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Ligand-induced differential cross-regulation of Toll-like receptors 2, 4 and 5 in intestinal epithelial cells

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Abstract

Toll-like receptors (TLR) 2, TLR4 and TLR5 are primary mucosal sensors of microbial patterns. Dissection of the cross-talk between TLRs in intestinal cells has thus far been hampered by the lack of functional TLR2 and TLR4 in *in vitro* model systems. Here we report that the mouse intestinal epithelial cell line mIC_{cl2} expresses these TLRs and that receptor expression and function are regulated by environmental TLR stimuli. Our results show that stimulation of TLR5 by bacterial flagellin resulted in upregulated *TLR2* and *TLR4* mRNA and concomitant sensitization of the cells for subsequent TLR2 (Pam_3CSK_4) and TLR4 (LPS) stimulation. Exposure to low amounts of either Pam_3CSK_4 or LPS in turn downregulated *TLR5* mRNA and attenuated subsequent flagellin-mediated NF-κB activation, pointing to a negative feedback mechanism. Pam_3CSK_4 and LPS also downregulated *TLR4* mRNA but upregulated *TLR2* mRNA and sensitized cells for subsequent TLR2 stimulation. Inhibition of the phosphatidylinositol-3-kinase/Akt pathway only affected LPS-mediated TLR cross-talk indicating that differential TLR cross-regulation was conferred via different mechanisms. Together, our results demonstrate that the expression and function of TLR in intestinal cells are highly dynamic and tightly regulated in response to encountered bacterial stimuli.

Keywords: Toll-like receptor; Cross-regulation; Mucosa

1. Introduction

The intestinal epithelium forms a primary barrier for pathogenic microorganisms and plays a major role in the control of immune homeostasis. Adequate protection against bacterial pathogens is achieved via a sophisticated innate host defence machinery. Activation of this system results in the production of mucus and luminal secretion of antimicrobial peptides as well as in basolateral release of cytokines and chemokines that stimulate the recruitment of inflammatory cells and modulate immune cell function (Mumy and McCormick, 2005). The activation status of the intestinal defence system is thought to be tightly controlled by cellular receptors that sense conserved structural motifs of microbial origin. One major class of receptors that recognize these microbe-associated molecular patterns is the family of Toll-like receptors (TLRs).

The members of the TLR family share a common architecture that consists of an ectodomain that carries leucine-rich repeats (LRR), a transducing membrane segment, and a cytoplasmic Toll/IL-1R (TIR) signalling domain. Activation of TLRs by microbial stimuli results in most cases in the recruitment of the cytoplasmic adaptor proteins MyD88 and TIRAP (Mal) followed by the formation of a complex with IRAKs and TRAF6 that ultimately results in the activation and nuclear translocation of NF-κB (Akira and Takeda, 2004). This transcription factor controls the expression of genes involved in the modulation of the innate and adaptive host defence (Akira and Takeda, 2004). TLR signalling also activates several MAP kinases, such as p38, JNK and ERK1/2, which play a modulatory role in TLR-induced pro-inflammatory gene expression (Akira and Takeda, 2004; Symons et al., 2006). Several TLRs also (TLR4) or exclusively (TLR3) signal via a MyD88-independent route via the adaptors TRAM (TLR4) and TRIF (TLR3 and 4) resulting in activation of interferon-regulatory factor 3 that stimulates the production of IFNβ and the expression of IFN-inducible genes (Akira and Takeda, 2004).

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The human and mouse gut mucosa express several TLRs including TLR2, TLR4, and TLR5 (Abreu et al., 2005; Ortega-Cava et al., 2003, 2006). These TLRs play a key role in the recognition of microbe-associated molecular patterns from Gram-positive and Gram-negative bacterial species. TLR2 is activated by bacterial lipoproteins, lipoteichoic acid, and, in conjunction with TLR1 or TLR6, by diacylated or triacylated lipopeptides, respectively (Aliprantis et al., 1999; Schwandner et al., 1999). TLR4 can form a complex with MD-2 and CD14 and signals the presence of bacterial lipopolysaccharide (LPS) (Hoshino et al., 1999; Poltorak et al., 1998). TLR5 can sense flagellin, the major subunit of bacterial flagella (Andersen-Nissen et al., 2005; Hayashi et al., 2001). Under circumstances such as DSS-induced colitis, bacterial infection, or prolonged exposure to high concentration of ligand, expression of TLR2 and TLR4 is subject to change (Ortega-Cava et al., 2003; Totemeyer et al., 2003). In fact, activation of TLR2 or TLR4 through commensal bacteria turns out to be a prerequisite for the protection against epithelial damage (Rakoff-Nahoum et al., 2004). For macrophages and endothelial cells, cross-talk between TLR2 and TLR4 resulting in alterations in TLR expression and/or function has been demonstrated (Fan et al., 2003; Matsuguchi et al., 2000; Nilsen et al., 2004; Totemeyer et al., 2003). Knowledge of the factors that control TLR expression and function in intestinal epithelial cells is still in its infancy but is crucial to facilitate future targeted modulation or restoration of intestinal homeostasis.

Understanding of the intestinal (cross) regulation of TLR2, TLR4 and TLR5 may have thus far been hampered by the fact that the most commonly used models of intestinal epithelial cells such as Caco-2, HT-29, and T84 cell lines, only respond to flagellin (Abreu et al., 2005). A novel epithelial (mIC_{cl2}) cell line derived from intestinal mouse crypts recently has been shown to respond to LPS (Bens et al., 1996; Hornef et al., 2002, 2003). mIC_{cl2} Cells form a tight monolayer separated by tight-junctions with a distinct brush-border (apical) and basolateral membrane compartment and have retained specific epithelial features such as dome formation, membrane-associated enzyme activities and expression of pIgRs (Bens et al., 1996). In the present study, we demonstrate that mIC_{cl2} cells carry a functional TLR2, TLR4 and TLR5 and exploited this system to investigate bacterial ligand driven cross-regulation of the TLR response in intestinal epithelial cells. Our results indicate the existence of several positive and negative feed back loops that control TLR receptor expression and TLR-mediated responses. The differential cross-regulation of TLRs was found to be conferred via different, phosphatidyl-inositol-3-kinase (PI3K)/Akt-sensitive (TLR4) and -insensitive (TLR2/TLR5) pathways.

2. Materials and methods

2.1. Reagents

The full-length *fliC* gene encoding flagellin from *S. enteriditis* strain 706 extended with a carboxy-terminal His-tag was cloned in front of an IPTG-inducible promoter in pT7.7 and expressed in *E. coli* BL21(DE3). Cells expressing His-tagged flagellin were lysed in 8 M urea, 100 mM NaH_2PO_4-Tris (pH 8.0) and subjected to nickel-affinity chromatography (Qiagen, Venlo, The Netherlands). Fractions were eluted from the column with 8 M urea, 100 mM NaH_2PO_4-Tris (pH 4.5) and analyzed on SDS-PAGE. Fractions with highest flagellin purity were extensively dialysed against PBS and subsequently stored in aliquots at −80 °C. For certain experiments, HPLC purified flagellin was used for TLR stimulation with similar results. LPS of *S. enteriditis* was isolated according to the hot phenol method (Westphal and Jann, 1965). Similar results were obtained with LPS repurified using the deoxycholate-phenol method (Hirschfeld et al., 2000). The synthetic lipoprotein Pam_3Cys-Ser-$(Lys)_4$ (Pam_3CSK_4) was purchased from Invivogen (Toulouse, France).

2.2. Cell cultures

The mouse intestinal epithelial cell line mIC_{cl2} (Bens et al., 1996; Hornef et al., 2002), stably transfected with an NF-κB luciferase reporter construct (Hornef et al., 2003), was cultured in DMEM/Ham's F-12 (1:l, v/v), 60 nM sodium selenite, 5 μg/ml bovine transferrin, 2 mM glutamine, 50 nM dexamethasone, 1 nM triiodothyronine, 5 μg/ml bovine insulin, 20 mM D-glucose, 10 nM mouse epidermal growth factor, 2% FCS, and 20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) (pH 7.4). Propagation of the mIC_{cl2} cell line in IMDM with 10% FCS for several passages to exclude possible effects of the additives present in the complex medium, yielded no noticeable differences in TLR function.

The following cell lines were routinely grown in 25 cm^2 tissue culture flasks in 5 ml of DMEM with 10% fetal calf serum (FCS): human embryonic kidney (HEK293) cells, the murine macrophage cell lines J774.1, C3H/HeJ (TLR4-deficient) and C3H/HeJ stably transfected with mTLR4 (Poltorak et al., 2000), and HeLa cells (clone 57A) stably transfected with an NF-κB luciferase reporter construct (Rodriguez et al., 1999).

Dendritic cells derived from murine bone marrow were generated as described (Lutz et al., 1999). Briefly, bone marrow cells harvested from tibiae and femurs of mice were cultured in 100 mm diameter suspension culture dishes (Corning Life Sciences, Schiphol-Rijk, The Netherlands) in 10 ml IMDM with 10% FCS, 50 μM 2-mercaptoethanol and penicillin–streptomycin (Invitrogen, Breda, The Netherlands), supplemented with 20 ng/ml granulocyte macrophage-colony stimulating factor (GM-CSF; Cytogen Research & Development Inc., West Roxbury, US). On day 2, 10 ml of culture medium containing 20 ng/ml GM-CSF was added to the dishes followed by another 200 ng GM-CSF on day 4. On day 8, non-adherent and loosely adherent cells were harvested, washed twice with PBS, and used for subsequent experiments. All cell lines were maintained at 37 °C in a 5% CO_2-95% air atmosphere.

2.3. cDNA Constructs

Full-length cDNAs encoding hTLR1 (U88540) and hTLR2 (BC033756) were amplified from HEK293 cells derived chromosomal DNA. Mouse mCD14 (NM_009841) and

Table 1
Real time RT-PCR primers

Product	Forward	Reverse
mTLR5	5′-TTCGCACGGCTTTATCTTCTC-3′	5′-GGCAAGGTTCAGCATCTTCAA-3′
mTLR4	5′-TGACAGGAAACCCTATCCAGAGTT-3′	5′-TCTCCACAGCCACCAGATTCT-3′
mTLR2	5′-TGTCTCCACAAGCGGGACTT-3′	5′-TTCGATGGAATCGATGATGTTG-3′
KC	5′-CAAGAACATCCAGAGCTTGAAGGT-3′	5′-GTGGCTATGACTTCGGTTTGG-3′
MIP-2	5′-GGGCGGTCAAAAAGTTTGC-3′	5′-TGTTCAGTATCTTTTGGATGATTTTCTG-3′
mTNFα	5′-GTACCTTGTCTACTCCCAGGTTCTCT-3′	5′-GTGTGGGTGAGGAGCACGTA-3′
mIP-10	5′-GACGGTCCGCTGCAACTG-3′	5′-CTTCCCTATGGCCCTCATTCT-3′
β-Actin	5′-CTGGCCTCACTGTCCACCTT-3′	5′-GGGCCGGACTCATCGTACT-3′

mTLR4 (NM_021297) were amplified from C3H/HeJ-mTLR4 macrophage DNA and mTLR5 (AF186107) was amplified from J774.1 macrophage DNA. Chromosomal DNA was isolated using the DNeasy Tissue kit (Qiagen, Venlo, The Netherlands). Genes were amplified in polymerase chain reactions (PCR) with primer sets flanked by the cDNA start and stop codon using DNA polymerase Pfu (Promega) and obtained PCR products were purified and cloned into pGEM-T-Easy (pGTE, Promega). For functional expression, human and mouse cDNAs were cloned into pTracer-CMV2 (Invitrogen, Breda, The Netherlands), except for mTLR5 which was cloned in pDR2EF1α (Charreau et al., 1994). A dominant-negative mTLR5 (mTLR5ΔTIR) was obtained by replacing a *Bam*HI-blunted *Not*I fragment (nucl. 128-2579) of pDR2EF1α-mTLR5 with a *Bam*HI-blunted *Tth*111I fragment (nucl. 128–2047) of pGTE-mTLR5. This resulted in deletion of the carboxy-terminal part of the molecule starting at amino acid 682. The expression construct pUNO-mMD2 was obtained from Invivogen (Toulouse, France). The vector pTK-LacZ was kindly provided by Dr. Bart van der Burg (Hubrecht Laboratory, Utrecht, The Netherlands).

2.4. Transfections

HeLa 57A cells were plated in a 24-well plate the day prior to transfection to reach a confluency of ∼90%. Cells were transfected with a total of 0.4 μg of various expression constructs plus 0.1 μg of pTK-LacZ using Fugene according to the instructions of the manufacturer (Roche-Diagnostics, Almere, The Netherlands). Empty pTracer-CMV2 was used for mock transfections. mIC_{cl2} Cells were transfected with 0.8 μg of pDR2EF1α-mTLR5ΔTIR and 0.2 μg of pTK-LacZ using Exgen500 (Fermentas, Germany). *In situ* staining for β-galactosidase activity revealed a mIC_{cl2} transfection efficiency of ∼50%.

2.5. Reporter gene assay

mIC_{cl2} Cells were grown in 24-well plates until 4–5 days post-confluence with refreshing the culture medium every 2 days. HeLa 57A cells were used 24 h post-transfection. After stimulation with TLR agonists, mIC_{cl2} and HeLa 57A cells were washed with PBS, lysed in 200 μl of Reporter Lysis buffer (Promega), and stored at −80 °C for 24 h. After centrifugation of the cell lysate, luciferase activity in 20 μl of supernatant was analyzed using 100 μl of Promega Luciferase Assay Reagent in a luminometer (TD-20/20, Turner Designs, Sunnyvale, US). Luciferase activity in transfected HeLa 57A was corrected for β-galactosidase activity (Promega). Data are expressed as mean ± S.D. values of one representative of at least three separate experiments. Statistical analysis of results was performed with a one-way ANOVA test with a Bonferroni post-test.

2.6. Real-time RT-PCR

Total RNA was extracted from mIC_{cl2}, BMDCs, C3H/HeJ and C3H/HeJ-mTLR4 cells with RNA-Bee (Tel-Test Inc.). Samples were diluted to exactly 1 μg/μl and treated with RNase-free DNase I (Invitrogen). Primer Express software (Applied Biosystems) was used to design forward and reverse primer sets to amplify 50–80 bp fragments of mTLR5, mTLR4, mTLR2, MIP-2, KC, TNFα, IP-10 or mouse β-actin (Table 1). RT-PCR was performed on 0.1 μg of DNase I-treated RNA with 1 μM of primers using ABI Prism 7000 (Applied Biosystems) and the Sybr Green RT-PCR kit (Eurogentec, Maastricht, The Netherlands). Real-time cycler conditions were 30 min at 48 °C, followed by 15 min at 95 °C, and then for 40 cycles at 95 °C for 15 s, 50 °C for 30 s, and 72 °C for 30 s. Threshold cycles (C_t) were calculated using ABI Prism 7000 SDS software (Version 1.1). The mRNA level of the target gene corrected to that of the housekeeping gene β-actin was calculated by subtracting their corresponding C_t before (1) and after (2) treatment using the formula: (1) $\Delta C_{t\ \text{control}} = C_{t\ \text{target gene control}} - C_{t\ \beta\text{-actin control}}$, (2) $\Delta C_{t\ \text{treated}} = C_{t\ \text{target gene treated}} - C_{t\ \beta\text{-actin treated}}$. The fold change in mRNA was determined by: Fold change = $2^{[\Delta C_t(\text{treated}) - \Delta C_t(\text{control})]}$. RNA was isolated from two independently performed experiments and each batch of RNA was analyzed twice by real time RT-PCR. Data shown are from one representative experiment.

3. Results

3.1. Specificity of the TLR agonists

Assessment of the cross-regulation of TLR function requires the use of highly pure ligands. The unique TLR stimulating activity of the compounds used in the present study was verified in multiple ways. The synthetic triacyl lipopeptide Pam_3CSK_4, purified *S. enteritidis* LPS, and recombinant *S. enteritidis* flagellin stimulated HeLa 57A cells only when transfected with their cognate receptor TLR2/TLR1, TLR4/CD14/MD2, or TLR5,

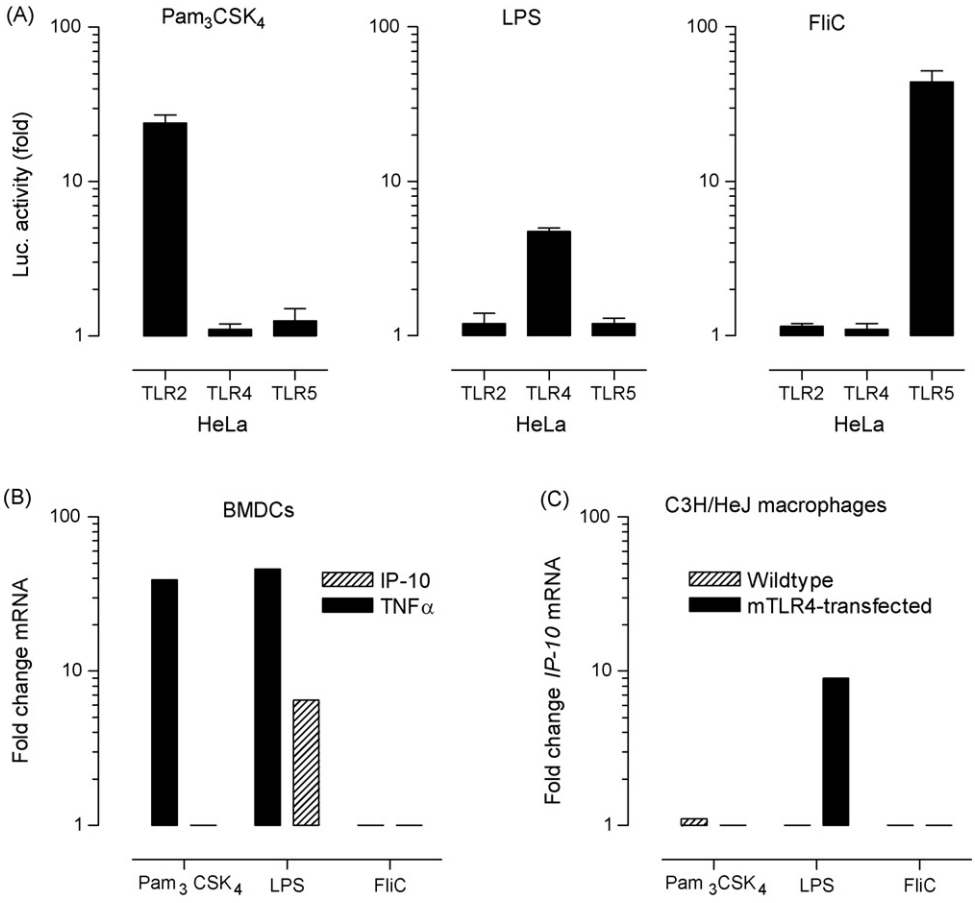

Fig. 1. Purity and specificity of TLR ligands (A) HeLa cells stably transfected with an NF-κB-luciferase reporter construct (clone 57A) were transfected with expression constructs encoding human TLR2 and TLR1, mouse TLR4 together with CD14 and MD2, or mouse TLR5. Twenty-four hours post-transfection, cells were stimulated (5 h) with 300 ng/ml Pam_3CSK_4, 10 ng/ml LPS or 2000 ng/ml flagellin, lysed, and analyzed for luciferase activity. Data (means ± S.D.) are a representative of at least three separate experiments and shown as fold difference compared to vector-transfected cells. (B) Bone marrow-derived dendritic cells (BMDCs) were stimulated (5 h) with 300 ng/ml Pam_3CSK_4, 10 ng/ml LPS, or 2000 ng/ml flagellin and analyzed for expression of *TNF α* and *IP-10* mRNA by real-time RT-PCR. (C) Wild-type C3H/HeJ (TLR4-deficient) and mTLR4 stably transfected C3H/HeJ macrophages were stimulated as in (B) and analyzed for expression of *IP-10* mRNA by real-time RT-PCR. Data are from one representative of four assays and shown as fold change in mRNA levels after treatment compared to non-treated cells.

respectively (Fig. 1A). Second, the lack of possible bacterial contaminants in flagellin was confirmed using bone marrow-derived dendritic cells (BMDCs) that carry functional TLR2, TLR3, TLR4 and TLR9, but lack TLR5 (Datta et al., 2003; Means et al., 2003). In these cells, purified flagellin yielded no response, while Pam_3CSK_4 and LPS expectedly induced *TNFα* mRNA (Fig. 1B). Third, the presence of possible LPS contaminants in Pam_3CSK_4 or flagellin was determined by investigation of the IP-10 (CXCL10) response, which is activated through the MyD88-independent signalling pathway of TLR4. Induction of *IP-10* mRNA was exclusively mediated through LPS in both BMDCs (Fig. 1B) and mTLR4-transfected C3H/HeJ macrophages (Fig. 1C). This confirms that Pam_3CSK_4 and flagellin lack TLR4 stimulating activity.

3.2. Functional analysis of TLR2, TLR4, and TLR5 in mIC_{cl2} cells

To investigate cross-regulation of TLR2, TLR4, and TLR5 in intestinal epithelial cells, the model cell lines Caco-2, T84, and HT-29 are unsuitable because they lack functional TLR2 and TLR4 (Abreu et al., 2005). The mouse intestinal epithelial cell line mIC_{cl2} (Bens et al., 1996) has been shown to express functional TLR4 (Hornef et al., 2002, 2003). Real-time RT-PCR demonstrated that mIC_{cl2} cells also express TLR2 and TLR5 (Table 1). Unlike Caco-2 cells (Lee et al., 2006), mIC_{cl2} cells do not express TLR9 (not shown). Expression of *TLR4* and *TLR5* mRNA in mIC_{cl2} cells is comparable to BMDCs and C3H/HeJ-mTLR4 macrophages, whereas expression of *TLR2* mRNA is 10-fold lower (Table 2).

Exposure of mIC_{cl2} cells to various concentrations of Pam_3CSK_4 for 3 h caused a small but consistent activation of NF-κB, indicating the presence of functional TLR2 (Fig. 2A). Assessment of TLR5 function by 3 h of exposure to recombinant flagellin yielded virtually no NF-κB response at 300 ng/ml, but a potent activation at 600 and 2000 ng/ml of flagellin (Fig. 2A). These concentrations of flagellin are within the physiological range as during systemic infection serum flagellin levels can reach concentrations of up to 1.5 μg/ml within 8 h of infection (Eaves-Pyles et al., 2001). Transfection of a dominant-negative

Table 2
Relative basal TLR mRNA levels in mIC_{cl2} cells compared to BMDCs and C3H/HeJ macrophages

		Fold[a]			
		β-Actin	TLR2	TLR4	TLR5
mIC_{cl2}		1	1	1	1
BMDCs		1	11.3	0.9	0.5[c]
C3H/HeJ	Wild-type	1	12.1	3.9[b]	0.03[c]
	+ mTLR4	1	12.8	2.2	1.9

[a] Fold = $2^{-\Delta\Delta C_t}$ ($\Delta\Delta C_t$ is the difference in TLR C_t value between mIC_{cl2} and BMDCs or C3H/HeJ cells). *Note*: The RT-PCR only allows comparison of the relative expression level of each TLR between different cell types but not of the relative expression of TLRs within a single cell type.

[b] TLR4 in wild-type C3H/HeJ cells is non-functional (Poltorak et al., 1998), but this defect is restored by stable transfection with a functional mouse *TLR4* cDNA (Poltorak et al., 2000).

[c] TLR5 in BMDCs and C3H/HeJ cells is non-functional.

mTLR5 construct into mIC_{cl2} cells (50% transfection efficiency), reduced NF-κB activation by 40–50% in the presence of flagellin but not TNFα (not shown). The addition of LPS (1–10 ng/ml) to mIC_{cl2} cells also caused a dose-dependent NF-κB activation (Fig. 2A), in agreement with earlier observations (Hornef et al., 2002, 2003). To ensure the relevance of the results obtained with the NF-κB luciferase reporter system, we analyzed the effect of TLR activation on the expression of the chemokines KC (CXCL1) and MIP-2 (CXCL2). KC and MIP-2 are mouse orthologs of human IL-8 (Bozic et al., 1994), which is one of the mainly secreted chemokines by epithelial cells upon inflammation (Rimoldi et al., 2005). Similar to stimulation of TLR4 (Hornef et al., 2002), activation of TLR2 (Fig. 2B) and TLR5 (Fig. 2C) induced *MIP2* and *KC* mRNA expression in mIC_{cl2} cells in a time-dependent fashion.

3.3. TLR5 activation positively regulates TLR2 and TLR4

To investigate the potential regulatory effects of TLR5 activation, mIC_{cl2} cells were exposed to flagellin (300 ng/ml) for up to 8 h and TLR mRNA levels were determined at regular intervals using real-time RT-PCR. Flagellin induced a persistent upregulation of *TLR2* mRNA transcript (Fig. 3A). Stimulation with flagellin transiently increased *TLR4* mRNA levels with peak levels at approximately 4 h of stimulation (Fig. 3B).

Attempts to verify the flagellin-induced upregulation of *TLR2* and *TLR4* mRNA at the protein level via immunoblot analysis yielded no consistent results due to the low amounts of receptor protein. Therefore, we fully focused on the more important functional consequences of the cross-regulatory effects of flagellin. Hereto, cells were primed with flagellin (or buffer alone) for 3 h and subsequently stimulated with Pam_3CSK_4 or LPS.

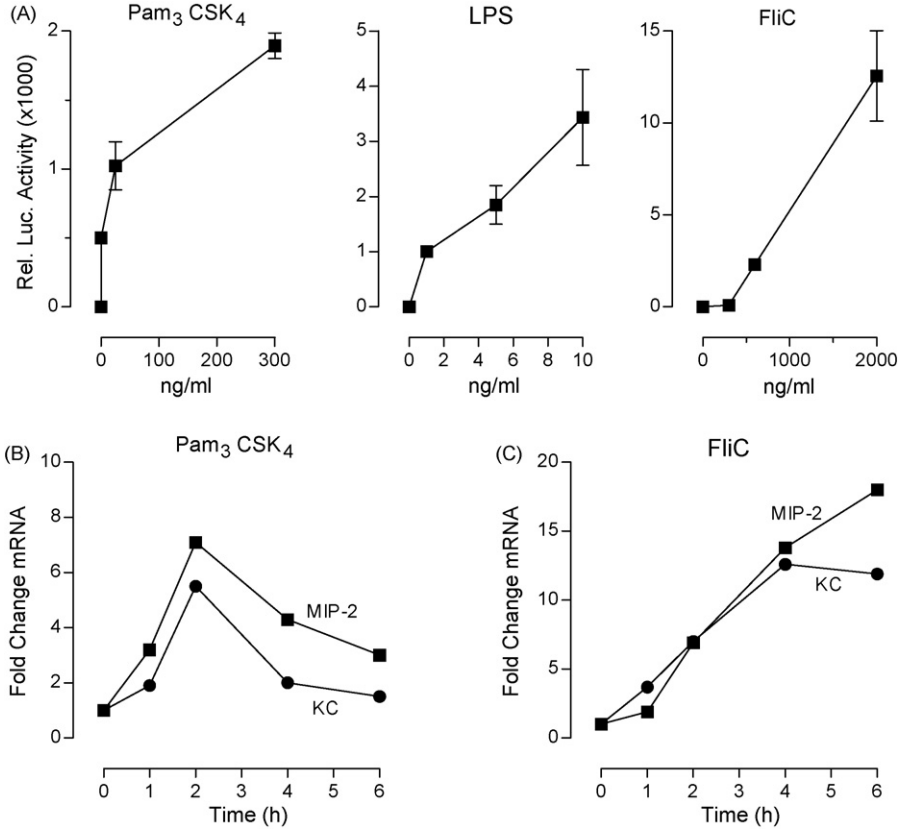

Fig. 2. Functional TLRs 2, 4 and 5 in mIC_{cl2} cells. (A) Confluent monolayers of mIC_{cl2} cells were incubated with 0.25–300 ng/ml Pam_3CSK_4, 1–10 ng/ml LPS, or 300–2000 ng/ml flagellin for 3 h, and analyzed for luciferase activity. Data (means ± S.D.) were corrected for background activity in untreated cells and are a representative of at least three separate experiments. Confluent monolayers of mIC_{cl2} cells were incubated with 300 ng/ml Pam_3CSK_4 (B) or 300 ng/ml flagellin (C) and mRNA for the chemokines MIP-2 and KC was quantified by real time RT-PCR at the indicated time points. Data are from one representative of four assays and shown as fold change in mRNA levels after treatment compared to non-treated cells.

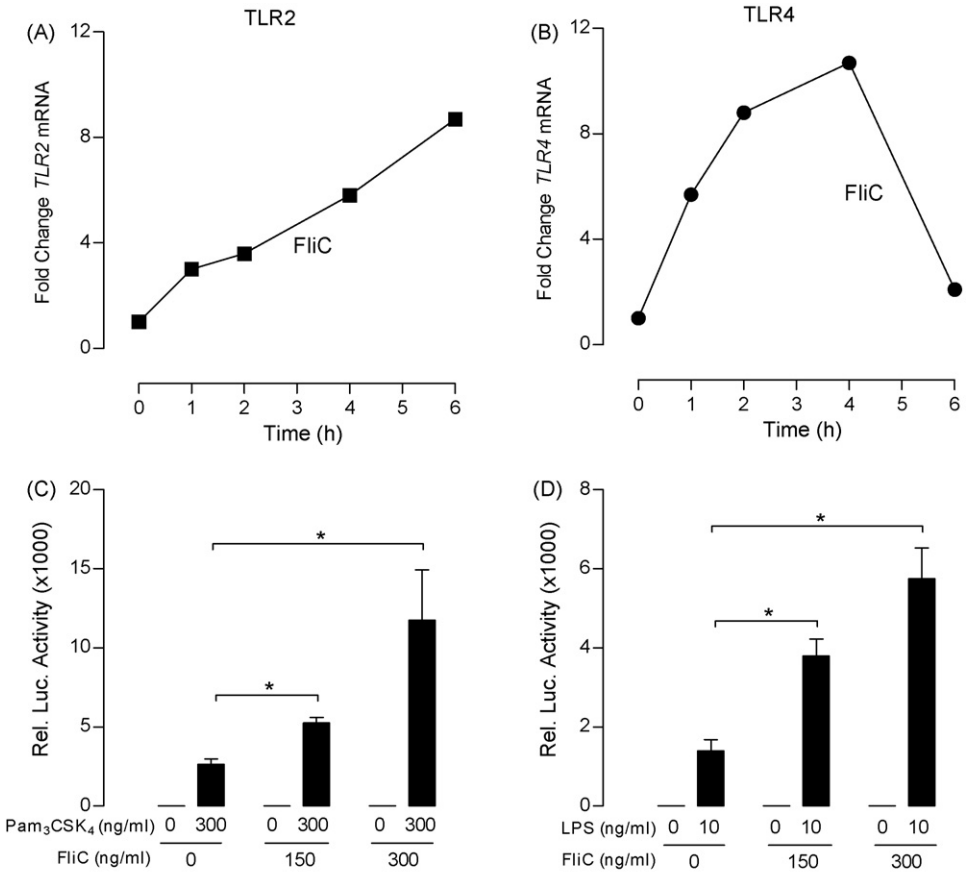

Fig. 3. Flagellin increases expression and function of TLR2 and TLR4. (A and B) Confluent monolayers of mIC_{cl2} cells were incubated with 300 ng/ml flagellin and mRNA for TLR2 (A) and TLR4 (B) was quantified by real time RT-PCR at the indicated time points. Data are from one representative of four assays and shown as fold change in mRNA levels after treatment compared to non-treated cells. (C and D) Confluent monolayers of mIC_{cl2} cells were primed with 0, 150 or 300 ng/ml flagellin for 3 h, washed, and subsequently incubated with 0, 300 ng/ml Pam_3CSK_4 (C) or 10 ng/ml LPS (D) for an additional 3 h. Cells were analyzed for luciferase activity. Data (means ± S.D.) were corrected for background activity in untreated cells and are a representative of at least three separate experiments. $^{*}p < 0.05$.

Flagellin priming was deliberately performed with a low concentration of flagellin (300 ng/ml) proven to be functional at the level of cytokine induction (Fig. 2C), but not sufficient to increase NF-κB luciferase enzyme activity beyond detection levels (Fig. 3C, see also Fig. 2A). Priming with low concentrations of flagellin strongly sensitized mIC_{cl2} cells for both Pam_3CSK_4- (Fig. 3C) and LPS-mediated NF-κB luciferase activity (Fig. 3D).

3.4. TLR2- or TLR4 activation negatively regulates TLR5

The apparent positive regulation of TLR2- and TLR4 expression and function by TLR5 activation led us to investigate the effect of TLR2- or TLR4 activation on TLR5. Stimulation of cells with Pam_3CSK_4 or LPS caused a steady decline of *TLR5* mRNA for up to 6 h (Fig. 4A). At the functional level, priming of mIC_{cl2} cells with low concentrations (0.1 and 10 ng/ml) of Pam_3CSK_4 (or buffer alone) attuenuated the NF-κB response to a high concentration (2000 ng/ml) of flagellin (Fig. 4B). Similar results were obtained when mIC_{cl2} cells were primed with LPS (Fig. 4C). The flagellin-mediated NF-κB response was not further enhanced by priming with a low concentration of flagellin (Fig. 4D). Thus, while priming of mIC_{cl2} cells with a TLR5 agonist sensitized the cells for subsequent stimulation of TLR2 and TLR4 (Fig. 3C and D), pre-exposure to TLR2 and TLR4 agonists had the opposite effect and attenuated the TLR5 response (Fig. 4 B and C).

3.5. Differential regulation between TLR2 and TLR4

To assess the possible cross-regulation between TLR2 and TLR4, we determined *TLR2* and *TLR4* mRNA levels in Pam_3CSK_4- and LPS-stimulated mIC_{cl2} cells. Analogous to the effect on *TLR5* mRNA (Fig. 4A), both Pam_3CSK_4 and LPS caused a persistent downregulation of *TLR4* transcript in mIC_{cl2} cells (Fig. 5A). In contrast, *TLR2* mRNA levels gradually increased after stimulation with Pam_3CSK_4 or LPS (Fig. 5B), indicating differential regulation of *TLR4* and *TLR5* versus *TLR2* mRNA levels by the different TLR agonists. To assess the effect of the apparent cross-regulation at the functional level, mIC_{cl2} cells were primed with low concentrations of LPS or Pam_3CSK_4 (or buffer alone) and subsequently with a high dose of Pam_3CSK_4. Priming of mIC_{cl2} cells with Pam_3CSK_4 sensitized the Pam_3CSK_4-mediated NF-κB luciferase activity (Fig. 5C). The same effect was found when mIC_{cl2} cells were primed with low concentrations of LPS (Fig. 5D).

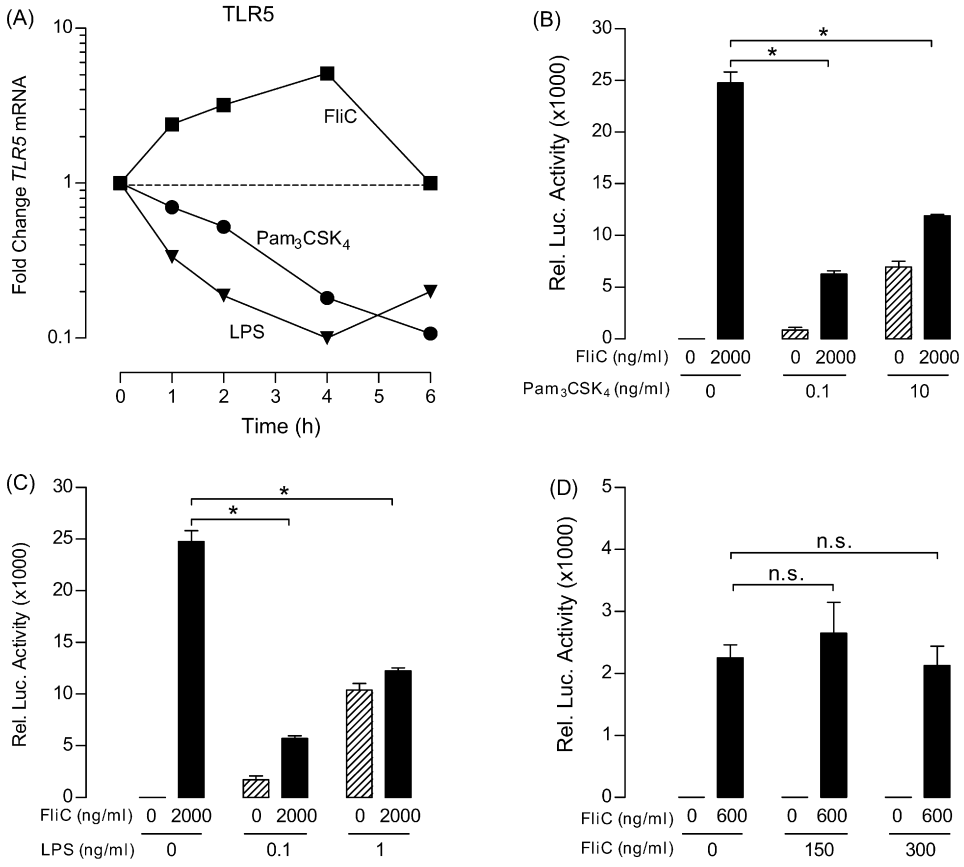

Fig. 4. Pam_3CSK_4 and LPS suppress expression and function of TLR5. (A) Confluent monolayers of mIC_{cl2} cells were incubated with 300 ng/ml Pam_3CSK_4, 10 ng/ml LPS or 300 ng/ml flagellin and expression of *TLR5* mRNA was quantified by real time RT-PCR at the indicated time points. Data are from one representative of four assays and shown as fold change in mRNA levels after treatment compared to non-treated cells. (B) Confluent monolayers of mIC_{cl2} cells were primed with 0, 0.1 or 10 ng/ml Pam_3CSK_4 for 3 h, washed, and subsequently incubated without or with 2000 ng/ml flagellin for 3 h. (C) Same as in B except that cells were primed with 0, 0.1 or 1 ng/ml LPS. (D) Cells were primed with 0, 150, or 300 ng/ml flagellin for 3 h, washed and subsequently incubated without or with 600 ng/ml flagellin for 3 h. Cells were analyzed for luciferase activity. Data (means ± S.D.) were corrected for background activity in untreated cells and are a representative of at least three separate experiments. $^{*}p < 0.05$.

3.6. Contribution of the PI3K/Akt pathway to TLR cross-regulation

The above results indicate that in mIC_{cl2} cells, flagellin acts as a positive regulator of TLR expression for all TLR tested, while Pam_3CSK_4 and LPS downregulate TLR5 and TLR4 but upregulate TLR2 expression. To further investigate the basis of the differential cross-regulation of the TLRs, we investigated the role of the phosphatidylinositol 3-kinase (PI3K/Akt) pathway implicated in the regulation of TLR signaling and cytokine production (Guha and Mackman, 2002; Martin et al., 2003). In mIC_{cl2} cells, specific inhibition of PI3K with wortmannin (Hazeki et al., 2006; Yano et al., 1993) caused an increase of NF-κB luciferase activity in response to Pam_3CSK_4, LPS as well as flagellin (Fig. 6A), indicating that PI3K has a dampening effect on the activation of NF-κB, irrespective of the ligand used. Comparison of TLR mRNA levels in the absence and presence of wortmannin showed that inhibition of PI3K enhanced the LPS-mediated upregulation of *TLR2* mRNA from 5 to 28-fold and prevented the downregulation of *TLR4* and *TLR5* mRNA (Fig. 6B). PI3K inhibition did not affect the flagellin- and Pam_3CSK_4-induced cross-regulation of TLR expression (not shown), Similarly, the PI3K inhibitor selectively enhanced the LPS-induced *MIP-2* response (Fig. 6C). These results suggest that the LPS- but not the Pam_3CSK_4- or flagellin-induced cross-regulation of TLR and stimulation of the MIP-2 response are sensitive to further fine tuning by the PI3K/Akt pathway.

4. Discussion

Intestinal homeostasis depends on the fine interplay between the microbial flora and the host response. Most bacterial pathogens carry multiple pathogen-associated molecular patterns (PAMPs) that are recognized by distinct pathogen recognition receptors such as TLRs. The strength and nature of the response will likely vary dependent on the available TLR repertoire. Here we provide evidence that mouse intestinal epithelial cells have positive and negative feedback mechanisms that regulate the expression and sensitivity of distinct TLRs towards their own as well as other TLR agonists. This cross-regulation of TLR expression and function was demonstrated for TLR2, TLR4, and TLR5 that recognize the bacterial TLR agonists triacylated lipopeptide, LPS, and flagellin, respectively. More specifically, exposure of the intestinal cells to low con-

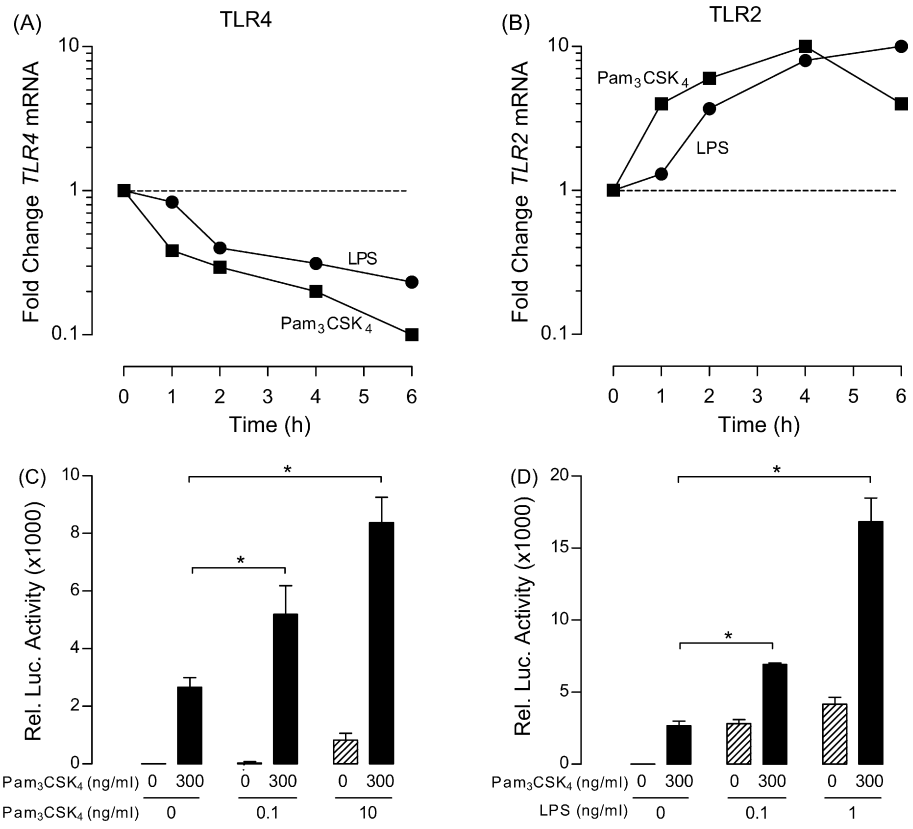

Fig. 5. Pam_3CSK_4 stimulates and LPS suppresses expression and function of TLR2 and TLR4. (A and B) Confluent monolayers of mIC_{cl2} cells were incubated with 300 ng/ml Pam_3CSK_4 or 10 ng/ml LPS and expression of *TLR2* (A) and *TLR4* mRNA (B) was quantified by real time RT-PCR at the indicated time points. Data are from one representative of four assays and shown as fold change in mRNA levels after treatment compared to non-treated cells. (C and D) Confluent monolayers of mIC_{cl2} cells were primed with 0, 0.1, 10 ng/ml Pam_3CSK_4 (C) or 0, 0.1, 1 ng/ml LPS (D) for 3 h, washed, and subsequently incubated without or with 300 ng/ml Pam_3CSK_4 for 3 h. Cells were analyzed for luciferase activity. Data (means ± S.D.) were corrected for background activity in untreated cells and are a representative of at least three separate experiments. $^{*}p < 0.05$.

centrations of flagellin upregulated *TLR5* as well as *TLR2* and *TLR4* mRNA, and enhanced the response to the triacyl peptide Pam_3CSK_4 and LPS. Exposure of the cells to low concentrations of Pam_3CSK_4 and LPS in turn caused a down-regulation of *TLR4* and *TLR5* mRNA and attenuated the response towards flagellin, but had opposite cross-regulatory effects on *TLR2* expression. This cross-regulation of TLR pathways (summarized in Fig. 7) suggests that the response of intestinal epithelial cells at a given time is not a static event but varies dependent on the relative concentrations of TLR agonist that are present in the microenvironment. This awareness is crucial to understand the intricate TLR regulatory network in intestinal cells and may open new avenues to directed modulation of the innate immune response to our benefit.

Cross-regulation of the TLR2, TLR4, and TLR5 signaling pathways in intestinal epithelial cells has thus far not been demonstrated, problably in part because of the variable presence of the different functional TLR pathways in the commonly used intestinal cell lines (Caco-2, HT-29, T84) (Abreu et al., 2005). A key aspect of our work was the use of a mouse intestinal mIC_{cl2} cell model system, derived from intestinal crypt cells (Bens et al., 1996). Like other epithelial cell models, such as Caco-2 and MDCKII cells, mIC_{cl2} cells form a tight monolayer separated by tight-junctions with a distinct brush-border (apical) and basolateral membrane compartment. They differ, however, from other epithelial cell lines with respect to specific enzymatic functions and the maintenance of functional TLR4 and transport of IgA through pIgRs (Bens et al., 1996; Hornef et al., 2002, 2003). Our finding that polarized mIC_{cl2} cells expressed *TLR2*, *TLR4,* and *TLR5* transcripts and responded to Pam_3CSK_4, LPS, and flagellin indicates that the cells carried functional TLR2, TLR4 and TLR5 complexes. This expression repertoire is in line with the reported *in vivo* expression of these receptors in mouse cecum (Ortega-Cava et al., 2003, 2006; Singh et al., 2005) and classify mIC_{cl2} cells as an excellent tool to study the cross-regulation of the TLR2, TLR4, and TLR5 pathways in intestinal epithelial cells.

Our results clearly indicate bacterial flagellin as a prime activator of the intestinal innate immune response. Flagellin activated NF-κB and caused upregulation of chemokine (*MIP-2* and *KC*) mRNA expression and a rapid increase in *TLR2*, *TLR4*, and *TLR5* transcripts. This effect was not caused by contaminating LPS or other TLR ligands as flagellin yielded no response in different types of TLR5-negative cells. Activation of TLR5 by flagellin has been well established and growing evidence exists for the contribution of the flagellin/TLR5 complex in gastrointestinal disorders, like Crohn's Disease (CD) (Begue et al., 2006; Marx, 2007; Ramos et al., 2004). New types of flagellin

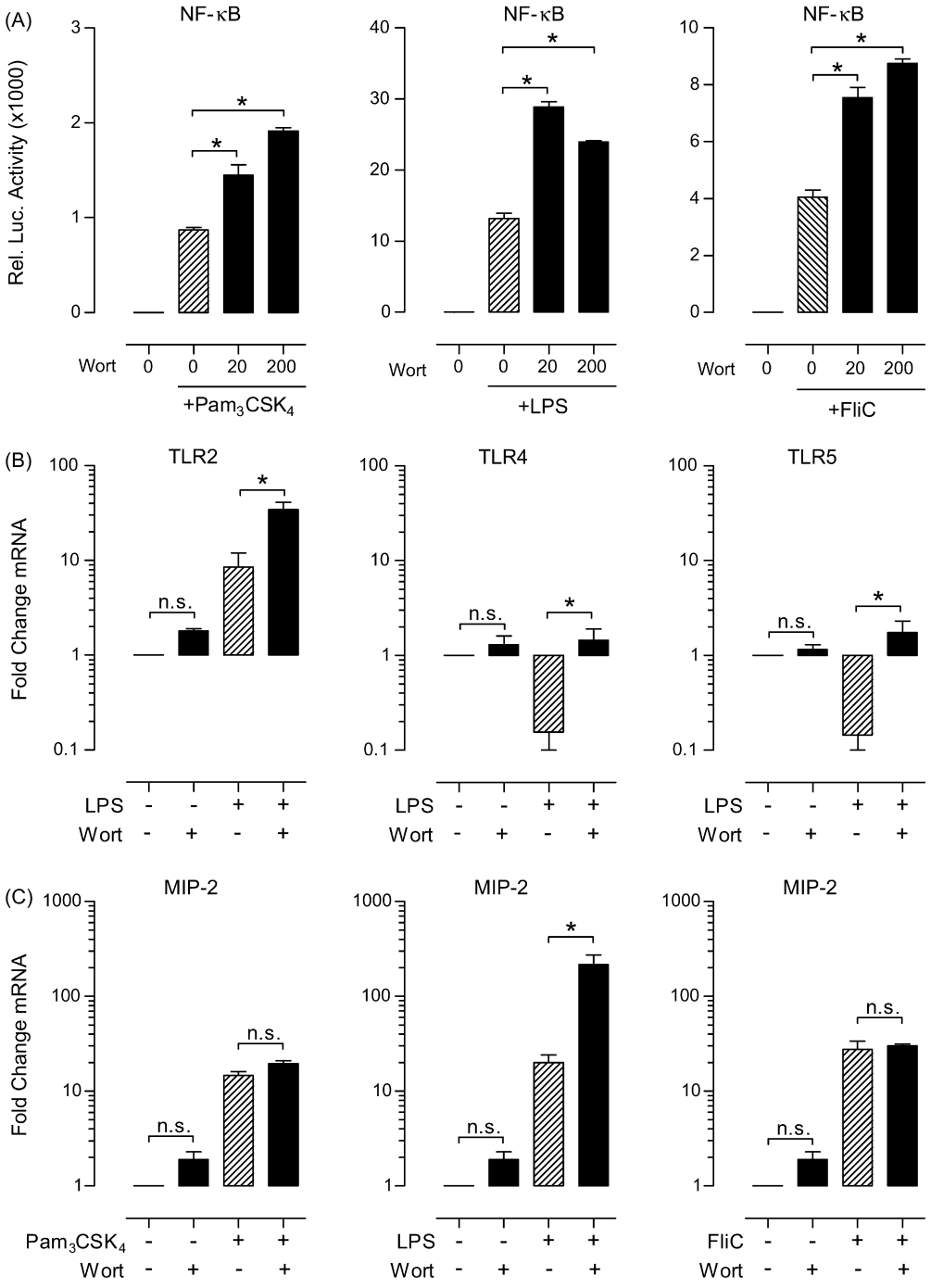

Fig. 6. Role of PI3K/Akt in TLR signalling. (A) Confluent monolayers of mIC_{cl2} cells were preincubated with 20 nM and 200 nM Wortmannin (Wort) or vehicle alone (0.05% DMSO) for 1 h followed by stimulation (4 h) without or with 300 ng/ml Pam_3CSK_4, 300 ng/ml flagellin, or 10 ng/ml LPS and analyzed for NF-κB luciferase activity. Data (means ± S.D.) were corrected for background activity in untreated cells and are a representative of at least three separate experiments. $^*p < 0.05$. (B) Confluent monolayers of mIC_{cl2} cells were preincubated with 200 nM Wortmannin (Wort) or vehicle alone (0.05% DMSO) for 1 h followed by stimulation (4 h) without or with 10 ng/ml LPS and analyzed for and expression of *TLR2, TLR4* and *TLR5* mRNA by real-time RT-PCR. (C) Confluent monolayers of mIC_{cl2} cells were treated similar as in A and subsequently analyzed for expression of *MIP-2* mRNA by real-time RT-PCR. Data (means ± S.E.) are from four assays and shown as fold change in mRNA levels after treatment compared to vehicle-treated cells. Statistical analysis: n.s., not significant; $^*p < 0.01$.

molecules have been identified in CD patients (Elson et al., 2006; Lodes et al., 2004), while carriage of a TLR5-stop codon may provide protection against CD (Gewirtz et al., 2006). The biological activity of flagellin, however, varies among bacterial species (Andersen-Nissen et al., 2005). In particular flagellins derived from bacterial species belonging to the alpha and epsilon Proteobacteria that successfully colonize or persist in the intestinal lumen, appear to lack the ability to activate TLR5 in contrast to flagellin of bacterial pathogens such as Salmonella. It has been speculated that this TLR5 evasion has evolved as a survival strategy to escape the innate host defence (Andersen-Nissen et al., 2005). Our finding that flagellin upregulates *TLR2*, *TLR4* and

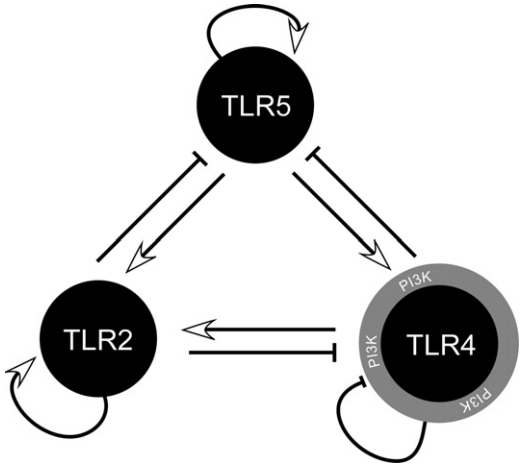

Fig. 7. Schematic representation of the cross-talk between TLRs 2, 4 and 5 in intestinal mIC_{cl2} cells. Activation of TLR5 by low amounts of flagellin enhances the response towards the TLR5 agonist (positive amplification) and increases the TLR2 and TLR4 response, putting the cells in maximal alert. Activation of TLR2 and TLR4 in turn attenuates TLR5 signalling (negative feedback). TLR2 agonists also induce a positive amplification loop resulting in an increased TLR2 response but attenuate TLR4 and TLR5 signalling. Activation of TLR4 stimulates TLR2-, but attenuates TLR4- and TLR5 signalling. The PI3K/Akt pathway modulates the TLR4-mediated cross-regulation, while the cross-talk of TLR2 and TLR5 proceeds via PI3K/Akt-insensitive route(s).

TLR5 mRNA levels and enhances the response towards other bacterial TLR agonists such as lipopeptides and LPS may further explain the apparent evolutionary need of intestinal bacteria to develop flagellins that avoid TLR5 activation.

Our results suggest that flagellin appears to bring the intestinal cells in a state of alert. We also show that exposure to TLR2 and TLR4 agonists have the opposite effect to flagellin and dampen the response towards the microbial stimuli, including flagellin. This negative feedback may serve to protect the cells from overstimulation and may be part of a tightly controlled system to maintain intestinal immune homeostasis. Recently, TLR9 has been proposed to play a key role in the regulation of intestinal immunity. TLR9 blocks signalling via other TLRs and provides protection against intestinal colitis by stimulation of the production of IFNs (Katakura et al., 2005; Lee et al., 2006). In mIC_{cl2} cells, we did not detect TLR9 mRNA under steady-state conditions, but rather found TLR5 as a key regulatory molecule. To our knowledge, our study is the first to show that TLR5 activation sensitizes intestinal epithelial cells to TLR2 and TLR4 ligands. Also, desensitization of the TLR5 response by TLR2 and TLR4 agonists in gut epithelial cells is novel. In human monocytes, TLR4 activation does not change TLR5 expression (Cabral et al., 2006), indicating that TLR cross-regulation may vary depending on the host species and on the cell subset.

Cross-talk between TLR2 and TLR4 signaling pathways have been reported in murine macrophages and endothelial cells (Fan et al., 2003; Matsuguchi et al., 2000; Nilsen et al., 2004; Totemeyer et al., 2003). Interestingly, the effects in these cell types are in agreement to that what we observed for intestinal mIC_{cl2} epithelial cells. Tolerance and cross-tolerance between TLR2 and TLR4 upon long-term (24 h) TLR activation has been attributed to changes in expression of TLRs, as well as to the function of several signaling molecules including IRAK-1, NF-κB and MAP kinases (Dobrovolskaia et al., 2003; Li et al., 2000, 2006; Medvedev et al., 2000, 2002; Otte et al., 2004; Wang et al., 2002). Whether differences in signalling molecules also contribute to the here observed TLR (cross)-regulation upon short-term TLR activation in mIC_{cl2} cells awaits detailed investigation of the cell signalling dynamics.

Important to future targeted manipulation of the intestinal homeostasis is understanding of the mechanisms that drive the positive and negative TLR feedback loops. With regard to the changes in TLR expression levels, it is evident from our results that in Pam_3CSK_4-, LPS-, and flagellin-stimulated mIC_{cl2} cells the expression of *TLR2* mRNA is upregulated (Figs. 3A and 5B), suggesting a common signalling pathway. Such a pathway may include the central TLR regulator NF-κB, which has been shown to play an essential role in the activation of the mouse *TLR2* promoter (Musikacharoen et al., 2001; Wang et al., 2001). The mouse *TLR4* promoter does not contain NF-κB sites but carries several other elements involved in positive (AP-1, Ets, PU.1) and negative (GATA-1 and Oct-1) regulation of *TLR4* transcription (Pedchenko et al., 2005; Roger et al., 2005). The *TLR5* promoter has not been studied in great detail but *in silico* analysis indicates similar elements as to those in the *TLR4* promoter. A resemblance in promoter architecture between *TLR4* and *TLR5* may also contribute to their apparent similar transcriptional regulation, i.e. their upregulation by flagellin (Figs. 3B and 4A) and downregulation by LPS and Pam_3CSK_4 (Figs. 4a and 5A). Seemingly at variance with this scenario of transcriptional regulation is that in RAW264.7 macrophages, LPS-induced downregulation of *TLR4* mRNA has been attributed to an increase in *TLR4* mRNA turnover (Roger et al., 2005). LPS is known to activate the PI3K/Akt pathway (Guha and Mackman, 2002) and this event may, through inactivation of MAPK p38 (Fukao and Koyasu, 2003), decrease mRNA stability at the 3′-UTR region of a variety of genes (Dean et al., 2004). In favor of this mechanism is our finding that inhibtion of the PI3K/Akt pathway enhances NF-κB activation as well as the LPS-mediated downregulation of *TLR4* and *TLR5* mRNA and upregulation of *TLR2* and *MIP-2* mRNA (Fig. 6). However, this modulatory effect of the PI3K/Akt pathway was not observed for the effects of Pam_3CSK_4 and flagellin on TLR expression and the *MIP-2* mRNA levels. Combined our data suggest that, in mIC_{cl2} intestinal cells, all TLR agonists tested may exert their regulatory effects on TLR mRNA by influencing gene transcription, but that, in the case of NF-κB luciferase activity and the crossregulation of TLR by LPS, the effects are dampened by activity of the PI3K/Akt pathway, possibly by influencing mRNA stability.

At this time, it is difficult to discern whether the observed regulation of TLR function in mIC_{cl2} cells also holds for human intestinal cells. As noted, commonly used human intestinal cell lines such as Caco-2 and T84 lack functional TLR2 and TLR4 and thus are not suitable for this type of studies. For T84 cells, the role of PI3K in the signaling of TLR5 has been studied, but results appear to vary dependent with the type of cytokine investigated (Rhee et al., 2006; Yu et al., 2006). A further complication of the commonly used human polarized intestinal cell

lines is that the cellular localization of TLR5 in the various cell types appears to be different. In some cells (such as T84) TLR5 appears to be expressed solely at the basolateral cell surface (Gewirtz et al., 2001), while in other cell lines (such as Caco-2) and in colon xenografts *in vivo*, TLR5 is also expressed at the apical membrane (Bambou et al., 2004; Cario and Podolsky, 2000; Miyamoto et al., 2006). As under natural conditions intestinal epithelial cells will be most frequently exposed to flagellin from the luminal side, we added flagellin to the apical side of mIC_{cl2} cells in this study, although we noted similar responses to flagellin when the flagellin was applied from the basolateral side. The basis for the apparent diversity in TLR receptor expression among the various cell types is unclear. We propose that the reported, sometimes controversial differences in TLR expression and associated responses among cell lines as well as mucosal tissues are a reflection of natural diversity caused by differences between species and/or stages of cell differentiation as well as the here reported dynamics of TLR expression and function in intestinal epithelial cells. Thus the intestinal TLR expression and cytokine responses *in vivo* are not stable characteristics but will vary dependent on the repertoire of environmental stimuli, i.e. the composition of the endogenous intestinal flora,

In conclusion, our study for the first time demonstrates the existence of an intricate regulatory network of cross-talk of the TLR2, TLR4, and TLR5 pathways in intestinal epithelial cells. The identified positive and negative amplification loops for the different TLR agonists and the involvement of different regulatory pathways indicate that the intestinal innate immune system is highly dynamic which may open avenues to target this system to our benefit.

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