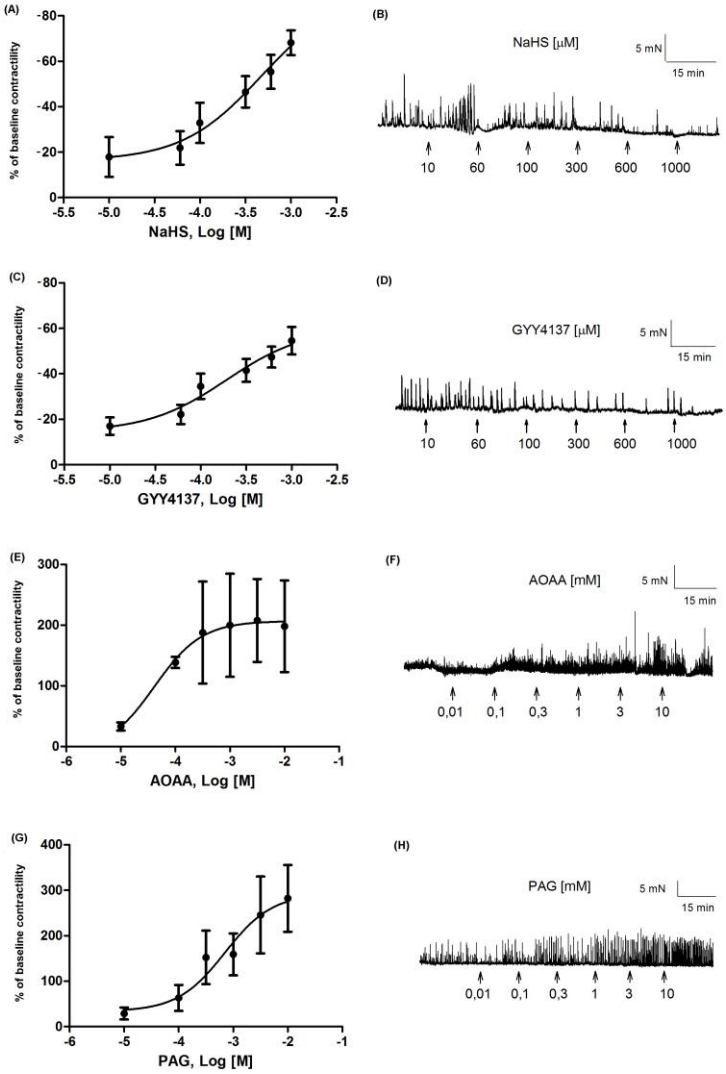

Figure 1 *In vitro* effect of H_2S donors and inhibitors of H_2S synthesis enzymes on spontaneous motility. Graphs and recordings of the spontaneous contractions of circular smooth muscle of mouse colon showing the effects of NaHS (10-1000 µM) (**A, B**), GYY4137 (10-1000 µM) (**C, D**) aminooxyacetic acid (AOAA, 0.01-10 mM) (**E, F**) and D-L Propargylglycine (PAG, 0.01-10 mM) (**G, H**). The results are the mean values of motor responses (AUC) to the different drugs, expressed as % of baseline contractility, and the vertical bars indicate SEM ($n \geq 4$ mice for each group). P<0.01 for AOAA; P<0.001 for NaHS, GYY4137 and PAG

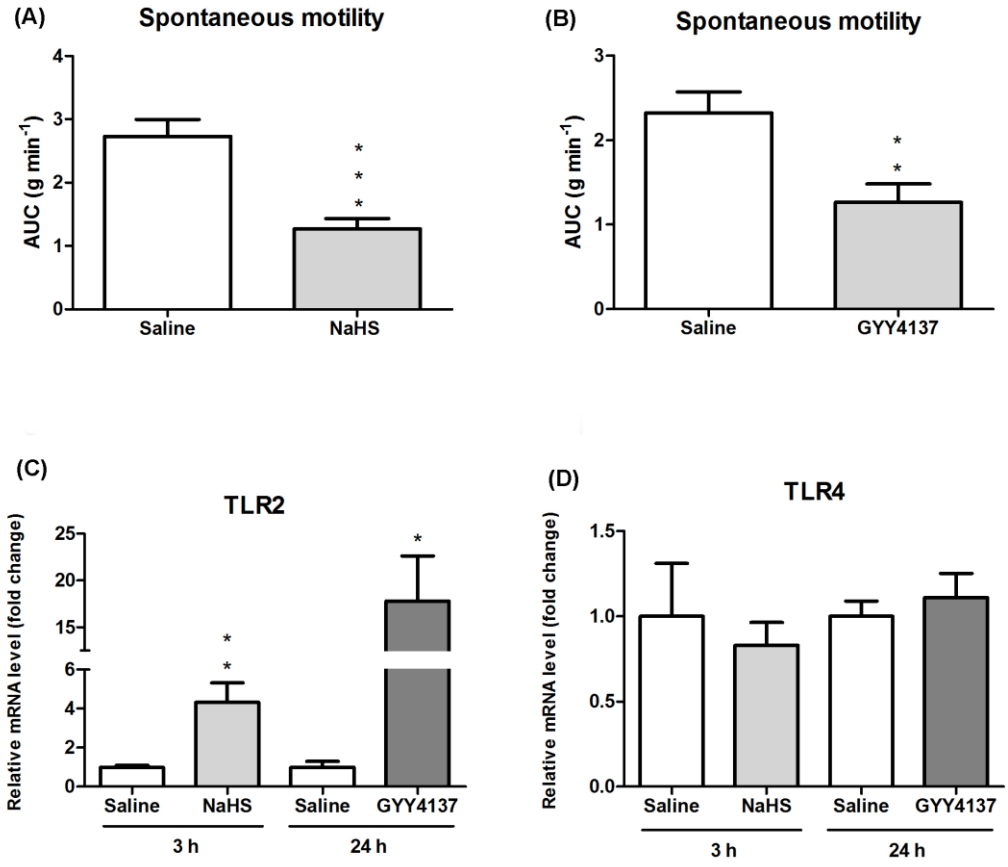

Figure 2 *In vivo* effect of H_2S donors on spontaneous motility and TLR gene expression. **A, B** Graphs showing the colonic spontaneous motility of mice treated with saline or NaHS 5 mg Kg^{-1} for 3 h or saline or GYY4137 5 mg Kg^{-1} for 24 h. The results are the mean values of spontaneous motility (AUC) expressed as $g \cdot min^{-1}$. **C, D** Real time-PCR analysis of TLR2 and TLR4 mRNA expression levels (fold change) in colon from mice treated with saline, NaHS or GYY4137. The results are the mean values. In all graphs the vertical bars indicate SEM ($n \geq 4$ mice for each group). *P<0.05; **P<0.01; ***P<0.001 vs. saline

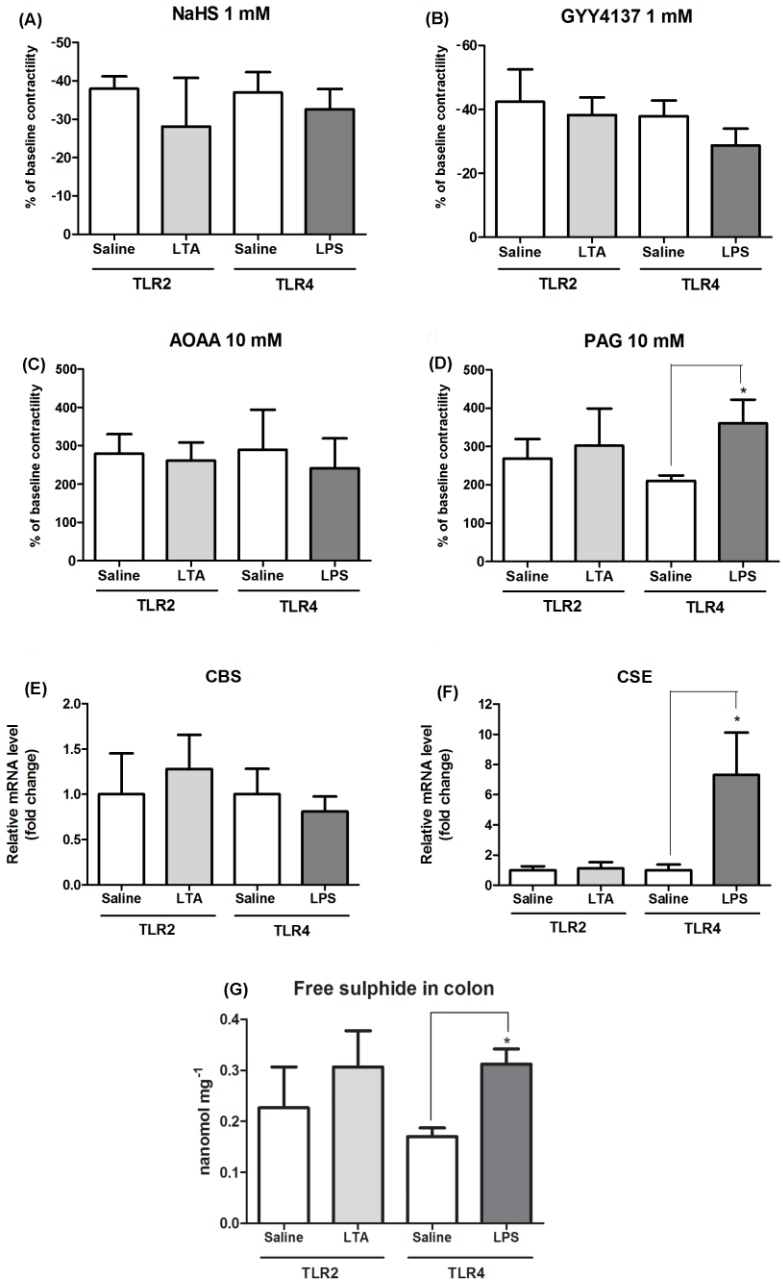

Figure 3 Effect of TLR2 and TLR4 activation on the H_2S system. Graphs showing the motor responses induced by NaHS (1 mM) (**A**) , GYY4137 (1 mM) (**B**), aminooxyacetic acid (AOAA, 10 mM) (**c**) and and D-L Propargylglycine (PAG, 10 mM) (**D**) in mice treated with saline or lipoteichoic acid (ligand of TLR2, LTA, 20 mg Kg^{-1}) for 3 h, or saline or lipopolysaccharide (ligand of TLR4, LPS, 5 mg Kg^{-1}) for 24 h. The results are the motor responses (AUC) to the different drugs, expressed as % of baseline contractility. **E, F** Real time-PCR analysis of CBS and CSE mRNA expression levels (fold change) in colon from mice treated with saline, LTA or LPS. **G** Free sulfide levels (nanomol mg^{-1}) in the colon from mice treated with saline, LTA or LPS. The results are the mean values and the vertical bars indicate SEM ($n \geq 5$ mice for each group). *P<0.05 vs. saline

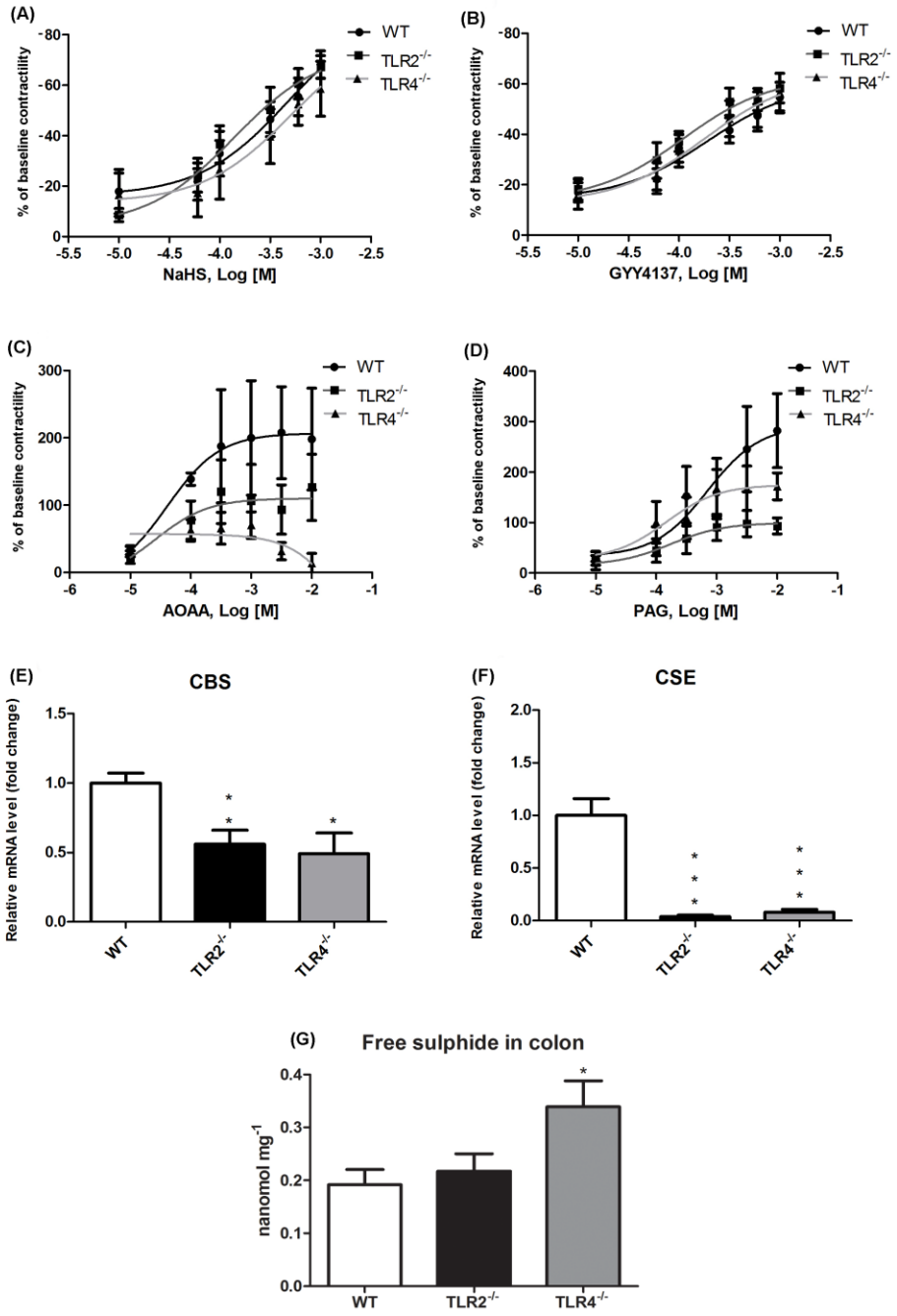

Figure 4 Effect of TLR2 and TLR4 deficiency on the H_2S system in colon. Graphs showing the motor responses induced by NaHS (10-1000 µM) (**A**), GYY4137 (10-1000 µM) (**B**), aminooxyacetic acid (AOAA, 0.01-10 mM) (**C**) and D-L Propargylglycine (PAG, 0.01-10 mM) (**D**) in WT, *Tlr2*$^{-/-}$ and *Tlr4*$^{-/-}$ mice. The results are the motor responses (AUC) to the different drugs, expressed as % of baseline contractility. **E, F** Real time-PCR analysis of CBS and CSE mRNA expression levels (fold change) in colon from WT, *Tlr2*$^{-/-}$ and *Tlr4*$^{-/-}$ mice. **G** Free sulfide levels (nanomol mg^{-1}) in the colon from WT, *Tlr2*$^{-/-}$ and *Tlr4*$^{-/-}$ mice. The results are the mean values and the vertical bars indicate SEM ($n \geq 4$ mice for each group). *P<0.05; **P<0.01; ***P<0.001 vs. WT

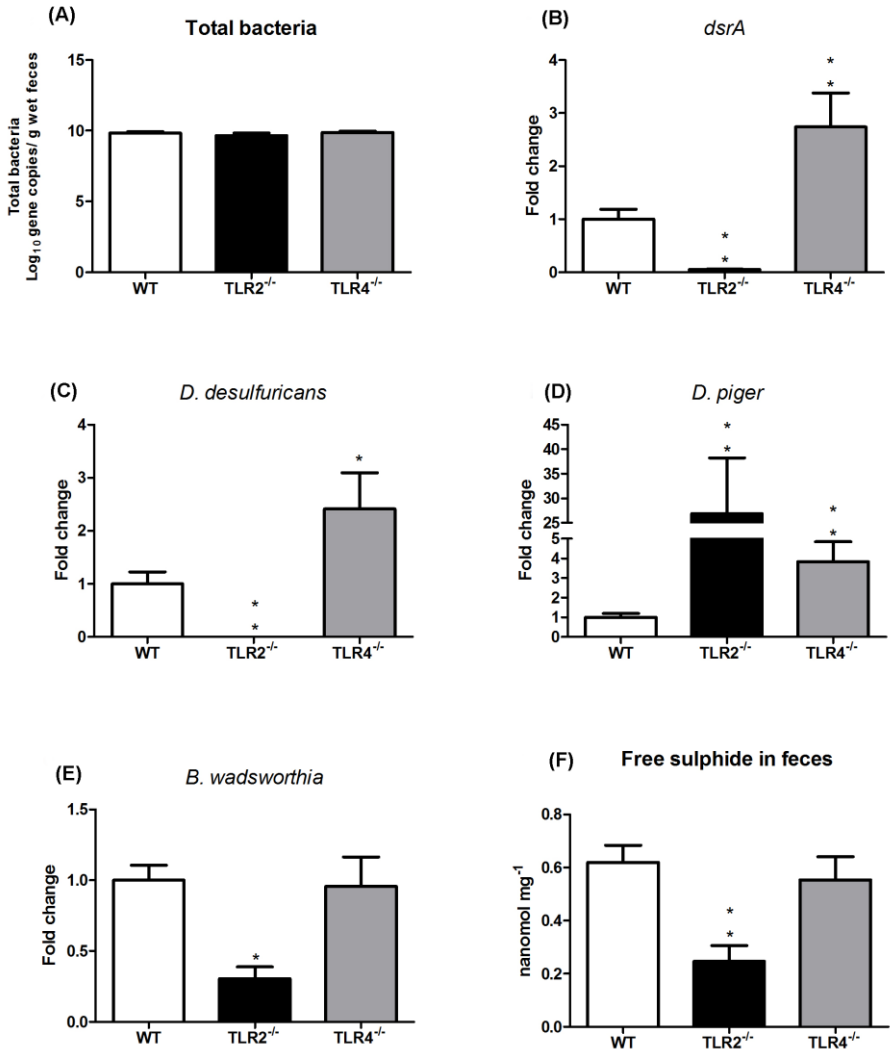

Figure 5 Effect of TLR2 and TLR4 deficiency on bacteria and H_2S levels in feces. Graphs showing the levels of total bacteria (**A**), dissimilatory sulfite reductase (*dsrA*) gene (**B**), and *Desulfovibrio desulfuricans* (**C**), *Desulfovibrio piger* (**D**) and *Bilophila wadsworthia* (**E**) bacteria in WT, *Tlr2*$^{-/-}$ and *Tlr4*$^{-/-}$ mice. The results are expressed as $\log_{10}$ 16S rRNA gene copies per gram of wet feces for total bacteria and as fold change for each specific bacteria. **F** Free sulfide levels (nanomol mg^{-1}) in the feces from WT, *Tlr2*$^{-/-}$ and *Tlr4*$^{-/-}$ mice. The results are the mean values and the vertical bars indicate SEM ($n \geq 6$ mice for each group). *P<0.05; **P<0.01 vs. WT