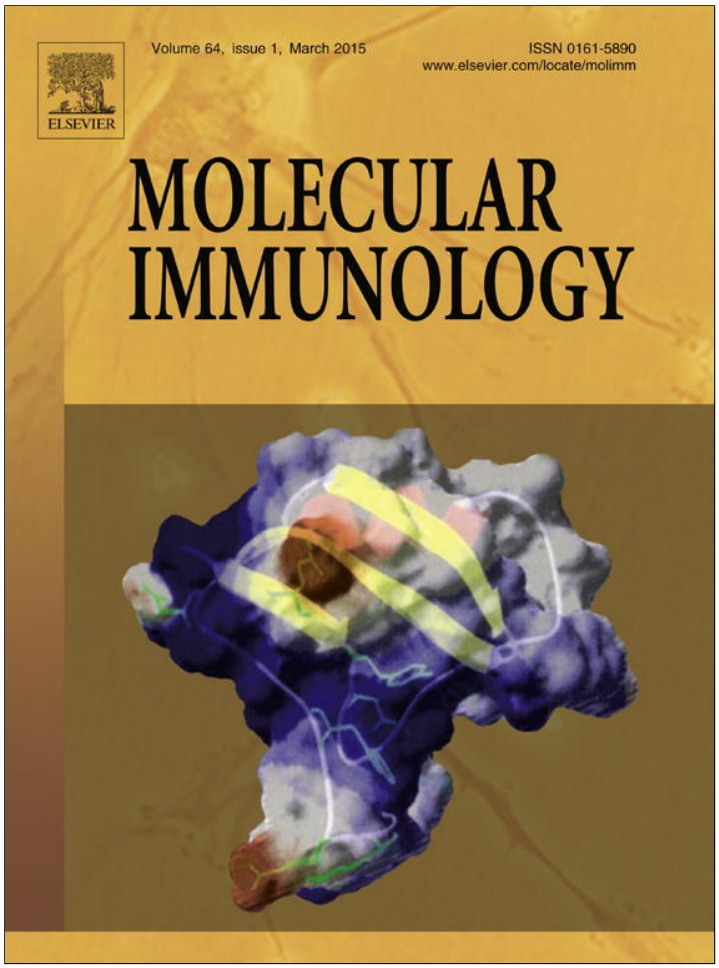

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The toll-like receptor family protein RP105/MD1 complex is involved in the immunoregulatory effect of exopolysaccharides from *Lactobacillus plantarum* N14

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ABSTRACT

The radioprotective 105 (RP105)/MD1 complex is a member of the Toll-like receptor (TLR) family. It was reported that RP105/MD1 cooperates with the lipopolysaccharide (LPS) receptor TLR4/MD2 complex and plays a crucial role in the response of immune cells to LPS. This work evaluated whether RP105, TLR4 or TLR2 were involved in the immunoregulatory capacities of *Lactobacillus plantarum* N14 (LP14) or its exopolysaccharides (EPS). EPS from LP14 were fractionated into neutral (NPS) and acidic (APS) EPS by anion exchange chromatography. Experiments with transfectant HEK$^{RP105/MD1}$ and HEK^{TLR2} cells demonstrated that LP14 strongly activated NF-κB *via* RP105 and TLR2. When we studied the capacity of APS to activate NF-κB pathway in HEK$^{RP105/MD1}$ and HEK^{TLR4} cells; we observed that APS strongly stimulated both transfectant cells. Our results also showed that LP14 and APS were able to decrease the production of pro-inflammatory cytokines (IL-6, IL-8 and MCP-1) in porcine intestinal epithelial (PIE) cells in response to enterotoxigenic *Escherichia coli* (ETEC) challenge. In order to confirm the role of TLR2, TLR4 and RP105 in the immunoregulatory effect of APS from LP14, we used small interfering RNA (siRNA) to knockdown these receptors in PIE cells. The capacity of LP14 and APS to modulate pro-inflammatory cytokine expression was significantly reduced in PIE$^{RP105-/-}$ cells. It was also shown that LP14 and APS were capable of upregulating negative regulators of the TLR signaling in PIE cells. This work describes for the first time that a *Lactobacillus* strain and its EPS reduce inflammation in intestinal epithelial cells in a RP105/MD1-dependend manner.

1. Introduction

The radioprotective 105 (RP105)/MD1 complex is a member of the Toll-like receptor (TLR) family of proteins that was discovered originally in mouse B cells (Miyake et al., 1995). However, RP105 is not B cell-specific. In fact, the expression of RP105 is similar to the expression of TLR4 on myeloid cells, including monocytes, macrophages, and dendritic cells (DC) (Nagai et al., 2005). Cloning human and mouse RP105 revealed a type I transmembrane protein that is structurally similar to TLRs, with extracellular leucine-rich repeat domains and the pattern of juxtamembrane

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cysteine residues conserved among the TLRs. In contrast to TLRs, however, RP105 lacks a TIR domain (Yoon et al., 2011). In addition, RP105 shares functional similarities to TLR4. Signaling through TLR4 is dependent on a secreted, extracellularly associated accessory protein, MD2. Similarly, surface expression and activity of RP105 are dependent on the MD2 homologue, MD1 (Miyake et al., 1998). The RP105/MD1 complex is also a negative regulator of TLR4/MD2 signaling. Divanovic et al. (2005a,b) demonstrated in HEK293 cells co-expressing TLR4/MD2 and RP105/MD1, that this last complex inhibits LPS–TLR4/MD2 signaling complex formation. Moreover, the authors reported that RP105 regulates LPS-driven TLR4 signaling in mouse DC and macrophages, inhibiting the production of cytokines dependent on both MAL/MyD88 and TRIF/TRIF pathways. Further, they demonstrated that RP105 is a physiological regulator of the *in vivo* responses to LPS. Experiments in RP105$^{-/-}$ mice showed that challenge with LPS significantly increased the systemic production of TNF-α and exhibit significant amplification of endotoxicity (Divanovic et al., 2005a,b).

In recent years, there has been a growing interest in the swine immune system because of its possible use as a model for the human immune system and because of the economic importance of swine as livestock (Dawson, 2011; Dawson et al., 2013). In addition, the swine gastrointestinal tract has many structural aspects that are more similar to those of the human system than the rodent system (Dawson et al., 2013; Mair et al., 2014). Therefore, we have focused on the swine immune system as a human model and have studied the immune responses to immunoregulatory probiotics (immunobiotics) *via* pattern recognition receptors (PRRs). In this regard, we isolated the cDNA sequences of porcine RP105 and porcine MD1 from ileal Peyer's patches (PP) and determined the full-length cDNA sequences of RP105 and MD1 (Tohno et al., 2007). The nucleotide and aminoacid sequences of porcine RP105 and MD1 were more similar to those of human than those of mouse, supporting the idea that swine is a better model than the mouse for human immune system extrapolations (Tohno et al., 2007). Subsequently, we constructed cells expressing both porcine RP105 and MD1, and demonstrated that the RP105/MD1 complex can recognize phosphopolysaccharide produced by immunobiotic strains such as *Lactococcus lactis* ssp. *cremoris*, resulting in the activation of nuclear factor-κB (NF-κB) through the phosphatidylinositol 3-kinase (PI3K) and Bruton's tyrosine kinase (Btk) signaling pathway (Tohno et al., 2007).

The immunobiotic *Lactobacillus plantarum* N14 (LP14) strain, produce exopolysaccharides (EPS) that exert anti-allergic and immunostimulatory Th1 abilities (Nagata et al., 2010; Hashiguchi et al., 2011). We have shown that EPS produced by LP14 can be fractionated into two types of EPS which had a different sugar composition. Neutral EPS (NPS), and acidic EPS (APS) from LP14 can be fractionated by anion exchange chromatography (Hashiguchi et al., 2011). However, the immunoregulatory activities of NPS or APS have not been evaluated before neither their contribution to the immunobiotic activity of LP14. Therefore, in this work we aimed to evaluate the immunoregulatory capacities of EPS from LP14 in intestinal epithelial and immune cells from pigs. In addition, we aimed to gain insight in the knowledge of the mechanisms involved in the immunoregulatory effect of LP14 and its EPS by studying the role of the RP105/MD1 complex, TLR4 and TLR2.

2. Materials and methods

2.1. Bacterial strains

L. plantarum N14 was isolated from pickled shallots (Nagata et al., 2010; Hashiguchi et al., 2011). The N14 strain was cultured at 30 °C for 24 h in deMan-Rogosa-Sharp (MRS; Sigma, St. Louis, MO, USA) medium pretreated with polymyxin B (Sigma, St. Louis, MO, USA) to remove endotoxin. After mass cultures, the bacteria were collected and washed by centrifugation, and lyophilized.

Enterotoxigenic *Escherichia coli* (ETEC) 987P was kindly gifted by National Institute of Animal Health (NIAH), Tsukuba, Japan. ETEC strain was plated in Tryptic soy agar (TSA, Becton, Dickinson and Company, New Jersey, USA) supplemented with 5% sheep blood (Nippon Biotest Laboratories Inc., Tokyo, Japan) for 24 h at 37 °C and then transferred single colony to tryptic soy broth (TSB, Becton, Dickinson and Company, New Jersey, USA) for 5 days at 37 °C without shaking to form a pellicle containing the pileated phase. Then, ETEC cells were collected from the pellicle and transferred to TSB and cultured 20 h at 37 °C with shaking (200 rpm). Finally, the subculture was centrifuged at 1900 × *g* for 10 min at 4 °C, washed with phosphate buffered saline (PBS) and heat-killed at 100 °C for 30 min. The heat-killed cells were then resuspended with DMEM medium for further experimental analysis (Shimazu et al., 2012).

2.2. Exopolysaccharides from L. plantarum N14

Exopolysaccharides (EPS) were extracted and fractionated by the method of Kitazawa et al. (1998). The acid EPS (APS) and neutral EPS (NPS) were fractionated from EPS by ion-exchange chromatography on DEAE-Toyopearl 650M (2 cm × 50 cm, Tosoh Co., Ltd., Tokyo, Japan) by linear gradient elution from 0 to 0.5 M of NaCl in 50 mM Tris–HCl buffer (pH 8.6). Each 5 mL of the eluate was monitored for neutral sugars at 490 nm by phenol–H_2SO_4 reaction and electrical conductivity. The fractionated polysaccharide was evaporated and dialyzed by SnakeSkin Dialysis Tubing (3500MWCO, Thermo) and lyophilized.

2.3. Isolation of immunocompetent cells from swine Peyer patches and mesenteric lymphoid nodes

Suspensions of porcine PPs or MLN immunocompetent cells were prepared from adult swine intestine as described previously (Shimazu et al., 2012). This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the Guidelines for Animal Experimentation of Tohoku University, Sendai, Japan. The present study was approved by the Institution Animal Care and Use Committee of Tohoku University with a permitted No. 2011-noudou-5 and all efforts were made to minimize suffering. Briefly, PPs or MLNs tissue pieces were manually cut into small pieces, as small as possible, to facilitate the disruption of tissue and cell separation. Tissue pieces were then gently pressed through a nylon mesh and washed three times in complete RPMI 1640 medium (Sigma, St. Louis, MO, USA) supplemented with 10% FCS (Sigma, St. Louis, MO, USA). Residual erythrocytes were lysed by resuspending in hypotonic salt solution (0.2% NaCl). Next, harvested PP or MLN cells were subjected to hypertonic rescue in an equal volume of 1.5% NaCl. Finally, immune cells were fractionated using Lympholyte-mammal (Cedarlane, Hornby, Ontario, Canada) density gradient centrifugation, and the isolated immune cells were suspended in complete RPMI 1640 (Sigma, St. Louis, MO, USA) supplemented with 10% FCS (Sigma), 50 μg/mL and 50 μg/mL penicillin–streptomycin (Gibco®, Life Tech., Carlsbad, CA, USA) (Fujie et al., 2011).

2.4. Mitogenicity assay

The mitogenicity assay was performed as described previously (Fujie et al., 2011). Briefly, PPs or MLN immunocompetent cells (2×10^5 cells/well) were placed in a round ninety-six-well microplate (SUMILON®, Sumitomo Bakelite Co. Ltd., Tokyo, Japan) and incubated at 5% CO_2, 37 °C for 48 h in complete RPMI 1640

medium (Sigma, St. Louis, MO, USA) supplemented with 2% FCS. Immune cells were stimulated with *L. plantarum* N14, NPS or APS for 48 h (100 μg/mL). During the final hour of culture, the cells were radiolabeled with 9.25 kBq per well of [methyl-^{3}H]-thymidine (GE Healthcare, Tokyo, Japan). The cells were then harvested with a glass fiber filter (PerkinElmer Japan, Kanagawa, Japan). The [methyl-^{3}H]-thymidine incorporation was quantitated in a liquid scintillation counter (Beckman Instruments, Palo Alto, CA, USA). Results are presented as the SI, calculated with the following equation (Fujie et al., 2011):

$$\frac{\text{counts per minute in treated culture} - \text{counts per minute in background}}{\text{counts per minute in control cultures} - \text{counts per minute in background}}$$

2.5. Porcine intestinal epithelial cells

The PIE cell line is a non-transformed intestinal cultured cell line, which was originally established at Tohoku University from intestinal epithelia of unsuckled neonatal swine (Moue et al., 2008). PIE cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen Corporation, Carlsbad, CA) supplemented with 10% fetal calf serum (FCS), penicillin (100 mg/mL), and streptomycin (100 U/mL) at 37 °C in a humidified atmosphere of 5% CO_2. The cells grow rapidly in culture growth conditions without any transformation or immortalization. The proliferative capability of PIE cells diminishes after 50 passages in culture. Therefore, the PIE cells between 20th and 40th passages were used in this study (Shimazu et al., 2012).

2.6. Quantitative analysis of expression with real-time quantitative PCR

We performed two-step real-time quantitative PCR (RT-qPCR) to characterize the expression of mRNAs in PIE cells and immune cells. Total RNA was isolated from each PIE or immune cell sample using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). To remove the genomic DNA, the isolated samples were treated with DNAse (PureLinkTM DNase, Invitrogen). All cDNAs were synthesized using a Quantitect reverse transcription (RT) kit (Qiagen, Tokyo, Japan) according to the manufacturer's recommendations. Real-time quantitative PCR was carried out using a 7300 real-time PCR system (Applied Biosystems, Warrington, UK) and the Platinum SYBR green qPCR SuperMix uracil-DNA glycosylase with 6-carboxyl-X-rhodamine (Invitrogen, Carlsbad, CA, USA). The primers for cytokines and negative regulators of the TLR signaling used in this study were previously described (Shimazu et al., 2012). The PCR cycling conditions were 5 min at 50 °C, followed by 5 min at 95 °C, and then 40 cycles of 15 s at 95 °C, 30 s at 60 °C, and 30 s at 72 °C. The reaction mixtures contained 2.5 μL sample cDNA and 7.5 μL master mix, which included the sense and antisense primers. Expression of β-actin in each sample was assessed, and the β-actin data were used as an internal control to normalize differences between samples and to calculate relative expression levels. According to the minimum information for publication of quantitative real-time PCR experiments guidelines, β-actin was used as a housekeeping gene because of its high stability across porcine various tissues (Bustin et al., 2009; Nygard et al., 2007). Relative index was calculated as the ratio of cytokine mRNA expression to β-actin. Then values were normalized by common logarithmic transformation for statistical analysis.

2.7. Immunomodulatory activity in immune cells

Porcine PPs or MLN immunocompetent cells (4×10^6/well) were placed in a forty-eight-well microplate (SUMILON®, Sumitomo Bakelite Co. Ltd., Tokyo, Japan) and stimulated with *L. plantarum* N14, APS or NPS at a final concentration of 100 μg/mL for 6 h. Expression of IL-6, IL-8 and TGF-β mRNA was evaluated by RT-qPCR method with the corresponding primers (supplementary Table 1).

Supplementary Table 1 related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.molimm.2014.10.027.

2.8. Immunomodulatory activity in PIE cells

For anti-inflammation experiments PIE cells were plated (3.0×10^4 cells/well) in twelve-well type I collagen coated plates (Iwaki, Tokyo, Japan) and cultured for 3 days. After changing media, *L. plantarum* N14, APS or NPS were added at a final concentration of 100 μg/mL. PIE cells were incubated for 6 or 48 h at 37 °C, 5% CO_2. After stimulation, cells were washed vigorously at least three times with fresh DMEM medium to eliminate all stimulants and subsequently stimulated with ETEC 987P (5×10^7 cells/mL) for 12 h. The expressions of IL-6, IL-8 or TGF-β mRNA were evaluated by RT-qPCR method described above. In some experiments, unlabeled anti-porcine TLR2-rabbit IgG or anti-porcine TLR4-rabbit IgG (Biolegend, San Diego, CA, USA) was used for blocking the receptors. Cultured porcine cells were incubated with the unlabeled anti-TLR2 or anit-TLR4 antibodies for 12 h before stimulation with *L. plantarum* N14, APS or NPS.

2.9. $HEK^{RP105/MD1}$ and HEK^{TLR2} cells

Transfected cells were established according to our previous report (Tohno et al., 2007). Briefly, HEK293 cells (4×10^5/well) were plated in six-well plates for 24 h prior to transfection. The cells were transfected with the pcDNA3 vector (Invitrogen, Carlsbad, CA, USA) encoding porcine RP105, or TLR2 (1 μg/well) using Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA, USA). The RP105, or TLR2 expressing transfectants were selected with G418 (Invitrogen, Carlsbad, CA, USA). One of RP105 expressing lines was further transfected with the pIRESpuro expression vector (Clontech, Palo Alto, CA, USA) encoding MD1. Cells expressing MD1 were selected with puromycin (Clontech, Palo Alto, CA, USA). Expression of transfected genes was confirmed by real-time RT-PCR with the corresponding primers (supplementary Table 1). Transfectants expressing both RP105 and MD1 were designated $HEK^{RP105/MD1}$ and the TLR2 transfectants were designated HEK^{TLR2} cells. HEK293 cells containing plasmid vectors without the inserted DNAs were used as controls.

2.10. RP105/MD1, TLR2, and TLR4 knockdown in PIE cells

For RNA interference, PIE cells were plated (3.0×10^4 cells/well) in twelve-well type I collagen coated plates (Iwaki, Tokyo, Japan) and cultured for 3 days. Then cells were transfected with 300 pmol of various RP105/MD1, TLR2 or TLR4 specific short interfering RNAs (siRNAs) using lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA, USA) for different time intervals (12, 24, 36, 48, 60 and 72 h). Primers used for RP105/MD1, TLR2 and TLR4 gene knockdown in PIE cells are listed in supplementary Table 1. Stealth RNAiTM siRNA Negative Control Med GC Duplex #3 (#12935-113, Invitrogen, Carlsbad, CA, USA) was used as control. RT-PCR was performed with corresponding primers for RP105/MD1, TLR2 and TLR4 (supplementary Table 1) to examine the inhibition of RP105/MD1, TLR2 and TLR4 expressions in knockdown cells.

2.11. Analysis of intracellular Ca^{2+} flux

Evaluation of intracellular calcium flux by lactobacilli, EPSs and TLR ligands in porcine RP105/MD1, TLR2 and TLR4 knockdown PIE cells were performed according to the method of Kitazawa

et al. (2007) with some modifications. Briefly, the knockdown cells (1×10^5 cells/100 μL medium) were plated on ninety-six-well Cell Culture Clear Bottom Black Half Area Plate (Corning Inc., NY, USA) at 37 °C for 24 h. After aspiration medium carefully, 100 μL lording buffer of Calcium Kit-Fluo 4 (Dojindo Laboratories, Kumamoto, Japan) was added and incubated at 37 °C for 1 h, following replacement of 100 μL recording medium. Then, the dyed cells were washed with PBS and stimulated with *L. plantarum* N14, APS or NPS; or TLR ligands (LPS and Pam3CSK4) by the dropping system ins a fluorescence spectrophotometer (ARVO™ X3, PerkinElmer Inc., MA, USA). The fluorescence intensity was recorded up to 150 s after stimulation.

2.12. Statistical analysis

Statistical analyses were performed using the general linear models (GLM) procedures available in the SAS computer program (SAS, 1994). Comparisons between mean values were carried out using one-way analysis of variance and Fisher's least-significant-difference (LSD) test. For these analyses, p values of <0.05 were considered significant.

3. Results

3.1. Effect of L. plantarum N14, APS, and NPS on PPs and MLN cells

A commonly used method to study the immunostimulatory properties of bacteria or bacterial compounds is the evaluation of mitogenic activity (Fujie et al., 2011). We used this method to assess the immunostimulatory capacity of *L. plantarum* N14 and its purified APS and NPS, using immunocompetent cells isolated from swine mesenteric lymphoid nodes (MLN) and Peyer's patches (PP) (Fig. 1A and B). *L. plantarum* N14, APS and NPS were able to significantly increase the mitogenic activity of MLN cells compared with the control group (Fig. 1A). *L. plantarum* N14 and APS increased mitogenic activity with concentrations higher than 10 μg/mL, in a concentration-dependent manner. On the contrary, MNL mitogenic activity was superior with the lower concentration of NPS (Fig. 1A). In addition, *L. plantarum* N14 was able to increase PP mitogenic activity with all the concentrations evaluated in a similar manner, while only the higher concentrations of APS and NPS induced this effect (Fig. 1B).

We also evaluated the effect of *L. plantarum* N14, APS and NPS on cytokine expression in MNL and PP cells (Fig. 1C). *L. plantarum* N14 increased the expression of IL-6 and IL-8 in PP cells while it significantly reduced the expression of TGF-β when compared to controls. In MLN cells, the N14 strain upregulated IL-8 mRNA while reduced the expression of IL-6 (Fig. 1C). NPS downregulated the expression of IL-6 and IL-8 in MNL and PP cells respectively, while it significantly increased the expression of TGF-β in both cells populations (Fig. 1C). APS increased IL-8 mRNA in MNL and PP cells and TGF-β mRNA in MLN cells (Fig. 1C).

3.2. Effect of L. plantarum N14, APS, and NPS on PIE cells

The effect of *L. plantarum* N14 and its purified APS and NPS were evaluated first in PIE cells without inflammatory challenge. For this purpose, PIE cells were stimulated with *L. plantarum* N14, APS or NPS, and the expression of the inflammatory cytokines IL-6 and IL-8, and the immunoregulatory cytokine TGF-β was evaluated. As shown in Fig. 2A, the effect on PIE cells was specific for each stimuli. *L. plantarum* N14 induced the expression of IL-6, IL-8 and TGF-β. APS increased IL-6 and IL-8 mRNAs while NPS upregulated IL-6 and TGF-β mRNAs. For the evaluation of the anti-inflammatory activities of *L. plantarum* N14 and its EPS, PIE cells were first stimulated with bacteria, APS or NPS for 48 h and then challenged with

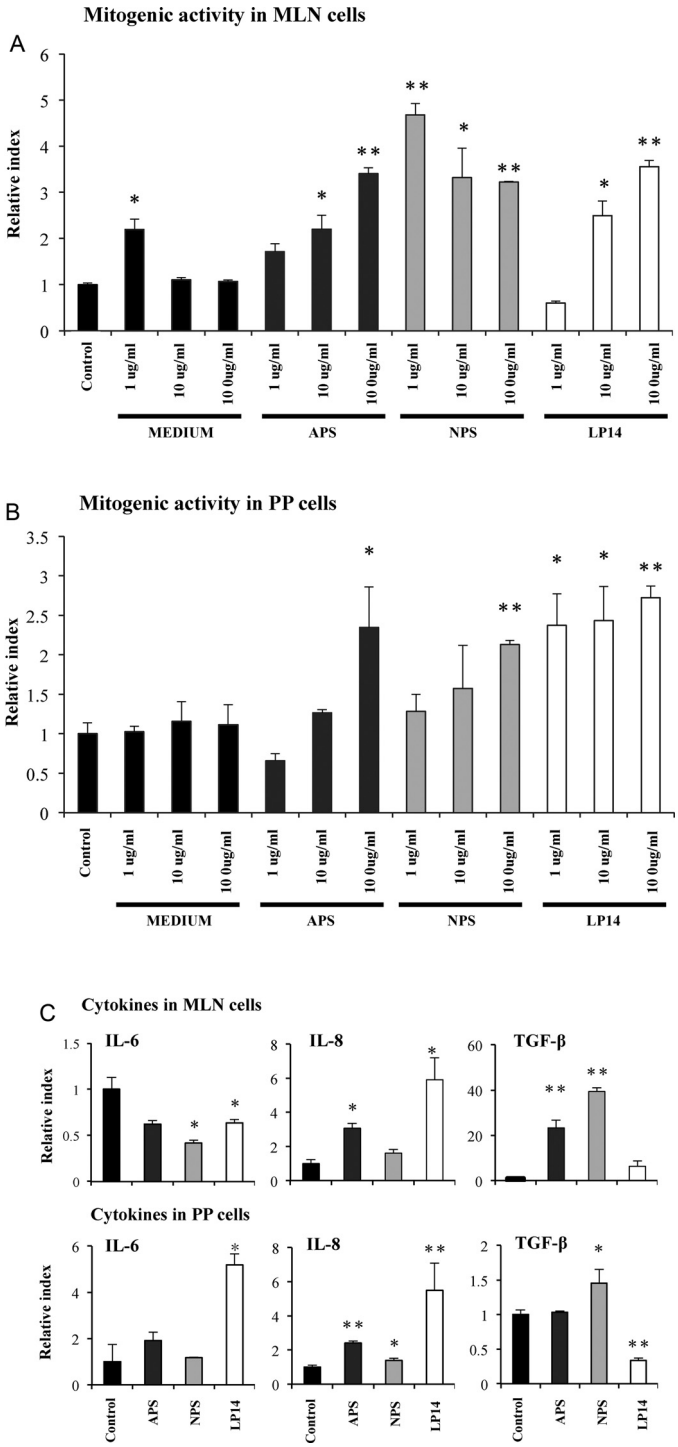

Fig. 1. Effect of *Lactobacillus plantarum* N14 (LP14), acidic extracellular polysaccharide (APS), and neutral extracellular polysaccharides (NPS) in porcine immune cells from Peyer's patches (PP) and mesenteric lymphoid nodes (MLN). Immune cells were stimulated with LP14, APS or NPS and mitogenic activity (A and B) and cytokine expression levels (C) were determined. Three or four independent experiments were conducted for each case and the average values (mean ± S.D.) are shown. The presence of various asterisks (* and **) indicates statistical differences with significant levels of $p < 0.05$ and $p < 0.01$ respectively.

ETEC 987P. The mRNA levels of IL-6, IL-8 and MCP-1 were determined after 12 h. As described before (Shimazu et al., 2012), PIE cells challenged with ETEC 987P significantly increased the expression pro-inflammatory mediators IL-6, IL-8 and MCP-1. Again, the effect on PIE cells was specific for each stimuli. IL-6 expression in PIE cells

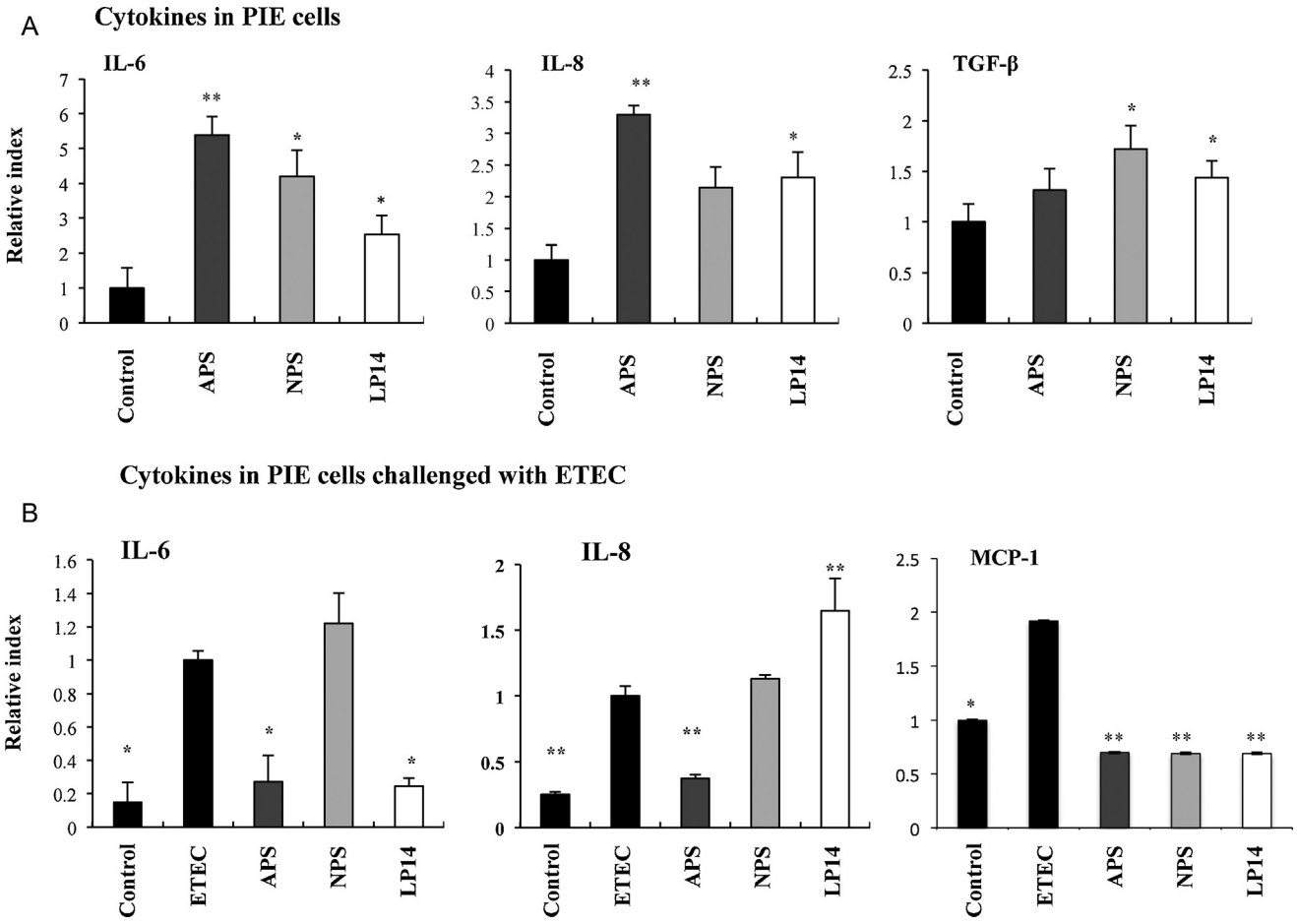

Fig. 2. Effect of *Lactobacillus plantarum* N14 (LP14), acidic extracellular polysaccharide (APS), and neutral extracellular polysaccharides (NPS) in porcine intestinal epitheliocyte (PIE) cells. (A) PIE cells were stimulated with *L. plantarum* N14, APS or NPS. (B) PIE cells were pre-stimulated with LP14, APS or NPS and then, re-stimulated with enterotoxigenic *E. coli* (ETEC) 987P for 12 h. PIE cells without any stimulations were used as cell controls. Three or four independent experiments were conducted for each case and the average values (mean ± S.D.) are shown. The presence of various asterisks (* and **) indicates statistical differences with significant levels of $p < 0.05$ and $p < 0.01$ respectively.

pre-stimulated with *L. plantarum* N14 was significantly lower than the observed in control cells, while levels of IL-8 mRNA were higher than controls (Fig. 2B). NPS did not induce significant changes in IL-6 or IL-8 expression in PIE cells after the challenge with ETEC when compared to controls. On the contrary, PIE cells pre-treated with APS showed significantly lower levels of IL-6 and IL-8 mRNAs than control cells (Fig. 2B). The three treatments were equally effective for reducing the expression of MCP-1 in ETEC-challenged PIE cells (Fig. 2B).

3.3. Effect of blocking anti-TLR2 and anti-TLR4 antibodies on L. plantarum N14, APS and NPS immunomodulatory activities

We next evaluated whether anti-TLR2 or anti-TLR4 blocking antibodies modified the effect of *L. plantarum* N14 and its purified APS and NPS in ETEC-challenged PIE cells (Fig. 3). Anti-TLR2 antibodies completely abolished the capacity of *L. plantarum* N14 to reduce the levels of IL-6 and MCP-1 mRNAs after the challenge with ETEC, while no modifications were observed in IL-8 mRNA levels. In addition, blocking TLR2 abolished the reduction of MCP-1 induced by NPS treatment (Fig. 3A). On the contrary, anti-TLR2 antibodies had no effect on the immunoregulatory capacity of APS in ETEC-challenged PIE cells (Fig. 3A). No effect was observed in the expressions of IL-6, IL-8 and MCP-1 mRNAs when anti-TLR4 blocking antibodies were used in ETEC-challenged PIE cells previously treated with *L. plantarum* N14 or NPS (Fig. 3B). However, anti-TLR4 antibodies significantly reduced the capacity of APS to down-regulate the expression of the three pro-inflammatory cytokines in ETEC-challenged PIE cells (Fig. 3B).

3.4. Effect of L. plantarum N14, APS, and NPS in HEK^{TLR2} and HEK$^{RP105/MD1}$ cells

The human HEK293 cell line was transfected with plasmids encoding porcine TLR2 or porcine RP105 and MD1 in order to obtain HEK^{TLR2} and HEK$^{RP105/MD1}$ cells (Fig. 4A). The NF-κB reporter assay was performed in these cells in order to evaluate the capacity of *L. plantarum* N14, APS, and NPS to stimulate TLR2 or RP105 (Fig. 4B). *L. plantarum* N14 was able to stimulate HEK^{TLR2} cells while no effect was observed in HEK$^{RP105/MD1}$ cells. On the contrary, APS stimulated HEK$^{RP105/MD1}$ cells while no effect was observed in and HEK^{TLR2} cells. No detectable effect was observed for NPS in HEK transfectants (Fig. 4B).

In order to further evaluate the role of RP105 and TLR2 in the immunomodulatory effects of *L. plantarum* N14, APS, and NPS; we next evaluated the induction of intracellular Ca^{2+} fluxes in HEK^{TLR2} and HEK$^{RP105/MD1}$ cells (Fig. 4C). Pam3CSK4 (TLR2 ligand) as well as *L. plantarum* N14 and NPS increased the mobilization of Ca^{2+} in HEK^{TLR2} cells, while in HEK$^{RP105/MD1}$ cells fluorescence peaks for Ca^{2+} mobilizations were not observed after stimulation with those treatments. On the contrary, the stimulation with APS or LPS did not induce changes in calcium mobilization in HEK^{TLR2} cells. However, both APS and LPS significantly increased the mobilization of Ca^{2+} in HEK$^{RP105/MD1}$ cells (Fig. 4C).

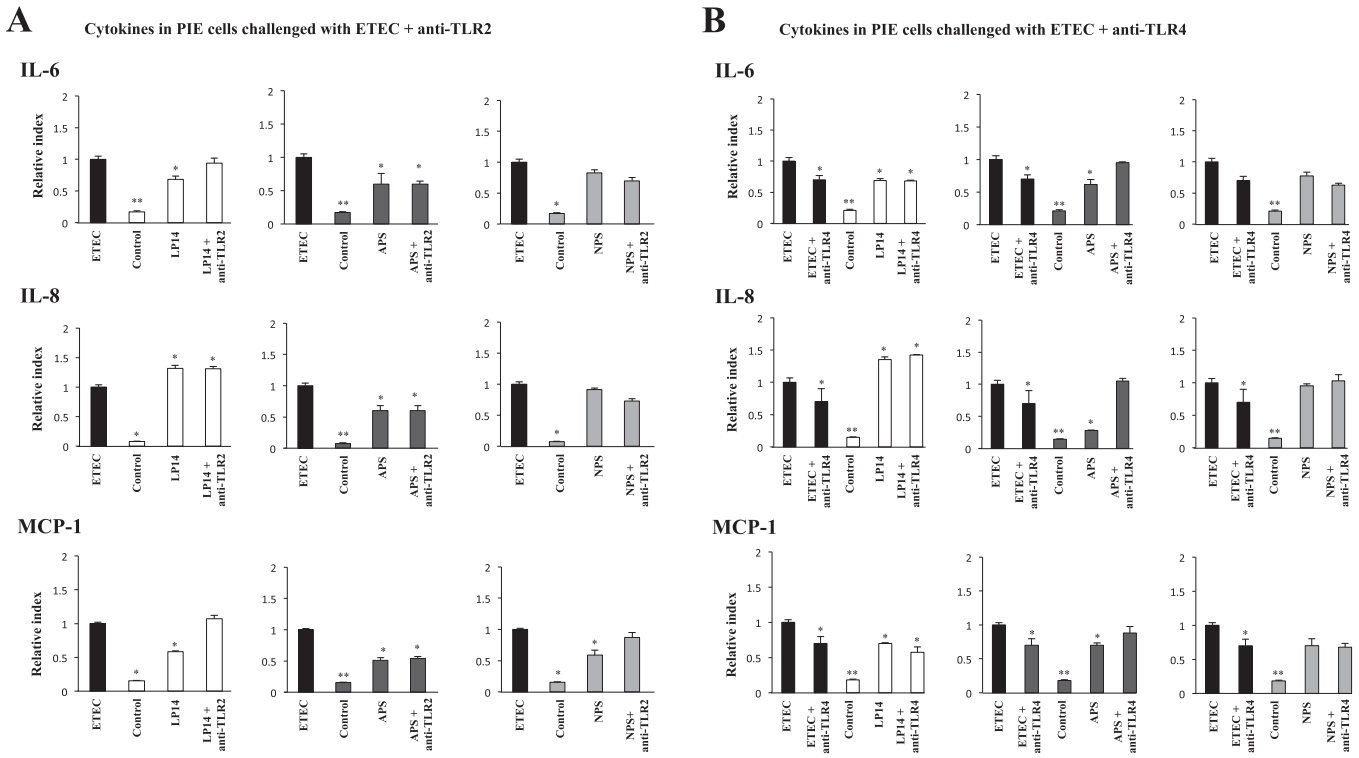

Fig. 3. Role of Toll-like receptor (TLR)-2 and TLR4 in the effect of *Lactobacillus plantarum* N14 (LP14), acidic extracellular polysaccharide (APS), and neutral extracellular polysaccharides (NPS) in porcine intestinal epitheliocyte (PIE) cells. PIE cells were pre-stimulated with LP14, APS or NPS in the presence and absence of anti-porcine TLR2 (A) and TLR4 (B) rabbit IgG antibodies and then, re-stimulated with enterotoxigenic *E. coli* (ETEC) 987P for 12 h. PIE cells without any stimulations were used as cell controls. Three or four independent experiments were conducted for each case and the average values (mean ± S.D.) are shown. The presence of various asterisks (* and **) indicates statistical differences with significant levels of $p < 0.05$ and $p < 0.01$ respectively.

3.5. *Effect of L. plantarum N14, APS, and NPS in PIE$^{TLR2-/-}$, PIE$^{RP105/MD1-/-}$ and PIE$^{TLR4-/-}$ cells*

We used RNA interference technology to knockdown RP105, TLR2 and TLR4 gene expression in PIE cells. For each receptor, three different siRNAs were used to transfect PIE cells and those with the higher capacity to inhibit receptors' expression were used in the subsequent experiments (supplemental Fig. 1). As shown in Fig. 5, the TLR2 ligand Pam3CSK4 significantly increased the expression of IL-8, IL-6 and TGF-β mRNAs in normal PIE cells. As expected, the capacity of Pam3CSK4 to increase these cytokines was abolished in PIE$^{TLR2-/-}$ cells while no effect was observed in PIE$^{RP1◦5/MD1-/-}$ and

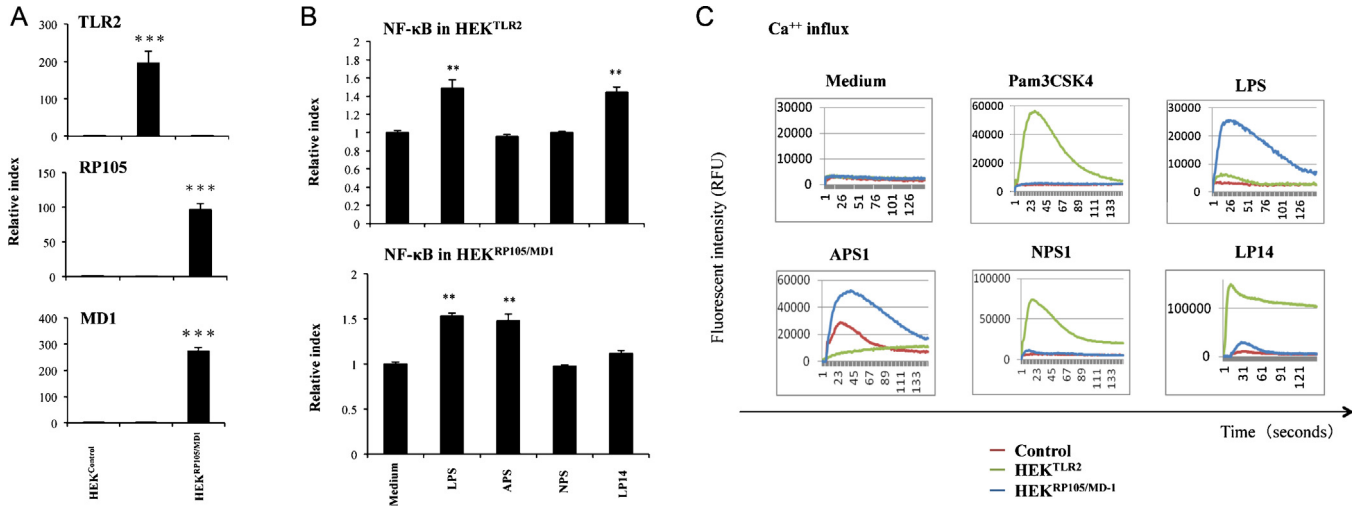

Fig. 4. Effect of *Lactobacillus plantarum* N14 (LP14), acidic extracellular polysaccharide (APS), and neutral extracellular polysaccharides (NPS) in transfectants expressing the toll-like receptor (TLR) family protein RP105/MD1 complex (HEK$^{RP105/MD1}$ cells) or TLR2 (HEK^{TLR2} cells). (A) Expression of transfected genes was confirmed by real-time RT-PCR. (B) The NF-κB reporter assay was performed in HEK$^{RP105/MD1}$ HEK^{TLR2} cells in order to evaluate the capacity of LP14, APS, and NPS to stimulate TLR2 or RP105. (C) Mobilization of intracellular calcium in HEK$^{RP105/MD1}$ HEK^{TLR2} cells after induction with LP14, NPS, APS, medium, LPS and Pam3CSK4 ligands. The induction of intracellular calcium mobilization was evaluated by recording fluorescence intensity with scanning at every 12 s time intervals. Three or four independent experiments were conducted for each case and the average values (mean ± S.D.) are shown. The presence of various asterisks (* and **) indicates statistical differences with significant levels of $p < 0.05$ and $p < 0.01$ respectively.

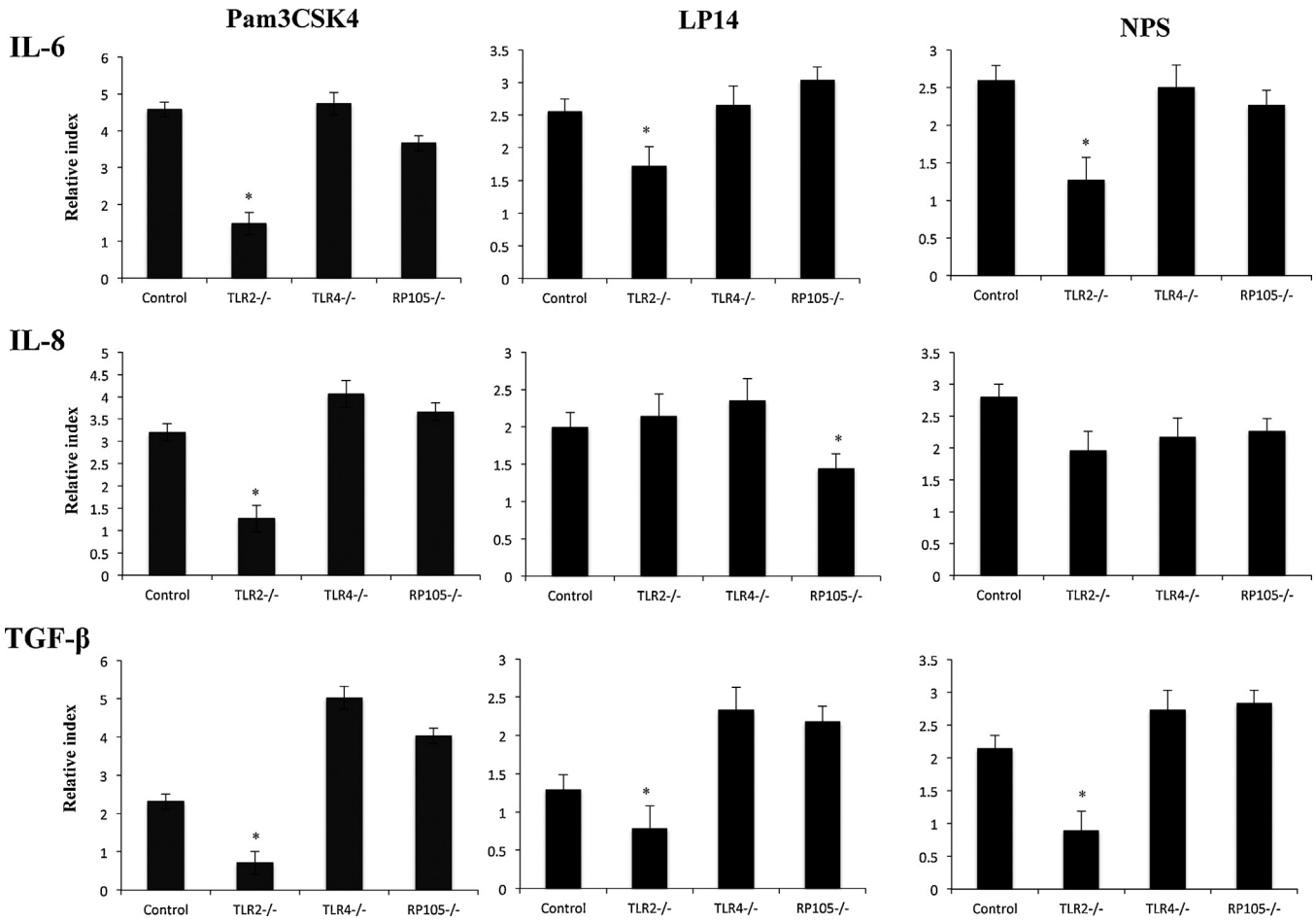

Fig. 5. Effect of *Lactobacillus plantarum* N14 (LP14), and neutral extracellular polysaccharides (NPS) and in Pam3CSK4 Toll-like receptor (TLR)-2, TLR4 and RP105 knockdown porcine intestinal epitheliocyte (PIE) cells. Normal PIE cells were used as cell controls. Three or four independent experiments were conducted for each case and the average values (mean ± S.D.) are shown. The presence of asterisks (*) indicates statistical differences with significant levels of $p < 0.05$.

$PIE^{TLR4-/-}$ cells when compared to normal PIE cells. The capacities of *L. plantarum* N14, and NPS to upregulate IL-6 and TGF-β mRNA were also reduced in $PIE^{TLR2-/-}$ cells. In addition, IL-8 mRNA was significantly reduced in $PIE^{RP105/MD1-/-}$ cells treated with *L. plantarum* N14 when compared with normal cells (Fig. 5).

Supplementary Fig. 1 related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.molimm.2014.10.027.

LPS significantly increased the expression of IL-8 and IL-6 normal PIE cells while slightly increased TGF-β mRNA. The capacity of LPS to augment IL-8 and IL-6 mRNAs was reduced in $PIE^{RP105/MD1-/-}$ and $PIE^{TLR4-/-}$ cells while no effect was observed in $PIE^{TLR2-/-}$cells when compared to normal PIE cells (Fig. 6). The capacity of APS to increase IL-8 and IL-6 was abolished in $PIE^{RP105/MD1-/-}$ and $PIE^{TLR4-/-}$ cells while no effect was observed in $PIE^{TLR2-/-}$cells when compared to normal cells (Fig. 6).

3.6. Effect of L. plantarum N14, APS and NPS on negative regulators of the TLR signaling pathway

Finally, we studied regulators that inhibit the TLR signaling pathway. To assess the expression of these negative regulators of TLRs, we previously cloned cDNAs corresponding to these proteins from swine (Shimazu et al., 2012). Then, expression of SIGIRR, Tollip, A20, Bcl-3, MKP-1, and IRAK-M mRNAs were evaluated in ETEC-challenged PIE cells. None of the treatments induced changes in expression of SIGIRR, or Tollip (Fig. 7). *L. plantarum* N14 was able to significantly increase the expression of A20, Bcl-3 and MKP-1 mRNAs while NPS treatment up-regulated the expression of A20 and Bcl-3 (Fig. 7). IRAK-M and MKP-1 mRNAs were significantly increased in ETEC-challenged PIE cells previously treated with APS (Fig. 7).

4. Discussion

In the past decade there was an enormous interest for a better understanding of the molecular processes underlying host–immunobiotic interactions. It was established that the molecular mechanism of probiotics immunomodulatory activity depends on the combination of distinct microbial-associated molecular patterns (MAMPs) that interact with various PRRs and the associated co-receptors that fine tune signaling (Lebeer et al., 2010). Therefore, host–immunobiotic interactions are not univocal but involve complex interactions among various microbial molecules, host receptors, and adaptor molecules. TLR2 is among the receptors that have been associated to beneficial probiotic effects. It was reported that bifidobacteria inhibit the production of IL-6 and TNF-α induced by immunostimulatory lactobacilli in blood immune cells *via* interaction with TLR2 (Zeuthen et al., 2008). In addition, anti-inflammatory probiotic bacteria induce much higher levels of IL-12 and lower IL-10 levels in bone marrow-derived DCs from TLR2 knockout mice compared with wild-type DCs (Zeuthen et al., 2008). More recently, Finamore et al. (2014) showed that *Lactobacillus amylovorus* DSM 16698T suppress the activation of TLR4

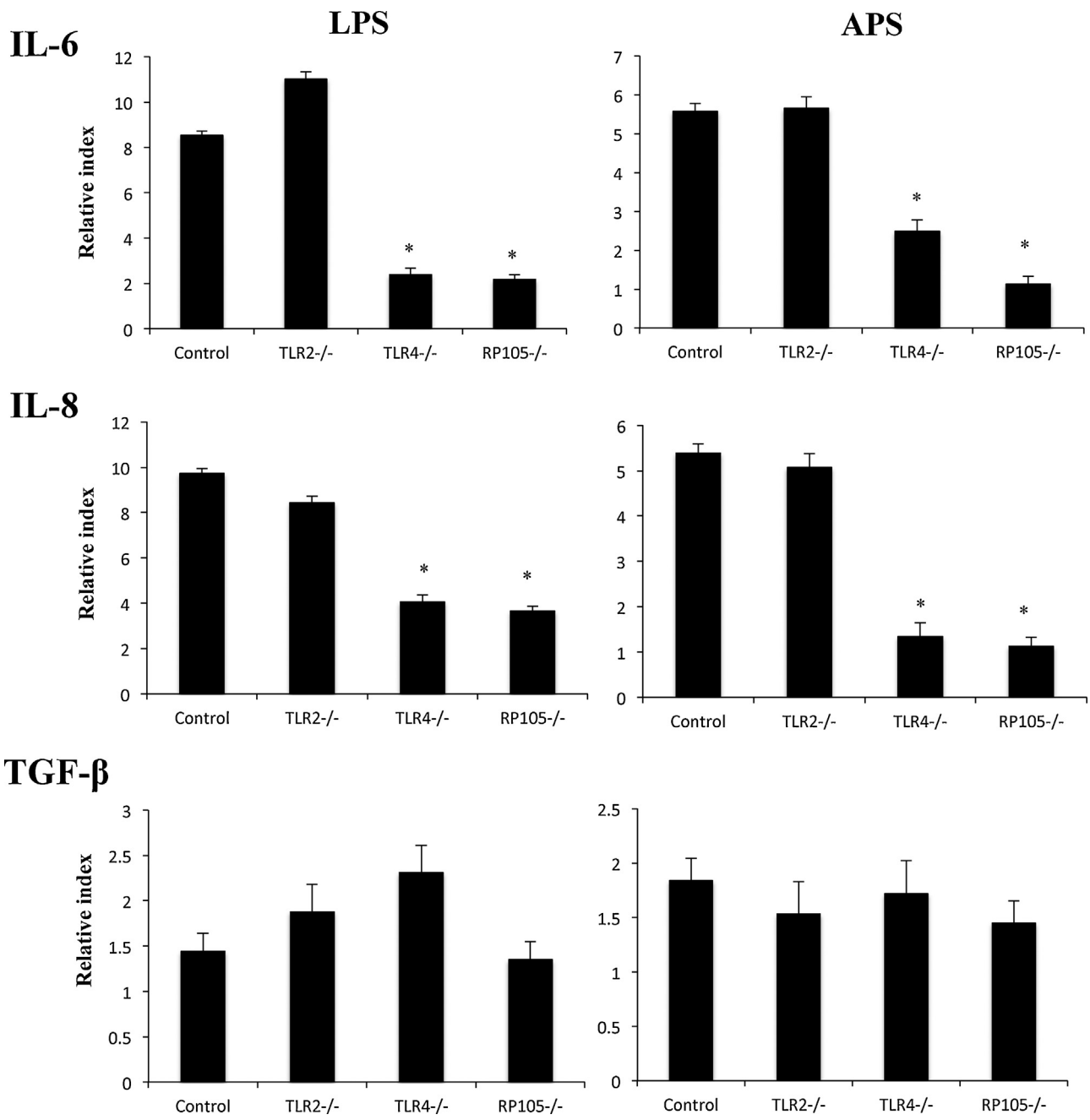

Fig. 6. Effect of acidic extracellular polysaccharide (APS) from *Lactobacillus plantarum* N14 and LPS in Toll-like receptor (TLR)-2, TLR4 and RP105 knockdown porcine intestinal epitheliocyte (PIE) cells. Normal PIE cells were used as cell controls. Three or four independent experiments were conducted for each case and the average values (mean ± S.D.) are shown. The presence of asterisks (*) indicates statistical differences with significant levels of $p < 0.05$.

signaling in Caco-2/TC7 cells through modulation of the negative regulators Tollip and IRAK-M and, that this effect was dependent on TLR2 signaling. Our previous studies in PIE cells and porcine immune cells also revealed that TLR2 plays an important role in the immunoregulatory activity of immunobiotic bacteria (Shimazu et al., 2012; Tomosada et al., 2013; Villena et al., 2012; Murata et al., 2014). Our studies of the anti-inflammatory activity of *L. jensenii* TL2937 in PIE cells showed that TLR2 have an important role in the immunoregulatory capacities of this strain. In fact, we showed that both this strain and the TLR2 ligand Pam3CSK4 increased expression of A20 and Bcl-3 mRNA and reduced the expression of pro-inflammatory cytokines in ETEC- and LPS-challenged PIE cells (Shimazu et al., 2012). Later, we also reported that bifidobacteria with the highest capacity to down-regulate the expression of inflammatory cytokines in response to heat-killed ETEC, were also capable of up-regulated A20 in PIE cells in a TLR2-dependent manner (Tomosada et al., 2013). Similarly, we demonstrated here that TLR2 has a significant role in the immunoregulatory effect of *L. plantarum* N14 in PIE cells (Fig. 8).

We used TLR2 knockdown PIE cells and calcium mobilization to evaluate the capability of *L. plantarum* N14 to modulate cytokines production in PIE cells. Calcium (Ca^{2+}) can be induced by variety of extracellular stimulants *via* TLRs and is essential for NF-κB activation and gene expressions (Gallo et al., 2006; Chun and Prince, 2006). TLR2 ligands induce intracellular Ca^{2+} mobilization in bone marrow derived macrophages and DCs (Aki et al., 2008). Pam3-Cys-Ser-Lys, a synthetic ligand of TLR2, also induce Ca^{2+} flux in murine macrophages and human airway epithelial cells (Chun

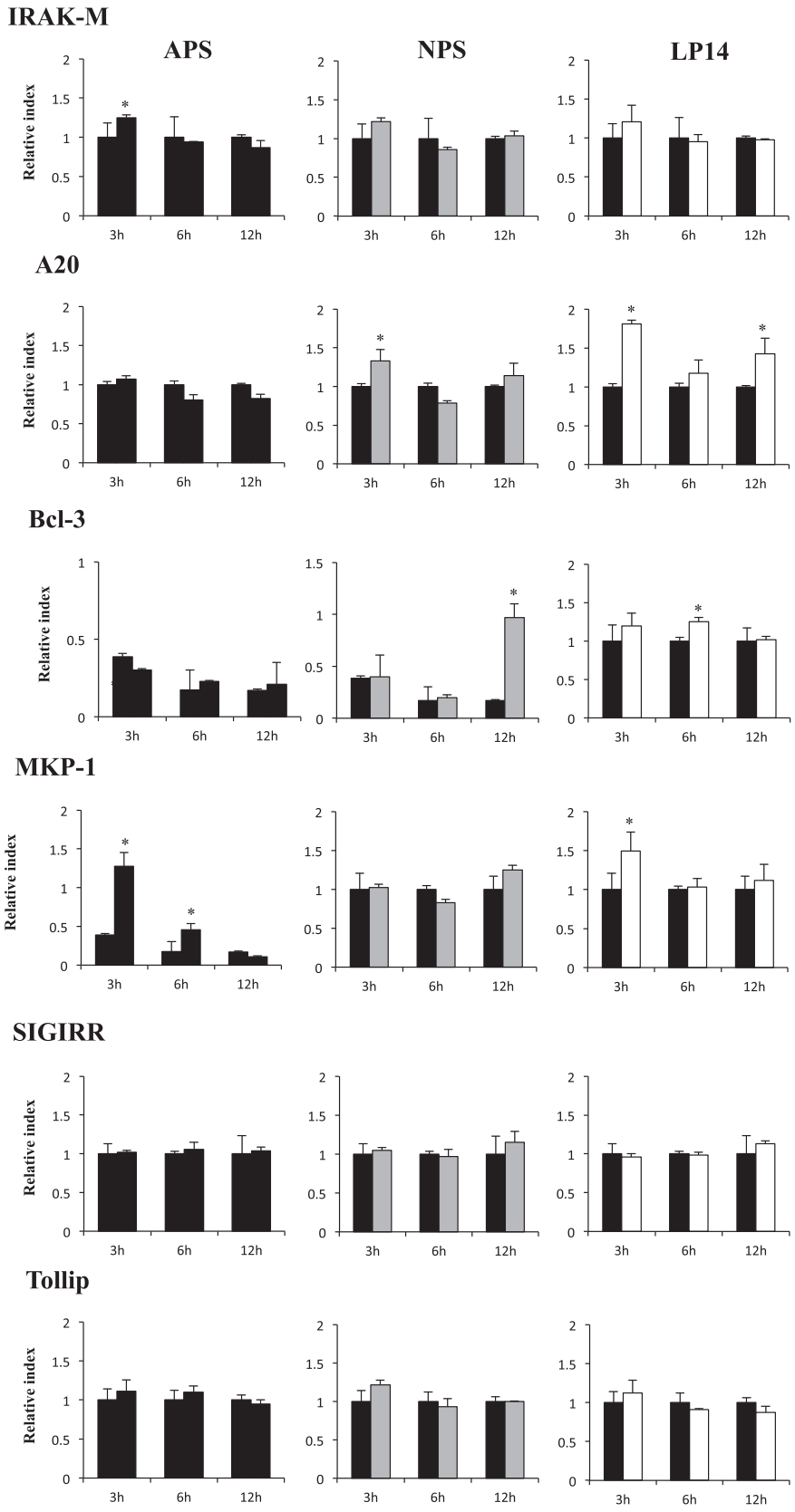

Fig. 7. Effect of *Lactobacillus plantarum* N14 (LP14), acidic extracellular polysaccharide (APS), and neutral extracellular polysaccharides (NPS) on the expression of Toll-like receptors (TLR) negative regulators in porcine intestinal epitheliocyte (PIE) cells. PIE cells were stimulated with LP14, APS or NPS and the expression of SIGIRR, Tollip, A20, Bcl-3, MKP-1 and IRAK-M negative regulators was evaluated after 3, 6 or 12 h. Three or four independent experiments were conducted for each case and the average values (mean ± S.D.) are shown. The presence of asterisks (*) indicates statistical differences with significant levels of $p < 0.05$.

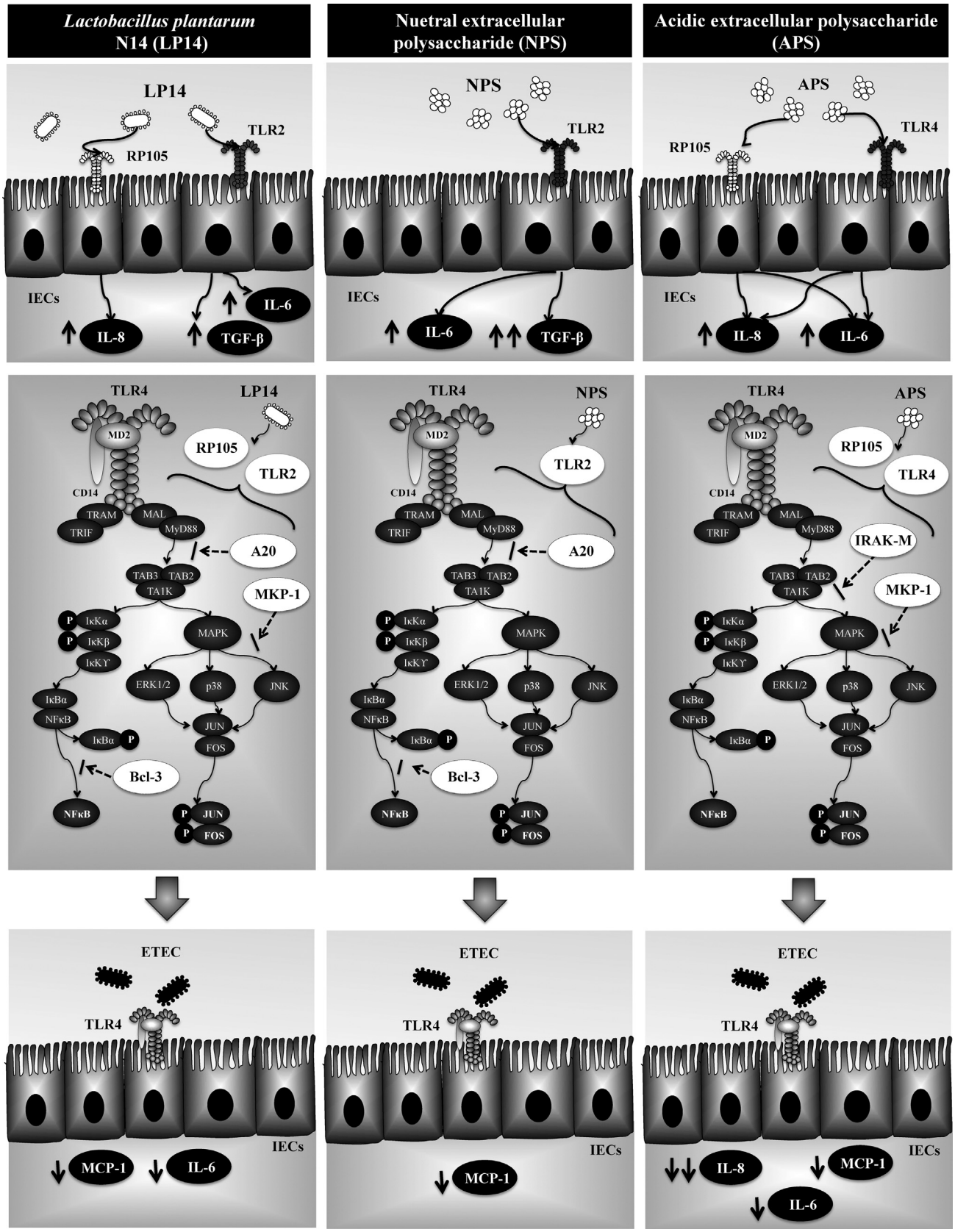

Fig. 8. Proposed mechanisms for immunomodulation by *Lactobacillus plantarum* N14 (LP14), acidic extracellular polysaccharide (APS), and neutral extracellular polysaccharides (NPS) in porcine intestinal epithelial (PIE) cells after stimulation with enterotoxigenic *E. coli* (ETEC).

and Prince, 2006; Aki et al., 2008). Then, by coupling the use of TLR2 knockdown PIE cells and calcium mobilization, we accurately demonstrated that the immunoregulatory effects of *L. plantarum* N14 depended on TLR2. In fact, the capacity of *L. plantarum* N14 to up-regulate the expression of IL-6 and TGF-β in normal PIE cells was reduced in $PIE^{TLR2-/-}$ cells. In addition, *L. plantarum* N14 was able to up-regulate A20 and Bcl-3 in PIE cells, which are known to depend on TLR2 activation in this cell line (Shimazu et al., 2012).

This work also demonstrates that EPS produced by *L. plantarum* N14 have important roles in the immunoregulatory capacity of this strain. The biosynthesis of EPS has been described in several species of *Lactobacillus*, as well as their role in their immunoregulatory capabilities (Patten et al., 2014; Wachi et al., 2014). It was reported that purified EPS produced by *Lactobacillus rhamnosus* RW-9595M, exerted immunosuppressive properties in macrophages by inducing high levels of IL-10 and low levels of TNF-α, IL-6 and IL-12 (Bleau et al., 2010). It was also demonstrated that mutant types of *L. casei* strain Shirota with defective cell wall-associated high molecular mass polysaccharides are potent inducers of IL-12, TNF-α, and IL-6, indicating that high molecular mass polysaccharides may give suppressor signals (Yasuda et al., 2008). In addition, studies evaluating the EPS-producing *Lactobacillus paraplantarum* BGCG11 strain and three Muc^- derivative strains demonstrated that EPS has an immunoregulatory effect since stimulation of human PBMC by the BGCG11 strain induced a Th2-Treg profile while the derivative strains stimulated a Th17 response (Nikolic et al., 2012). These works clearly show the immunoregulatory potential of EPS produced by lactobacilli. However, the cellular and molecular mechanism(s) involved in the immunoregulatory affects of EPS have not been studied in detail.

We demonstrated here that NPS was able to modulate the expression of cytokines in PIE cells in a TLR2-dependent manner (Fig. 8). Similarly to *L. plantarum* N14, the capacity of NPS to upregulate the expression of IL-6 and TGF-β in normal PIE cells was reduced in $PIE^{TLR2-/-}$ cells. Some works have described the ability of TLR2 to recognize bacterial polysaccharides (Lin et al., 2011; Graveline et al., 2007). The extracellular polysaccharide from the biofilm of *Thermus aquaticus* is recognized by TLR2. This was confirmed by the induction of IL-6 production in peritoneal macrophages from wild-type mice but not from $TLR2^{-/-}$ mice (Lin et al., 2011). The recognition of the capsular polysaccharide from *Streptococcus suis* also depends on TLR2 (Graveline et al., 2007). The stimulation of human monocytes with *S. suis* capsular polysaccharide triggered the release of IL-1, IL-6, IL-8 and MCP-1, which was significantly reduced by antibody-mediated blocking of TLR2. Then, our results indicate that NPS would be partially responsible of the immunomodulatory effects of *L. plantarum* N14 through its ability to stimulate TLR2. To further support this hypothesis we observed here that purified NPS was able to up-regulate A20 and Bcl-3, which are negative regulators that are expressed in PIE cells after TLR2 activation (Shimazu et al., 2012).

In addition, we demonstrated that APS from *L. plantarum* N14 has anti-inflammatory effects in PIE cells. Treatment of porcine IECs with APS up-regulated the expression of IRAK-M and MCP-1; and significantly reduced the expression of IL-6, IL-8 and MCP-1 after the challenge with ETEC. Moreover, we demonstrated that those effects depend on TLR4 and RP105 (Fig. 8).

Some works reported immunoregulatory/anti-inflammatory effects of polysaccharides and oligosaccharides through TLR4 activation (Capitán-Cañadas et al., 2014; Kovacs-Nolan et al., 2013). The effects of fructooligosaccharides (FOS) and inulin were evaluated in splenocytes from $TLR4^{-/-}$ and wild-type mice (Capitán-Cañadas et al., 2014). Both carbohydrates evoked cytokine secretion (TNF-α, IL-6, and IL-10) by mouse splenocytes but inhibited LPS-induced IFN-γ and IL-17 release. Interestingly, splenocytes from $TLR4^{-/-}$ mice showed a markedly depressed response, indicating that both carbohydrates act as TLR4 ligands. It was also described, using an LPS-induced model of nonlethal endotoxemia in mice, that the non-digestible disaccharide β-1,4-mannobiose (MNB) has anti-inflammatory effects. Authors also reported that MNB act as a TLR4 agonist (Kovacs-Nolan et al., 2013). In addition to their effects in immune cells, carbohydrates are also able to modulate immunity in IECs. It was documented that oligosaccharides reduce pro-inflammatory cytokine expression (IL-12p35, IL-8, and TNF-α) in Caco-2 cells, indicating that they can modulate IECs immune response by activation of PPARγ and inhibition of NF-κB (Zenhom et al., 2011). Moreover, it was described recently that inulin, goat's milk oligosaccharides or galactooligosaccharides were able to modulate the production of pro-inflammatory mediators in IECs lines. Interestingly, this work demonstrated for the first time that these prebiotic oligosaccharides act as TLR4 ligands in IECs and that the immunoregulatory effects were dependent on TLR4, Myd88, and NF-κB (Ortega-González et al., 2014). The results presented in this work are in line with these previous findings, by demonstrating a significant role for TLR4 in the immunoregulatory/anti-inflammatory activity of APS from *L. plantarum* N14 in PIE cells.

Of note, we have described for the first time here that EPS from *L. plantarum* N14 are able to reduce inflammation in PIE cells and that this effect was partially dependent on RP105/MD1 activation. In the presence of RP105 the activation of the TLR4 signaling pathway in myeloid cells is directly reduced, making RP105 one of the most important accessory molecules acting as a regulator of TLR4 signaling (Divanovic et al., 2005a,b). On the other hand, $RP105^{-/-}$ mice as well as $MD1^{-/-}$ mice show reduced LPS-dependent proliferation and CD86 up-regulation in B cells, indicating that the RP105/MD1 complex functions as a complementary receptor, and augments TLR4/MD2-mediated LPS responses. Moreover, RP105/MD1 is thought to bind LPS and other TLRs ligands; therefore this complex has been also linked to several inflammatory processes (Tada et al., 2008; Watanabe et al., 2013). Responses to synthetic TLR2 agonists, such as the lipopeptides Pam3CSK4 and MALP-2, have been reported to be impaired in $RP105^{-/-}$ B cells, however, in $RP105^{-/-}$ macrophages and DCs, TNF-α production in response to these TLR2 agonists was not different from that in wild type cells. Then, cell-specific differences seem to determine the different roles for RP105 in mediating cell activation. This hypothesis is supported by the finding that in B cells, cross-linking of RP105 by a specific antibody induced proliferation (Miyake et al., 1994), whereas the same antibody failed to activate macrophages or DCs (Divanovic et al., 2005a,b). However, the role of RP105/MD1 in the immunobiology of IECs has not been described. In addition, the involvement and possible role of RP105/MD1 in immunobiotic activities was not reported before. We demonstrated here that RP105/MD1 is necessary for the production of pro-inflammatory cytokines by PIE cells in response to LPS and ETEC, indicating that this receptor complex is functional in this porcine cell line. In addition, we demonstrated that RP105/MD1 has an important role in the anti-inflammatory effect of from *L. plantarum* N14 in PIE cells.

In conclusion, our results demonstrate that not only TLR2 but also TLR4 and RP105 are involved in the immunoregulatory effects of the immunobiotic strain *L. plantarum* N14. TLR2, TLR4 and RP105 cooperate to induce the expression of negative regulators of the TLR signaling (A20, Bcl-3, IRAK-M and MKP-1) and reduce the production of inflammatory cytokines in intestinal epithelial cells in response to ETEC challenge. Our work also demonstrates that both NPS and APS play unique roles in the immunomodulatory effect of *L. plantarum* N14. Moreover, purified APS seems

to have stronger anti-inflammatory effects than *L. plantarum* N14 since it was the only treatment able to reduce the expression of the three pro-inflammatory cytokines studied. Therefore *L. plantarum* N14 or purified APS are good candidates for *in vivo* studying the protective effect against intestinal inflammatory damage induced by ETEC infection or ETEC PAMPs challenge in the porcine host.

Conflict of interest

The authors declare no conflicting financial interests.

Acknowledgements

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