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TLR ligands and butyrate increase *Pyy* expression through two distinct but inter-regulated pathways

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Summary

The intestinal epithelium is an active barrier separating the host from its microbiota. It senses microbial compounds through expression of a wide range of receptors including the Toll-like receptors (TLRs). TLRs have been shown to regulate epithelium permeability or secretion of defensin by Paneth cells. However, the expression and function of TLRs in enteroendocrine L-cells, a specific subtype of intestinal cells secreting PYY and GLP-1, have not yet been assessed. PYY and GLP-1 are implicated in regulation of gut motility, food intake and insulin secretion, and are of great interest regarding obesity and type 2 diabetes. Using a cellular model of human L-cells and a reporter system for NF- κ B activation pathway, we reported functional expression of TLRs in these cells. Stimulation with specific TLR-agonists increased expression of *Pyy* but not *Proglucagon* in an NF- κ B-dependent manner. Moreover, the effect of TLR stimulation was additive to butyrate, a product of bacterial fermentation, on *Pyy* expression. Additionally, butyrate also increased *Tlr* expression, including *Tlr4*, and the NF- κ B response to TLR stimulation. Altogether, our results demonstrated a role of TLRs in the modulation of *Pyy* expression and the importance of butyrate, a product of bacterial fermentation in regulation of microbial TLR-dependent sensing.

Introduction

Enteroendocrine cells (EEC) are important cells of the intestinal epithelium that secrete hormones regulating a wide range of host functions including digestion, glucose and lipid metabolism and behaviour (Rehfeld, 1998; Karra and Batterham, 2010; Gunawardene *et al.*, 2011; Camilleri, 2015). Sensing of luminal content is mediated through expression of a great variety of receptors and transporters, including receptors that recognise fatty acids, peptones, bile acids as well as glucose and ion transporters, leading to hormone secretion (Cani *et al.*, 2007; Sternini *et al.*, 2008; Reimann *et al.*, 2012; Parker *et al.*, 2014). Individual EECs express specific pattern of hormones, even if recent finding showed an overlap in the expression of most of them (Gunawardene *et al.*, 2011; Habib *et al.*, 2012; Svendsen *et al.*, 2014). EECs that produce high quantity of GLP-1, GLP-2 and PYY are referred as L-cells and are of particular interest regarding the regulation of insulin secretion (Rondas *et al.*, 2013); intestinal permeability and differentiation (Janssen *et al.*, 2012; El-Jamal *et al.*, 2014); and food intake and intestinal motility (Tough *et al.*, 2011; De Silva *et al.*, 2011) respectively. EECs represent less than 1% of intestinal epithelial cells, limiting the access to enriched enteroendocrine sub-populations. Only a few cell lines issued from human adenocarcinoma, phenotypically corresponding to different enteroendocrine types, have been developed so far. NCI-h716 cells express specific L-cells differentiation factors and have been used as a human model to study GLP-1 secretion by L-cells in response to different luminal nutrients (Reimer, 2001; Hirasawa *et al.*, 2005; Jang *et al.*, 2007; Le Nevé and Daniel, 2011).

Intestine harbors the most important microbial community of the host, namely microbiota, which includes bacteria, archaea, yeasts and viruses, with the highest concentration in the colon. Among its many roles, the microbiota participates in the regulation of host immunity and in fiber digestion leading to the production of short chain fatty acids (SCFAs). Acetate, propionate and butyrate are the main SCFAs present in the colon lumen where they are used by the host as energetic substrate and as signalling molecules (Cummings, 1981; den Besten *et al.*, 2013). Part of the cross talk between microbiota and eukaryotic cells is mediated through recognition of conserved microbial associated molecular patterns (MAMPs) by a super-family of receptors called Pattern Recognition Receptors (Akira *et al.*, 2006). Among them, Toll-like receptors (TLR) have a major role in the

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innate immune responses, mainly by activating the well-described NF- κ B signalling pathway and inducing pro-inflammatory genes expression (Kawai and Akira, 2007). Expression of TLRs in intestinal epithelium cells is associated with the regulation of barrier functions and Paneth cell degranulation (Abreu, 2010; Santaolalla *et al.*, 2011; Rumio *et al.*, 2012; Graves *et al.*, 2014). Expression of TLR1, 2, 4, 5, 6 and 9 was reported in the mouse EEC line STC-1 (Palazzo *et al.*, 2007; Bogunovic *et al.*, 2007), and their stimulation increased secretion of cholecystokinin, a hormone regulating food intake secreted by duodenal enteroendocrine I-cells. TLRs are also expressed in enterochromaffin cells, a particular type of EEC, and their stimulation induced serotonin secretion (Bogunovic *et al.*, 2007; Kidd, 2009). L-cells are mainly present in the colon where microbial density and consequently concentration and diversity of MAMPs are the highest. Specific expression of TLRs in these cells has not been addressed to our knowledge. We hypothesised that TLRs may participate in the regulation of L-cells functions, including hormone production and

secretion. In this study, we reported the expression of TLRs in human NCI-h716 cell line and the impact of another bacterial product, butyrate, on their expression. We questioned their implication in the regulation of gut hormone production such as PYY, as they may participate in the cross talk between microbiota and host in the regulation of gut functions and food intake.

Results

NCI-h716 cells expressed functional Tlrs at the exception of Tlr4 and Tlr8

To decipher TLR expression in L-cells, we assessed by qRT-PCR their presence in a human cell line modelling L-cells, the NCI-h716 cells. We detected the expression of human *Tlr 1, 2, 3, 5, 6, 7* and *9* but not *Tlr4* nor *Tlr8* (Fig. 1A). *Tlr* expression was much lower than in THP-1 cells, a monocyte cell line, at the exception of TLR6, highly expressed in both cell lines and TLR3 and TLR7, receptors for virus RNA which are barely detectable in both cell lines

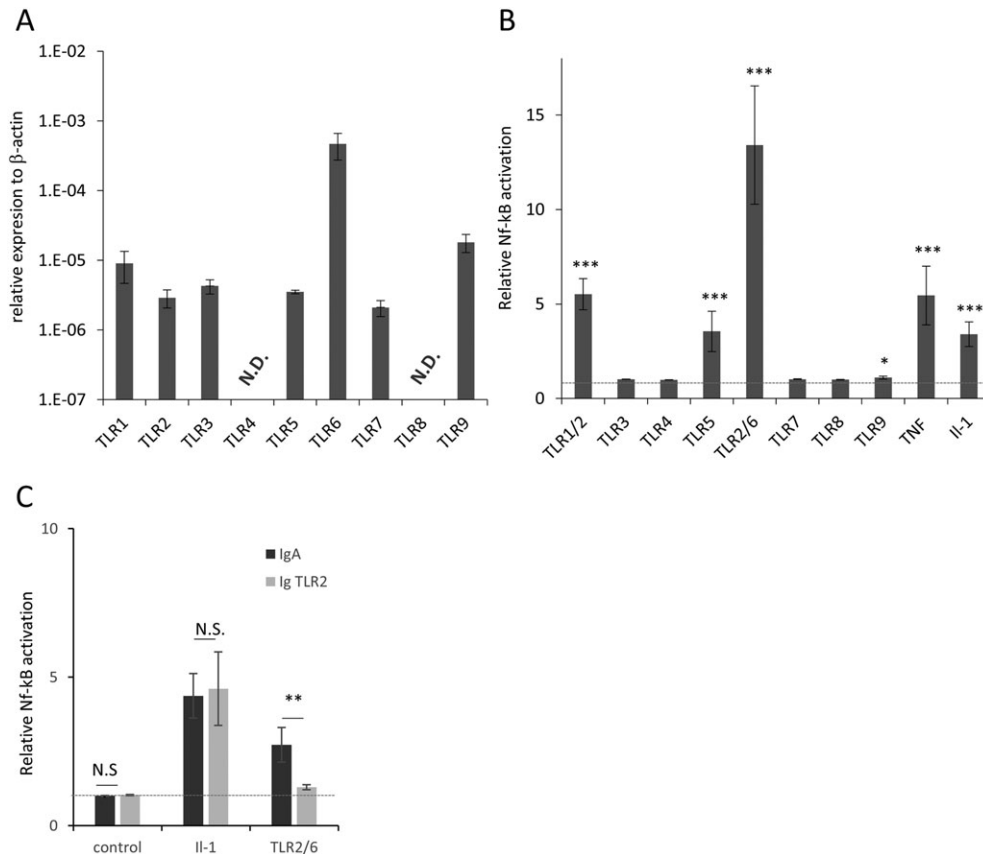


Fig. 1. NCI-h716 cells express functional TLRs

A. Expression of *Tlr 1–9* was detected by qRT-PCR in NCI-h716 cells. Data are represented as expression relative to β -actin determined by the $2^{-\Delta Ct}$ method, and expressed as means \pm sem on at least four distinct experiments performed in duplicate. N.D.: not detected

B. NF- κ B activation in NCI-h716 cells by TLR ligands relative to non-treated cells measured by quantification of secreted alkaline phosphatase (SEAP, OD 655 nm) after 24 h stimulation.

C. NF- κ B activation in NCI-h716 cells pre-incubated 1 h with blocking TLR2 antibody or the control isotype prior to TLR2/6 ligand or Il-1. Data are means \pm sem of at least three distinct experiments performed in duplicate.

(Fig. S1A). Interestingly, whereas TLR6 is supposed to function only in heterodimers with TLR2, its expression was much higher than *Tlr2* in NCI-h716 cells. TLR presence is not dependent on the cell line used as these receptors are expressed by another cell line modelling EECs, namely the HuTu-80 (Fig. S2A).

To test if these TLRs were functionally active in this cell line, we engineered NCI-h716 cells stably expressing a NF- κ B reporter system. Stimulation of most TLRs by their specific ligands induced a NF- κ B response in these cells without impacting cell viability (Fig. 1B and Fig. S1D). The highest responses were monitored with the specific ligands of the TLR2/6, and the TLR1/2 heterodimers and TLR5 (Fig. 1B). Moreover, TLR3, TLR7 and TLR9 stimulation barely activated NF- κ B response in NCI-h716, similarly to the results found in THP1 cells (Fig. S1B). TLR4 and TLR8 ligands had no effect on NF- κ B, in accordance to the lack of expression of these receptors in these cells. NCI-h716 cells also responded to Il-1 β and TNF- α by increasing NF- κ

B activity, as they express their receptors (Fig. S1C). We further confirmed that the TLR activation was responsible for the observed NF- κ B response. We pretreated cells with either an antibody antagonist of one TLR (TLR2) or its isotype control prior TLR2 stimulation. The anti-TLR2 blocking antibody specifically inhibited NF- κ B response to TLR2/6 ligand but not to Il-1 β (Fig. 1C). We could therefore demonstrate that NCI-h716 cells expressed functional TLRs that can respond to microbial motifs by inducing the NF- κ B pathway.

TLR agonists induced a NF- κ B-dependent expression of Pyy

Promoter gene analysis using Jaspar database (Mathelier *et al.*, 2014) revealed that *Pyy* promoter contains two putative NF- κ B binding domains in position -978 (GGGGTACCTCCC) and -833 (TGGGGTCCCCCA). Thus, we hypothesised that *Pyy* expression may be increased consequently to NF- κ B activation. By qRT-PCR, we showed that *Pyy* expression was increased by 80–100% after

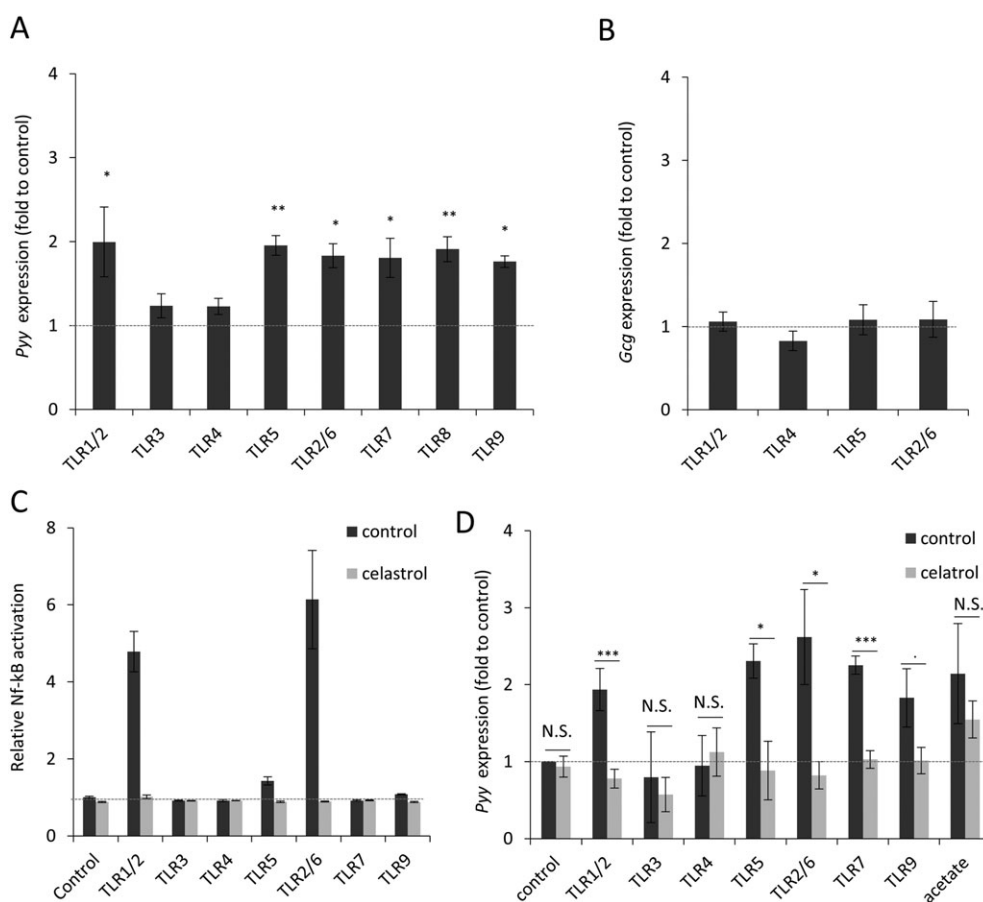


Fig. 2. TLR stimulation increased the expression of *Pyy* in a NF- κ B-dependent manner

A, B. *Pyy* (A) and *Proglucagon* (*Gcg*) (B) expression after stimulation with TLR ligands for 24 h.

C. Activation of NF- κ B in NCI-h716 cells by TLR agonists relative to non-treated cells measured by quantification of secreted alkaline phosphatase (SEAP, OD 655 nm) after 24 h stimulation in DMSO (control, dark grey) or celastrol 2 μ M (light grey) treated cells.

D. Relative expression of *Pyy* in control (dark grey) or celastrol (light grey) -treated NCI-h716 cells. Relative expression of genes is determined by the $2^{-\Delta\Delta Ct}$ method using β -actin expression for normalisation. Data are means \pm sem of at least three distinct experiments. (***: $p < 0.001$; **: $p < 0.01$; *: $p < 0.05$).

treatment with TLR agonists known to activate the NF- κ B pathway (Fig. 2A). Interestingly, TLR2/6 stimulation, which was one of the highest NF- κ B activators in these cells, induced a similar increased expression of *Pyy* as other TLR stimulations, indicating that the levels of NF- κ B activation and *Pyy* expression were not correlated. TLR3 and TLR4 activation, which did not induce detectable NF- κ B activation, had no effect on *Pyy* expression. Surprisingly, TLR7 and TLR8 agonists induced no detectable NF- κ B response in these cells but increased *Pyy* expression similarly as other TLR agonists. TLR ligands increased similarly *Pyy* gene expression in HuTu-80 cells (Fig. S2B). Interestingly, TLR stimulation did not increase expression of *proglucagon*, another gene coding for two hormonal peptides secreted by L-type EEC, which was anticipated as no NF- κ B binding site was detected in its promoter (Fig. 2B).

The NF- κ B inhibitor, celastrol, attenuated the TLR-dependent NF- κ B activation in the NF- κ B reporter cell-line without impacting the cell viability (Fig. 2C and Fig. S1D). Inhibition of NF- κ B signalling by this drug abolished the *Pyy* gene expression increased by all TLR agonists, including TLR7 and TLR8 agonists which did not have detectable effect on our NF- κ B reporter system (Fig. 2D). Moreover, this drug did not affect the NF- κ B independent increase of *Pyy* gene expression mediated by acetate. Thus, *Pyy* gene expression was increased by NF- κ B activation induced by TLR stimulation.

Butyrate and TLRs synergistically increased PYY expression

We showed that SCFAs had strong effect on *Pyy* expression (Fig. 3A and Larraufle et al., unpublished data). As SCFAs are present at high concentration in the lumen, we tested if

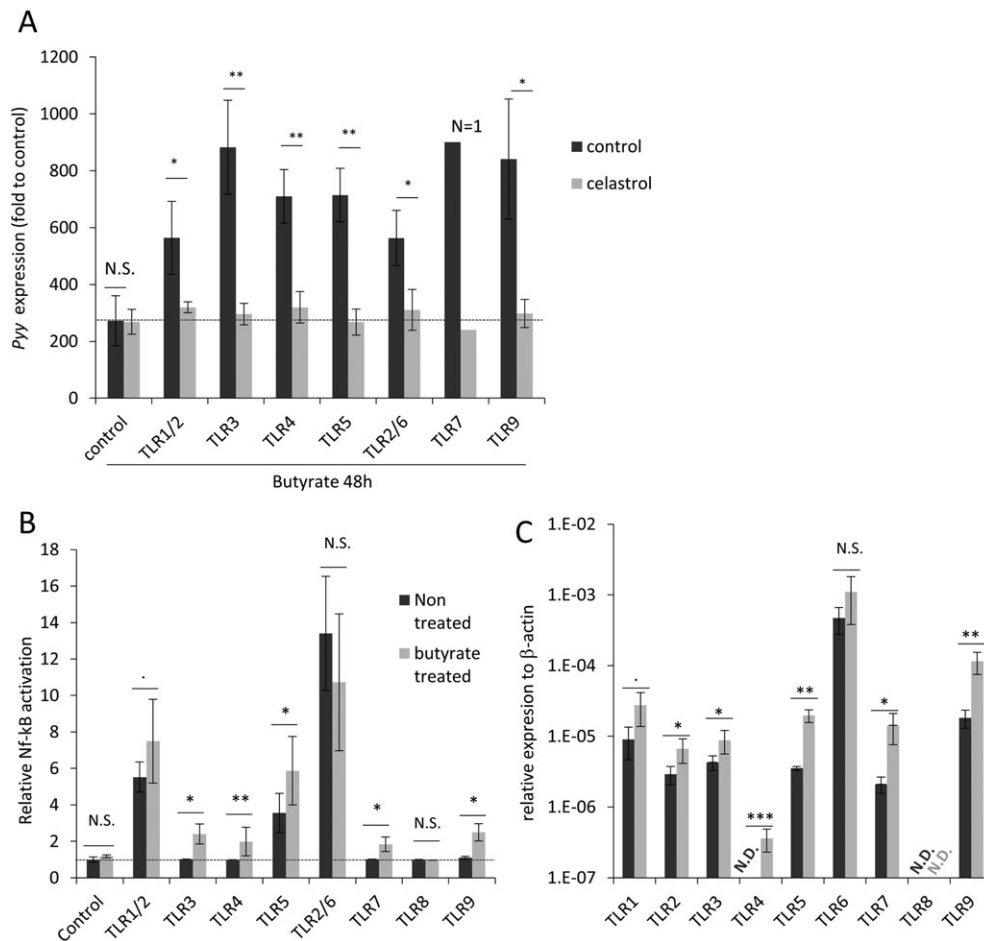


Fig. 3. Butyrate increased the TLR-dependent responses by increasing *Tlr* expression

A. Relative expression of *Pyy* in control (dark grey) or celastrol (light grey)-treated NCI-h716 cells after 48 h butyrate incubation and stimulation of the TLR for the last 24 h.

B. Activation of NF- κ B by TLR ligands in untreated (dark grey) or butyrate-stimulated (light grey) NCI-h716 cells measured by quantification of SEAP at OD655 nm. Results are expressed as fold increase relative to non-treated cells.

C. Expression of *Tlr* 1–9 was determined by qRT-PCR in untreated NCI-h716 (black bars) or stimulated with butyrate 2 mM for 24 h (grey bars). Data are normalised expression relative to β -actin determined by the $2^{-\Delta\Delta Ct}$ method, represented as means \pm sem on at least three distinct experiments, N.D.: not detected. (***: $p < 0.001$; **: $p < 0.01$; *: $p < 0.05$).

both TLR signalling and butyrate stimulation had additional effect on *Pyy* expression. Butyrate alone increased *Pyy* expression by a fold of about 300 after 48 h of stimulation, and addition of TLR agonist for the last 24 h of treatment with butyrate increased *Pyy* expression to a fold of about 500 compared to untreated cells (Fig. 3A). Thus, effect of TLR stimulation on *Pyy* expression was similar in treated and untreated cells, increasing mRNA levels by a fold of 1.8–2 in both conditions compared to control. Moreover, inhibition of the NF- κ B pathway by celastrol totally impaired the effect of stimulation of TLRs, but not of butyrate. Thus, the two stimulations occurred through different signalling pathways, both increasing *Pyy* expression additionally. Interestingly, stimulation by TLR3 or TLR4 agonists increased *Pyy* expression in butyrate-stimulated cells in an NF- κ B dependent manner whereas they had no effect on untreated cells (Fig. 2A). This indicated that butyrate might also regulate MAMP recognition.

Butyrate increased the expression of TLR and consequently the TLR-dependent NF- κ B activation

Using a NF- κ B reporter system, we showed that induction with TLR3 and TLR4 ligands significantly increased NF- κ B signalling only in butyrate treated cells. Moreover, butyrate stimulation also increased an NF- κ B response to all other TLR agonists excepted for TLR2/6 and TLR8 agonists, while butyrate alone had no effect on NF- κ B signalling. Butyrate also had a direct effect on *Tlr* expression, increasing mRNA levels of all of them except *Tlr6* and *Tlr8* (Fig. 3C). This higher expression of the receptors might participate in the increased NF- κ B response to TLR stimulation in butyrate-treated cells. Interestingly, only cells treated with butyrate expressed *Tlr4* and were thus sensitive to LPS. Similarly, butyrate, by increasing *Tlr3* expression, induced cell sensitivity to Poly(IC). These results explained the effect of LPS and Poly(IC) on *Pyy* expression found in butyrate treated cells but not in untreated cells.

Altogether, our results demonstrated that NCI-h716 cells, a model of colonic EEC L-cells, expressed functional TLRs except TLR8 and TLR4. TLR expression and consequently TLR-dependent responses are increased by butyrate, a compound usually found at high concentration in the lumen of the colon. We therefore concluded that butyrate and TLR stimulation synergistically increased *Pyy* expression which might mime what occurs in the physiological colon environment.

Discussion

PYY is a central hormone implicated in the regulation of food intake and gut motility, requiring a tight regulation of its expression. In this study, we demonstrated that PYY secreting cells, namely the enteroendocrine L-cells, sensed microbial molecules through the expression of a variety of

TLRs. *Tlr* expression was similar to intestinal epithelial cells and enterochromaffin cells except for *Tlr4* which could not be detected by qRT-PCR in untreated NCI-h716 cells (Fig. 1A and (Palazzo *et al.*, 2007; Bogunovic *et al.*, 2007; Graves *et al.*, 2014)). Moreover, we showed that L-cells are also stimulated by another bacterial product derived from fiber degradation, butyrate, present at high concentration in the colon. In addition, we showed a butyrate-dependent increase of TLR expression leading to an amplified cellular response to MAMPs and consequently a higher NF- κ B response. This indicates a potential process in which L-cells respond to microbial fermentation by increasing specifically MAMP receptor expression, increasing the sensing of the microbiota. In addition, we observed a butyrate-dependent *Tlr4* expression and a butyrate-dependent upregulation of other TLRs leading to a higher NF- κ B response to their respective ligands. We therefore proposed that butyrate might participate to the immune responses in L-cells by increasing microbial sensitivity via the upregulation of TLR expression. The impact of butyrate on *Tlr* expression in other intestinal epithelial cell types is unknown, but comparison between expression of *Tlrs* in small intestine and colon, where butyrate concentration is higher, shows that colonic epithelial cells expressed a broader range of *Tlrs* (Abreu, 2010). Small intestinal and colonic cells have different stem cell origin, but their environment and in particular the presence of high butyrate concentration might be a plausible hypothesis to explain differences in *Tlr* expression (Abreu, 2010). However, L-cells have been mainly studied for PYY and GLP-1 secretion, but their role in immunity is not known. Some evidences suggested a potential role of these hormones on the immune responses *via* the expression of hormone receptors in immune cells (Hogan *et al.*, 2011; Macia *et al.*, 2012; Yusta *et al.*, 2015). The scarcity of EECs in the intestinal epithelium makes difficult the study of the inflammatory response of these cells. However, in enterochromaffin cellular models, TLR stimulation induced an increase of expression of pro-inflammatory genes, suggesting that EEC might participate in immune response (Selleri *et al.*, 2008).

In our model in which cells were not polarised, we could not assess the TLR localisation precisely which would have given us clues of their role in these cells: to sense commensal microorganisms in the colon lumen or microorganisms that crossed the epithelial barrier and are potential threats for the host. Indeed, the two localisations have not the same role as the first one participate in the sensing and regulation of a commensal population which activates continuously the TLRs and should not induce a strong inflammatory response whereas the second participates in sensing of potential infection and therefore should induce an acute inflammatory response. Moreover, stimulation of the same TLR can induce different responses depending of its localisation (Lee *et al.*, 2006). *Ex vivo* studies are therefore required to

validate expression of TLRs in human L-cells all the long of the intestinal track. Moreover, functional and transcriptomic studies may decipher the precise role of expression of TLRs in these cells.

Promoter analysis revealed two potential NF- κ B binding domains in *Pyy* promoter but not in *proglucagon* promoter suggesting that TLR stimulation might regulate *Pyy* expression (Fig. 2A). Our results confirmed this hypothesis as *Pyy* but not *proglucagon* expression was increased after TLR stimulation in an NF- κ B-dependent manner. Interestingly, TLR3 and TLR4 ligands induced NF- κ B activation and consequently increased *Pyy* expression only in butyrate-treated cells (Fig. 3). However, TLR7 and TLR8 agonists did not induce any detectable activation of NF- κ B but increased *Pyy* expression. We cannot exclude that TLR7 and TLR8 ligands induced a weak NF- κ B activation, sufficient for the increase of *Pyy* expression that was below the detection threshold of our NF- κ B reporter system. Indeed, inhibition of NF- κ B pathway by celastrol prevented TLR7 and TLR8 ligand effects. The effect of TLR8 stimulation was surprising, as we could not detect any expression of *Tlr8* in these cells. However, human TLR8 and TLR7 recognise similar MAMPs, single strand RNAs (Heil *et al.*, 2004) and stimulation overlaps have been reported (Heil *et al.*, 2004). We therefore could not exclude that TLR8 ligand also activated with lower potency TLR7, and thus effects monitored might be mediated through TLR7.

Moreover, the increase of *Pyy* expression by TLR ligands was not dependent on the NF- κ B response, and only a very low NF- κ B response seemed to increase *Pyy* expression. We therefore hypothesised that *Pyy* expression may be regulated depending on a threshold of NF- κ B activation in a binary manner. Moreover, we showed that the TLR-dependent and butyrate-dependent activation of *Pyy* expression were synergistic, highlighting the important role of microbiota in increasing PYY production.

Interestingly, only *Pyy* but not *Proglucagon* expression was increased by MAMPs, indicating a potential unique feature of PYY as a sensor of microbiota among gut peptides. Butyrate also increased *Pyy* but not *proglucagon* expression (Larraufle *et al.*, submitted). Indeed, PYY is the hormone for which pattern of expression correlates to the density of microbiota (Adrian *et al.*, 1985). Moreover, its functions may also be important to indirectly regulate microbiota as it modulates food intake and small intestine and colonic motility revealing a potential important crosstalk between microbiota and host through this hormone (Ferrier *et al.*, 2000; Batterham and Bloom, 2003). Increased expression of *Pyy* during colonic infection because of TLR stimulation of L-cells could participate in host response by decreasing food intake and modulating intestinal motility, two important functions of PYY. PYY production has been reported to be increased after sepsis, in some infections, or in patients with inflammatory bowel disease (Adrian *et al.*, 1986; Higashiguchi *et al.*,

1994; Pfluger *et al.*, 2007; Kim *et al.*, 2010; El-Salhy *et al.*, 2012; Khosravi *et al.*, 2015). Anorexia associated to infection has been shown to be an important mechanism of host defence, and increased PYY circulating levels could participate in it (Murray and Murray, 1979; Exton, 1997). Another gut peptide also implicated in food intake control, namely CCK, can also be regulated during the inflammatory response to some parasitic nematode and induces hypophagia (Worthington *et al.*, 2013). Moreover, PYY has different roles on motility because of different receptor activation after peptide maturation. PYY matured form, PYY 3–36 decreases motility by inhibiting neuronal activity (Tough *et al.*, 2011) whereas PYY 1–36 increases motility directly increasing muscle contractions (Ferrier *et al.*, 2000; Ferrier *et al.*, 2002). Therefore, increasing PYY may lead to a local increased colonic motility, a physical way for expelling pathogens and limiting nutrient availability in the concerned area by impairing transit upstream, and therefore permitting increased nutrient absorption in non-pathogen infected area. Moreover, colitis has been associated with PYY plasma levels, and elevated PYY plasma levels may participate in deregulated intestinal motility observed in these pathologies. Therefore, we could hypothesise that PYY may be an actor of host response to colonic infection or inflammation as production is increased by TLR stimulation and potential other pathways.

Altogether, our results highlighted a role of TLR in EEC functions by regulating *Pyy* expression, which may be an important mechanism implicated in regulation of microbiota and in host defence during colonic infection. However, *in vivo* studies are required to confirm these *in vitro* results and precise the mechanisms implicated. A few studies described a role of TLRs in metabolism. Mice deficient for TLR5 had important microbiota modifications. Increased food intake was also found in these mice compared to wild type mice (Vijay-Kumar *et al.*, 2010). Implication of PYY was not assessed in this study, but it is possible that PYY plasma level decreased because of the lack of TLR5 induced *Pyy* expression. Modification of microbiota has also been associated with intestinal motility modifications, and stimulation of TLR4 and TLR5 inhibited ileal induced motility in a mechanism similar to PYY (Anitha *et al.*, 2012; Grasa *et al.*, 2015). Considering our results, it may be hypothesised that PYY regulates these functions by sensing gut microbiota and therefore modulates host responses. Interestingly, TLR-dependent responses were modified by another bacterial product, butyrate, indicating a relationship between different bacterial products.

Experimental procedures

Cell culture

NCI-h716 cell-line (a kind gift from C. Roche, Lyon, France) were cultivated in RPMI (Fischer Scientific) supplemented

with 10% SVF, 2 mM L-glutamine and 50 IU ml⁻¹ penicillin and 50 µg ml⁻¹ streptomycin in a humidified incubator at 37°C, 5% CO₂. Clones stably expressing the NF-κB reporter system pNifty2 SEAP (Invivogen) were selected and maintained in Zeocin (50 µg ml⁻¹, Invivogen).

TLR agonists, antibodies and cytokines

All TLR ligands were purchased from Invivogen and were used at recommended concentrations: Pam3CSK4 (TLR1/2): 500 ng ml⁻¹; Poly(I:C) (TLR3): 10 µg ml⁻¹; LPS (TLR4): 1 µg ml⁻¹; Ultra-Pure Flagellin (TLR5): 100 ng ml⁻¹; Pam2CSK4 (TLR2/6): 10 ng ml⁻¹; Imiquimod (TLR7): 1 µg ml⁻¹; CI075 (TLR8): 1 µg ml⁻¹, ODN2395 (TLR9): 5 µg ml⁻¹. Human recombinant Il-1β (10 ng ml⁻¹) and human recombinant TNFα (10 ng ml⁻¹) were purchased from Peprotech. Celatrol (Invivogen) was used as an NFκB inhibitor at 2 µM with 0.01% DMSO and was preincubated with cells 30 min prior TLR stimulation. The blocking antibody anti-TLR2 and its isotype control (IgA2, both from Invivogen) were used at 1 µg ml⁻¹, incubated 1 h prior TLR2/6 stimulation and for the remaining of the experiment.

RNA extraction and qRT-PCR

10⁶ NCI-h716 cells were seeded 48 h before lyse in 12 well plates. TLR agonists were added 24 h before RNA extraction if not indicated otherwise. RNA was extracted using a Qiagen RNeasy minikit with a DNase treatment (Qiagen) according to the manufacturer's instructions. Two micrograms of RNA was used for RT using High capacity cDNA Reverse transcription kit (Applied Biosystems). qPCR was performed on a AbiPrism 7000 system with Taqman gene expression assay probes for TLR1–9, Pyy, proglucagon, β-actin GAPDH, Il1rap, Il1r1, TNF1a, Tnf1b (resp Hs00413978_m1; Hs01872448_s1; Hs01551078_m1; Hs00152939_m1; Hs01920773_s1; Hs01039989_s1; Hs01933259_s1; Hs00152972_m1; Hs00370913_s1; Hs00373890_g1; Hs01031536_m1; Hs01060665_g1; Hs02758991_g1; Hs00895050_m1; Hs00991010_m1; Hs01042313_m1; Hs00153550_m1 (Lifetechnologies)), and Taqman gene expression master mix (Lifetechnologies). Data was analysed using 7000 System SDS software (Applied Biosystems) normalised to β-actin, and Student's t-test analysis was performed for statistical analysis.

NF-κB activity and cell viability

NCI-h716 pNiftySEAP2 cells were seeded at 5.10⁴ per well in 96 well-plates and incubated for 24 h in 100 µl RPMI with TLR agonist or control. Secreted alkaline phosphatase (SEAP) in supernatant was quantified by reaction with Quantiblue reagent (Invivogen) during 6 h incubation at 37°C by measuring OD 655 nm using a microplate reader (Infinite 200, Tecan) accordingly to the manufacturer's instructions. Data were normalised to non-treated cells for each experiment and analysed using Student's *t* test. Cell

viability was assayed using the CellTiter 96 Aqueouscell proliferation assay (Promega) following the manufacturer's instructions.

Author contribution

Conceived and designed the experiments: PL, JD, NL and HMB; performed the experiments: PL and NL; analysed the data: PL, NL and HMB; wrote the manuscript: PL, NL and HMB; reviewed the manuscript: JD, NL and HMB.

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Disclosures

The authors disclose no conflict of interest.

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Supporting information

Additional supporting information may be found in the online version of this article at the publisher's web-site:

Fig. S1. (A): Expression of *Tlr* 1–9 was detected by qRT-PCR in THP1 cells. Data are expression relative to β -actin determined by the $2^{-\Delta Ct}$ method, represented as means \pm sem on at least four distinct experiments performed in duplicate. N.D.: not detected (B): NF- κ B activation in THP1 cells by TLR ligands relative to non-treated cells measured by quantification of SEAP secreted after 24 h stimulation. (C): Expression of $Il1$ and $TNF-\alpha$ receptors was determined by qRT-PCR in NCI-h716 cells. (D): Normalised to control viability measured by MTS after lysis of 10^5 cells in 100μ l after different treatments. Data is mean \pm sem on four distinct experiments. Data are means \pm sem of at least three distinct experiments performed in duplicate.

Fig. S2. (A): Expression of *Tlr* 1–9 was determined by qRT-PCR in untreated HuTu-80 (black bars) or stimulated with butyrate 2 mM for 24 h (grey bars). Data are normalised expression relative to β -actin determined by the $2^{-\Delta Ct}$ method, represented as means \pm sem on at least three distinct experiments, N.D.: not detected. (***: $p < 0.001$; **: $p < 0.01$; *: $p < 0.05$). (B): *Pyy* expression after stimulation with some TLR ligands for 24 h in HuTu-80 ($n = 2$).