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BRIEF COMMUNICATION

Advanced application of porcine intestinal epithelial cells for the selection of immunobiotics modulating toll-like receptor 3-mediated inflammation

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KEYWORDS
Anti-inflammatory response;
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Purpose: In this study, we aimed to characterize toll-like receptor (TLR)-3-mediated inflammatory immune response in porcine intestinal epithelial (PIE) cells and in PIE-immune cell co-cultures and, to evaluate if these in vitro systems are useful for selecting immunomodulatory lactic acid bacteria.
Results: We demonstrated that these systems are valuable tools for the *in vitro* study of the inflammatory response triggered by TLR3 in intestinal epithelial cells (IECs) and of the interaction between IECs and immune cells. In addition, we showed that PIE cells could be used for

Abbreviations: B-cell lymphoma 3-encoded protein (Bcl-3), Chemokine (C-C motif) ligand 2 (CCL2); Chemokine (C-X-C motif) ligand 8 (CCL8), Interleukin (IL); Interleukin-1 receptor-associated kinase M (IRAK-M), Intestinal epithelial cell (IEC); Lactic acid bacteria (LAB), Microbe-associated molecular patterns (MAMPs); Mitogen-activated protein kinase phosphatase 1 (MKP-1), Pathogen-associated molecular patterns (PAMPs); Peyer's patches (PPs), Porcine intestinal epitheliocyte cell line (PIE cells); Single immunoglobulin IL-1R-related molecule (SIGIRR), Toll interacting protein (TOLLIP); Toll-like receptor (TLR), Ubiquitin-modifying enzyme A20 (A20).

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the selection of immunobiotic lactobacilli strains with anti-inflammatory activities. We found that *Lactobacillus casei* MEP221114 is an immunobiotic candidate for modulation of TLR3-mediated inflammatory responses.
Conclusion: The present study deepened our understanding of the mechanisms of immunobiotic action by demonstrating that the interaction between some lactobacilli strains and IECs can up-regulate the mRNA expression of TLR negative regulators and that this effect could help to regulate the production of inflammatory mediators during the generation of a TLR3-mediated immune response.
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Introduction

Toll-like receptor (TLR) signaling has been shown to be involved in intestinal epithelial cell (IEC) proliferation, IgA production, maintenance of tight junctions, and antimicrobial peptide expression, which are crucial functions for maintaining a healthy epithelial barrier. Epithelial TLR expression is also thought to be important for host defense against bacterial and viral pathogens through initial recognition of pathogen or microbe-associated molecular patterns (PAMPs/MAMPs). Despite this clear beneficial role for TLR signaling in IECs, TLRs can also trigger proinflammatory responses and, if dysregulated, may precipitate the development of inflammatory tissue damage.[1–3] In this regard, it has been shown that the inflammatory response induced by viral interaction with TLR3 can contribute to host tissue damage during viral infections.[2–5] Thus, in some cases, TLR3 responds to viral infection, but does not display a protective role. In addition, recent studies also demonstrated that probiotic lactic acid bacteria (LAB) can modulate virus induced-inflammatory responses in IECs,[6] suggesting the possibility that probiotics might regulate immune responses to viral infection.

In recent years, there has been a growing interest in the immune system of swine because of their economic importance as livestock and their possible use as a model for the human immune system.[7–9] We have recently developed a clonal porcine intestinal epitheliocyte (PIE) line for *in vitro* analyses.[10–13] Using real time RT-PCR and immunohistochemical analysis we observed an abundant expression of TLR3 in this cell line.[10–13] This result was in agreement with studies that demonstrated that IECs preferentially express TLR3 compared to other cell types in many organs including the airways and the gastrointestinal tracts.[14] Therefore, the aim of the present study was to evaluate whether PIE cells could be a useful tool for the study of TLR3 activation in IECs and for the selection of LAB strains that can modulate TLR3-mediated inflammation.

Materials and methods

PIE cell monocultures

PIE cells are nontransformed intestinal cell lines originally derived from intestinal epithelia isolated from a presuckling neonatal swine.[10] When PIE cells are cultured they assume a monolayer with a cobblestone and epithelial-like morphology and with close contact between cells. In addition, PIE cells are strongly positive for cytokeratin K8.13, a marker for porcine intestinal epithelial cells.[10] PIE cells were passaged by treatment with a sucrose/EDTA buffer for 4 min, detached using 0.04% trypsin in PBS, and then plated at a density of 1.5×10^4 cells/cm^2 in a type I collagen-coated dish (SUMILON, Tokyo, Japan) at 37 °C in an atmosphere of 5% CO_2. PIE cells were cultured in 10% fetal calf serum (FCS) Dulbecco's modified Eagle medium (DMEM)and passaged every 3 or 4 days. In the present study, we used PIE generations from 20 to 30.

Co-cultures of PIE and immune cells

Single-immune cell suspensions of porcine Peyer's patches (PP) were prepared from the ileum of adult swine as described previously.[11] Briefly, after cutting the specimens into small fragments, they were 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). Residual erythrocytes were lysed by resuspension in hypotonic salt solution (0.2% NaCl), followed by hypertonic rescue in an equal volume of 1.5% NaCl. Finally, immune cells were fractionated using Lympholyte-Mammal (Cedarlane, Hornby, Canada) density gradient centrifugation and the obtained cells were suspended in complete DMEM (Invitrogen, Tokyo, Japan) supplemented with 10% FCS (Sigma), 50 μg/ml penicillin/streptomycin and 50 μg/ml gentamicin (Nacalai Tesque, Kyoto, Japan).This mononuclear cell suspension contains a mixed population of T ($CD4^+$ and $CD8^+$), B ($CD21^+$) and antigen presenting cells ($CD4^-$ $CD8^-$ $MHCII^+$) (11).

For the co-culture experiments in the Transwell culture system, PIE cells were seeded on the apical surface of the transwell at a concentration of 1.5×10^5 cells/well in 12-well tissue culture plate [Transwell-Col. (PTFE), pore size 0.2 mm] while porcine PP immunocompetent cells were seeded in the basolateral compartment at a concentration of 2×10^7 cells/well.

Immunomodulatory activity of lactobacilli towards PIE cells

The following lactobacilli strains were used in this study: *Lactobacillus reuteri* MEP221101 and MEP221102; *L casei* MEP221103, TL2768, MEP221104, MEP221105, MEP221106, MEP221107, MEP22b1108, MEP221109, MEP221114 and MEP221115; *L rhamnosus* MEP221110, MEP221111, MEP221112

and GG; *L salivarius* MEP221113; *L jensenii* TL2937 and *L gasseri* MEP221117. The lactobacilli strains were isolated from human feces and obtained from the library of Meiji Co. (Odawara, Japan). The lactobacilli strains were grown in MRS medium (Difco, Detroit, MI, USA) for 16 h at 37 °C and washed with PBS. Lactobacilli were re-suspended in DMEM, enumerated under a microscope using a Petroff-Hausser counting chamber, and stored at −80 °C until use.

PIE cells were plated at a concentration of 1.5×10^4 cells/well on type I collagen-coated 24 well plates (SUMILON), and cultured for three days. After changing the medium, lactobacilli (5×10^7 cells/ml) were added and, 48 h later, each well was washed vigorously with medium at least three times to eliminate all of the stimulants, and the cells were then stimulated with poly(I:C) for the indicated times.

Quantitative real-time reverse transcription polymerase chain reactions analyses of mRNA expression in PIE and immune cells

Two-step real-time quantitative reverse transcription (qRT-PCR) was used to characterize the expression of mRNAs in PIE cells.[10–13] Total RNA from each sample was isolated from the PIE cells using TRIzol reagent (Invitrogen). All cDNAs were synthesized using a Quantitect Reverse Transcription 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, Cheshire, UK) using Platinum SYBR Green qPCR SuperMix UDG with ROX (Invitrogen). The primers used in this study were described previously.[10,11] The PCR cycling conditions were 5 min at 50 °C; followed by 2 min at 95 °C; then 40 cycles of 15 sec at 95 °C, 30 sec at 60 °C and 30 sec at 72 °C. The reaction mixture contained 5 μl of the sample cDNA and 15 μl of the master mix including 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 index.

TLR negative regulators

This study focused on the six TLR negative regulators [single immunoglobulin IL-1R-related molecule (SIGIRR); AB490122, toll interacting protein (Tollip); AB490123, Ubiquitin-modifying enzyme A20 (A20); AB490119, B-cell lymphoma 3-encoded protein (Bcl-3); AB490120, mitogen-activated protein kinase phosphatase 1 (MKP-1); AB490121 and Interleukin-1 receptor-associated kinase M (IRAK-M); partial] whose open reading frames or partial cDNAs have already been cloned.[12] Their mRNA expression was analyzed using real time qRT-PCR with equivalent designed primers.[12] Real time qRT-PCR was performed as described above.

Statistical analysis

The statistical analysis for all data was performed using GLM of the SAS computer program (SAS, 1994). Relative index was calculated as the ratio of mRNA expression of cytokines to one of β-actin normalized by common logarithmic transformation of expression. Comparisons among mean values for relative mRNA expressions of cytokines and chemokines in cells were carried out using one-way ANOVA. This test was performed considering the effect of time in Fig. 1, and the effect of stimuli in Fig. 2. In addition, the effect of stimuli for each time was considered in Figs. 3 and 4. Fisher's least significant difference multi comparison test (LSD) against the adjusted controls was then carried out. For these analyses, p-values <0.05 and <0.01 were considered significant, and shown as * and **, respectively.

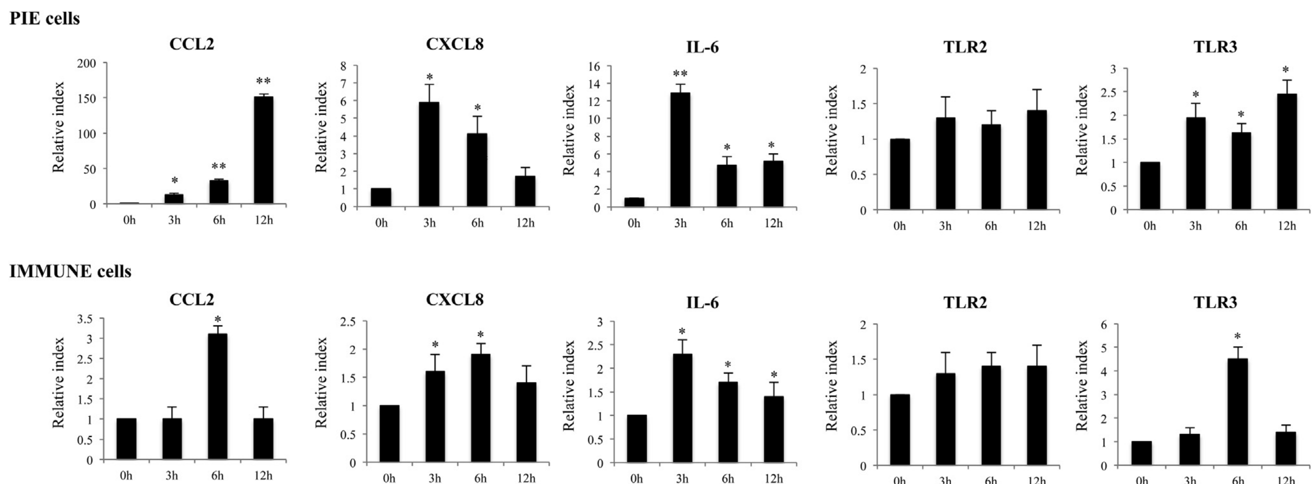

Figure 1. Expression of cytokines and Toll-like receptors (TLRs) in porcine intestinal epithelial (PIE) and immune cells after stimulation of monocultures and co-cultures with poly(I:C). PIE cells in monoculture or co-culture were pretreated with different lactobacilli strains (5×10^7 cells/ml) for 48 h, stimulated with poly(I:C) (60 ng/ml) and the mRNA expression of cytokines and TLRs was then analyzed at the indicated times post-stimulation. Expression of CCL2, CXCL8, IL-6, TLR2, and TLR3 in PIE cells (monocultures) and in immune cells (co-cultures) are shown. Values shown are means, and error bars indicate standard deviations. Values are shown relative to the value at 0 h, which was assigned a value of 1. The results are means of three measures repeated three times in independent experiments. **$p<0.01$ and *$p<0.05$ vs. cells cultured in the absence of poly(I:C).

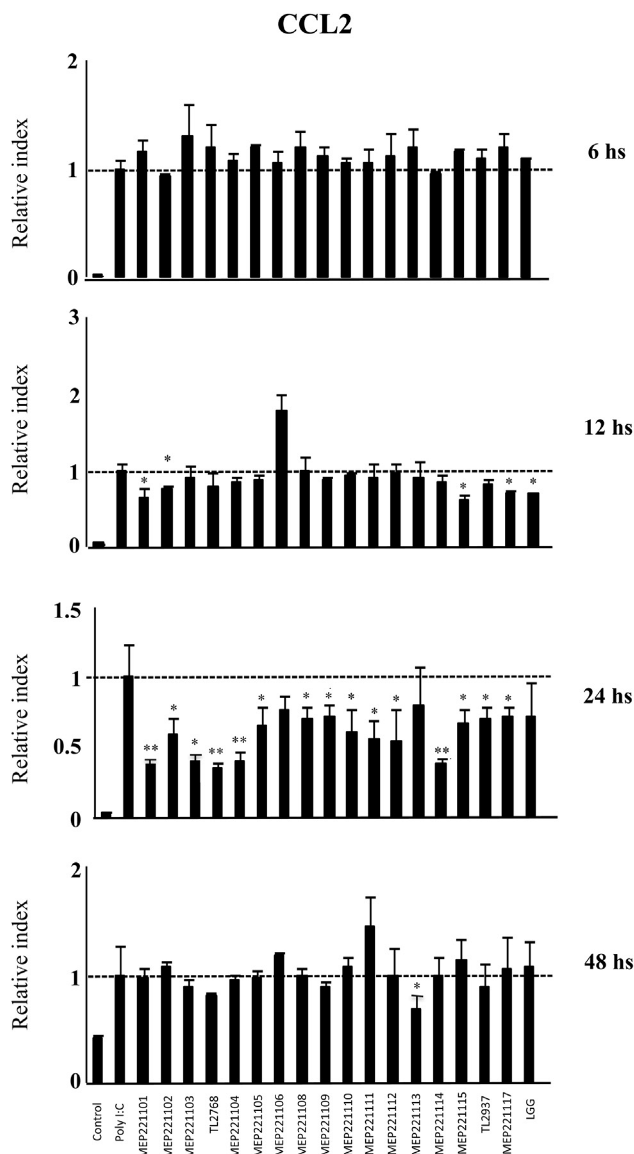

Figure 2. Selection of immunomodulatory lactobacilli. Porcine intestinal epithelial (PIE) cells were pretreated with the indicated lactobacilli strains (5×10^7 cells/ml) for 6, 12, 24, or 48 h, were then stimulated with poly(I:C) (60 ng/ml)for 12 h, following which the mRNA expression of CCL2 was analyzed. Values shown are means and error bars indicate standard deviations. Values are shown relative to the value of cells stimulated with only poly(I:C), which was assigned a value of 1. The results are means of three measures repeated three times in independent experiments.**$p<0.01$ and *$p<0.05$ vs. cells stimulated with poly(I:C) without previous treatment with bacteria.

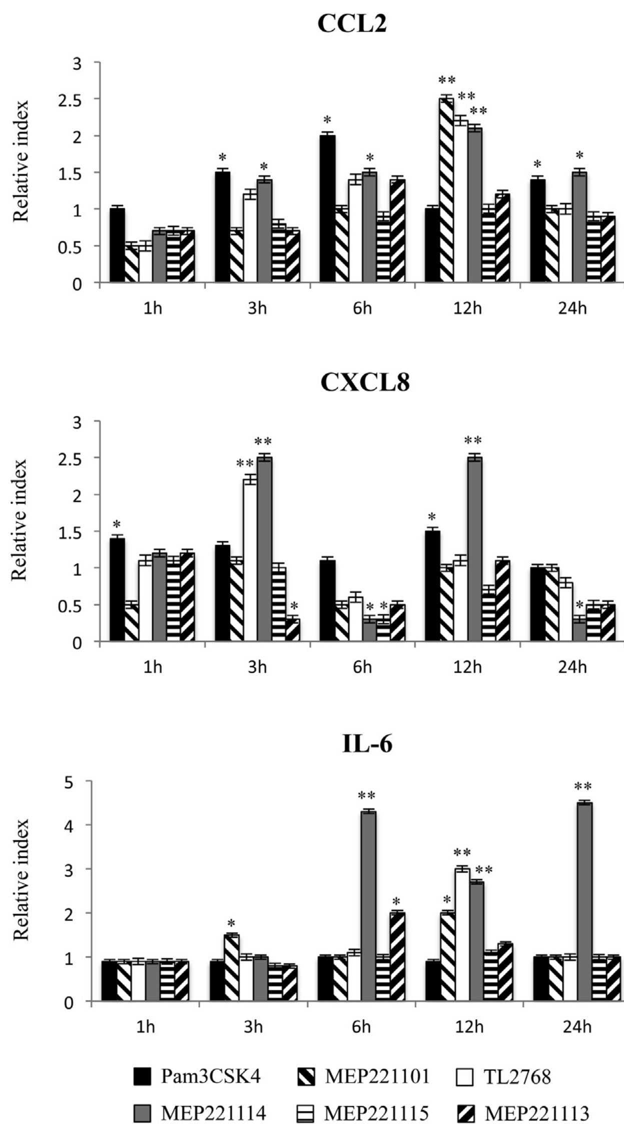

Figure 3. Expression of cytokines in porcine intestinal epithelial (PIE) after stimulation with selected lactobacilli strains. PIE cells were stimulated with *L reuteri* MEP221101, *L casei* TL2768, *L casei* MEP221114, *L casei* MEP221115, or *L salivarius* MEP221113(5×10^7 cells/ml) or with the TLR2 agonist Pam3CSK4 for 1, 3, 6, 12, or 24 h and the mRNA expression of CCL2, CXCL8, and IL-6 was then analyzed. Values shown are means and error bars indicate standard deviations. Values are shown relative to the value of nonstimulated cells, which was assigned a value of 1. The results are means of three measures repeated three times in independent experiments. **$p<0.01$ and *$p<0.05$ vs. cells cultured in the absence of lactobacilli.

Results

The first aim was to characterize the inflammatory immune response triggered by TLR3 activation in PIE cells. For this purpose, PIE cells were stimulated with a TLR3 ligand, poly(I:C), and the mRNA expression of proinflammatory cytokines was analyzed. Challenge with the TLR3 agonist significantly increased the expression of IL-6, CCL2/MCP-1 and CXCL8/IL-8 in PIE cells (Fig. 1). Levels of IL-6 and CXCL8 mRNA increased rapidly after poly(I:C) stimulation, reaching a maximum value at 3 hours. In contrast, the CCL2 level increased more gradually and did not reach its highest level of expression until 12 hours. The mRNA expression of TLR2 and 3 was also evaluated, and it was found that only that of TLR3 was significantly up-regulated with poly(I:C) challenge (Fig. 1). Since activation of innate immune signaling in IECs

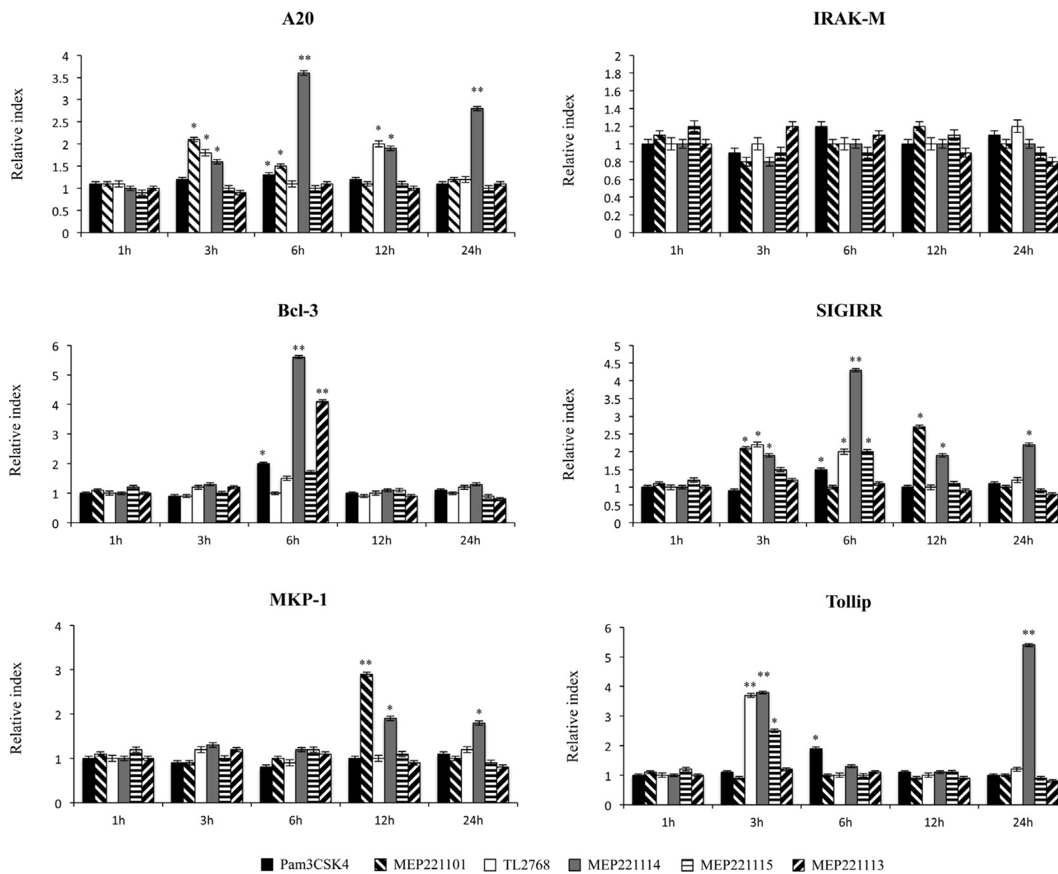

Figure 4. Expression of toll-like receptor (TLR) negative regulators in porcine intestinal epithelial (PIE) cells after stimulation with selected lactobacilli strains.PIE cells were stimulated with *L reuteri* MEP221101, *L casei* TL2768, *L casei* MEP221114, *L casei* MEP221115, or *L salivarius* MEP221113 (5×10^7 cells/ml) or with the TLR2 agonist Pam3CSK4 for 1, 3, 6, 12, or 24 h and the mRNA expression of the TLR negative regulators A20, IRAK-M, Tollip, Bcl-3, SIGIRR, and MKP-1 was analyzed. Values shown are means and error bars indicate standard deviations. Values are shown relative to the value of nonstimulated cells, which was assigned a value of 1.The results are means of three measures repeated three times in independent experiments. **$p<0.01$ and *$p<0.05$ vs. cells cultured in the absence of lactobacilli.

modulates adaptive immunity through cross talk with regulatory and effector immune cells in the *lamina propria*, the expression of cytokines in immune cells was analyzed in co-cultures of PIE cells and immune cells from swine PPs. Expression of IL-6, CCL2, and CXCL8 by PIE cells in co-cultures following stimulation with poly(I:C) (data not shown) was similar to that shown in Fig. 1 for the mono-cultures of PIE cells stimulated with poly(I:C). On the other hand, poly(I:C) increased the expression of proinflammatory cytokines and TLR3 in immune cells (Fig. 1), indicating that the factors released by PIE cells can modulate immune cell functions.

PIE cells were stimulated with each of the 18 different lactobacilli strains for 6, 12, 24, or 48 hours and were then challenged with poly(I:C). The levels of CCL2 were analyzed at 12 hours postchallenge. The strongest down-regulation of CCL2 that was induced by lactobacilli stimulation was observed following 24 hours of stimulation (Fig 2). In addition, the ability of lactobacilli to down-regulate CCL2 induction was strain-specific. Of the strain tested, *L casei* MEP221114 showed the highest ability to reduce CCL2 induction in PIE cells, followed by *L reuteri* MEP221101 and *L casei* TL2768. Other strains such us *L casei* MEP221115 had a moderate effect (Fig. 2). In addition, we aimed to evaluate the effect of lactobacilli strains on PIE cells without challenge with poly(I:C). Therefore, the effect of MEP221114, MEP221101, and TL2768 was further investigated, as was that of MEP221115 (moderate effect on CCL2 induction) and MEP221113 (negative control) strains and the TLR2 agonist, Pam3CSK4, on the induction of cytokines by PIE cells (Fig. 3). Stimulation of PIE cells with the different strains for 1 to 24 hours, resulted in up-regulation of the expression of CCL2, CXCL8, and IL-6 in PIE cells only by strains that were positive for CCL2 induction in the presence of poly(I:C) (MEP221114, MEP221101, and TL2768) and the effect of each of these lactobacilli was specific to that strain. *L casei* MEP221114 induced the most significant changes in CXCL8 and IL-6 levels (Fig. 3). In addition, it was found that CCL2 and CXCL8 were up-regulated in Pam3CSK4-stimulated PIE cells although the kinetics of upregulation and the levels of both chemokines were different to those found in lactobacilli treated cells (Fig. 3).

The expression levels of the TLR negative regulators (SIGIRR, Tollip, A20, Bcl-3, MKP-1, and IRAK-M) was evaluated in PIE cells treated with the selected lactobacilli strains (Fig. 4). Both the MEP221101 and the TL2768 strains

increased the levels of SIGIRR, Tollip, and A20 in PIE cells from 3 to 12 hours. The increases in the mRNA levels of the three TLR negative regulators induced by the *L casei* MEP221114 strain were significantly greater than those induced by the MEP221101 and TL2768 strains (Fig. 4). Moreover, the MEP221114-induced mRNA increases were sustained for longer than with the other strains, since SIGIRR, Tollip, and A20 mRNAs remained up-regulated at 24 hours only with MEP221114. In addition, MEP221114 also increased the mRNA levels of MKP-1 between 12 and 24 hours. The negative strain MEP221115 increased the levels of Bcl-3 and SIGIRR at 6 h, while MEP221113 only increased the levels of Bcl-3 (Fig. 4).

Discussion

In the present study, we characterized the inflammatory immune response triggered by poly(I:C) stimulation in PIE cells and found that challenge with this TLR3 agonist significantly increased the expression of IL-6, CCL2/MCP-1, and CXCL8/IL-8. Moreover, the mRNA of these three cytokines was up-regulated in immune cells that were co-cultured with PIE cells and stimulated with poly(I:C), indicating that the factors released by PIE cells can modulate immune cell functions. These changes in the expression of cytokines induced by poly(I:C) correlate with those previously observed in different intestinal viral infections in swine and other hosts. Increased levels of RANTES, IP-10, CXCL8, and CCL2 were observed in rotavirus-infected IECs.[15,16] Therefore, our results indicate that PIE cells may be a good tool to study *in vitro* immune responses triggered by TLR3 in IECs as well as the interaction between IECs and immune cells.

Proinflammatory mediators produced by IECs are known to contribute to viral clearance since they can induce the chemotaxis of various types of immune cells into inflammatory sites and their activation. However, proinflammatory mediators may play a detrimental role in viral infections. It has been suggested that genomic double-stranded (ds)RNA and TLR3-induced signaling may be involved in enterocyte destruction during rotavirus infection.[1] Indeed, Zhou et al[2] provided direct evidence that aberrant activation of TLR3 signaling induced by purified rotavirus genomic dsRNA or poly(I:C) causes a breakdown of mucosal homeostasis, leading to mucosal damage. Moreover, levels of IL-6, CXCL8, and tumor necrosis factor-α correlated with the severity of fever and diarrhea observed in children and pigs infected with rotavirus.[4,5] These studies show that it is imperative to generate an adequate inflammatory response against virus infection, accompanied by efficient regulation of the levels of inflammatory mediators, in order to achieve protection against pathogenic microorganisms without damaging host tissues. Therefore, one of the main aims of our study was to select potential immunoregulatory lactobacilli strains that could modulate TLR3-mediated inflammatory response in PIE cells. To investigate the potential protective effect of lactobacilli strains, we chose to assess the levels of CCL2, which is a chemokine with potent monocyte-activating and attracting properties. CCL2 has been suggested to play a major role during intestinal inflammation induced by rotavirus infection.[17–19] In addition we observed that the levels of CCL2 mRNA gradually increased after stimulation of PIE cells with a TLR3 agonist, suggesting that this chemokine may be a useful marker of potentially detrimental inflammatory responses. We evaluated the capacity of different lactobacilli strains to modulate the poly(I:C)-mediated inflammatory response in PIE cells and found that the ability of lactobacilli to down-regulate CCL2 expression was strain-specific. Moreover, we determined that the strain *L. casei* MEP221114 had a high ability to reduce CCL2 expression in poly(I:C)-challenged PIE cells.

Several reports have demonstrated that an important aspect of probiotic immune modulation is the regulation of pro- and anti-inflammatory cytokine production by direct interactions of probiotic bacteria with IECs and with immune cells.[7,8] Moreover, probiotic bacterial function may be mediated through stimulation of TLRs, and certain effects exerted by some probiotic strains on IECs and immune cells have been reported to be mediated through interactions with TLR2.[7,8] In support of this finding, *in vitro* and *ex vivo* studies using a primary culture of mouse IECs showed that probiotic bacteria interact with the TLR2 expressed in these cells and induce release of IL-6.[20] We therefore further investigated the effect of *L casei* MEP221114 on the induction of cytokines by PIE cells and compared the cytokine profile obtained with that obtained in PIE cells that were stimulated with the TLR2 agonist Pam3CSK4. CCL2, and CXCL8 mRNAs were both up-regulated in *L casei* MEP221114- and Pam3CSK4-stimulated PIE cells. However, IL-6 was up-regulated in *L casei* MEP221114-stimulated cells, but not in Pam3CSK4-tretated PIE cells. These results indicate that TLR2 might contribute to the induction of cytokines mediated by *L. casei* MEP221114, although its exact contribution remains to be confirmed.

Negative regulation of TLR signaling can be achieved at multiple levels. Regulation may occur through a reduction in the number of TLRs by down-regulation of the transcription and translation of TLR genes, or by degradation of TLR proteins.[21] When we evaluated the effect of the selected lactobacilli strains on the expression levels of TLR3 in PIE cells we did not find any variation in basal levels with different lactobacilli (data not shown). In addition, once the TLR and its ligand have interacted, TLR signaling can be further controlled by intracellular regulators, which can inhibit TLR signaling pathways.[21] Of the TLR signals that we evaluated in PIE cells treated with the selected lactobacilli strains (SIGIRR, Tollip, A20, Bcl-3, MKP-1, and IRAK-M) we found that *L casei* MEP221114 induced increases in the mRNA levels of SIGIRR, Tollip, MKP-1, and A20.

The ubiquitin-modifying enzyme A20 is not only indispensable for restriction of inflammation in response to bacterial infection, but also seems to control the immune response to viral infection. A20 regulates TLR3 signaling via interaction with the adaptor TIR-domain-containing adapter-inducing interferon-β (TRIF) as well as with Sendai virus-induced TLR3-dependent activation of genes with an ISRE in their promoters.[22] Moreover, it was shown that A20 inhibits dimerization of interferon regulatory factor 3 following engagement of TLR3 by dsRNA or Newcastle disease virus infection, leading to suppression of the IFN stimulation response.[23] On the other hand, production of IL-6 and tumor necrosis factor-α was significantly reduced in mice deficient in the TLR adaptor protein Tollip in

response to IL-1 and LPS when compared with wild-type mice, suggesting that Tollip controls the magnitude of inflammatory cytokine production.[21] To date, regulation of TLR3 activation in the gut by Tollip has not been studied. However, studies of normal human small airway epithelial cells showed that poly(I:C) induced an elevated expression of TLR3 and up-regulation of the TLR3 adaptor protein TRIF, but that it induced downregulation of Tollip expression.[24] This transcriptional regulatory mechanism was assumed to promote TLR3 signaling and to contribute to the strong inflammatory effects induced by poly(I:C). In addition, SIGIRR, which is a subtype of the IL-1 receptor family, is highly expressed in the gut and might therefore be involved in the regulation of intestinal inflammation. Indeed, SIGIRR-deficient mice developed more severe dextran sulfate sodium-induced colitis than wild-type mice.[25] Therefore, the sustained up-regulation of A20, Tollip, and SIGIRR would have an important role in the anti-inflammatory activity of *L casei* MEP221114.

In addition, the phosphatase MKP-1 is also an important negative feedback regulator that attenuates inflammatory responses to TLR signals transduced via the p38 MAPK pathway. When activated through TLR3 signaling, bone marrow-derived MKP-1$^{-/-}$ macrophages exhibit increased proinflammatory cytokine production and elevated expression of differentiation markers such as CD86 and CD40.[26] Moreover, recent studies by Wang et al[27] of cultured enterocytes showed that a ligand of TLR3 induces MKP-1 up-regulation, coincident with dephosphorylation of p38 following the peak of TLR3 ligand-induced phosphorylation. The NF-κB-dependent expression of MKP-1 contributes, by desensitization of p38, to the rapid establishment of unresponsiveness to TLR3 ligands in enterocytes. Therefore, the up-regulation of MKP-1 at 24 hours would explain the greater anti-inflammatory effect observed with *L casei* MEP221114 than with other lactobacilli.

We recently demonstrated that *L jensenii* TL2937 can beneficially modulate inflammatory immune response triggered by enterotoxigenic *Escherichia coli* and lipopolysaccharide in PIE cells and that this effect is related to an up-regulation of the TLR negative regulators, A20, Bcl-3, and MPK-1.[12] Moreover, we demonstrated that TLR2 has an important role in the anti-inflammatory activity of this bacterial strain through the modulation of A20 and Bcl-3, but not of MKP-1.[12] *L casei* MEP221114 appears to have similar effects since this strain strongly up-regulated A20, Bcl-3, Tollip, SIGIRR, and MKP-1 levels in PIE cells, whereas Pam3CSK4 induced changes in A20, Bcl-3, SIGIRR, and Tollip, but not in MKP-1. In addition, we observed that the levels of Bcl-3, SIGIRR, and Tollip were higher in PIE cells treated with the MEP221114 strain than in those treated with PamC3SK4, indicating that other PRRs could be involved together with TLR2 in the upregulation of these TLR negative regulators. We previously developed a porcine TLR2-expressing transfectant (HEK^{pTLR2} cells) method for evaluation of immune responses to immunobiotic microorganisms and we demonstrated that this system could accurately detect TLR2 activation by lactobacilli.[28] We showed that the ability of *L casei* MEP221114 to activate TLR2 was much lower than that of TL2768 or MEP221101 or even than that of strain MEP221113 that was negative for CCL2 upregulation.[28] Therefore, one or more pattern recognition receptors other than TLR2 might be important for the anti-inflammatory effect of *L casei* MEP221114. Further investigations are necessary to clarify the exact mechanism of action of this strain.

In this study, we characterized the TLR3-mediated inflammatory immune response in PIE cells and in PIE-immune cell co-cultures, and demonstrated that these systems could provide valuable tools for the study of *in vitro* inflammatory responses triggered by TLR3 in IECs and of the interaction between IECs and immune cells. In addition, we showed that PIE cells could be used for the selection of suitable immunobiotic lactobacilli strains with anti-inflammatory activities. *L casei* MEP221114 is one such immunobiotic candidate that could potentially be used to modulate TLR3-mediated inflammatory responses *in vivo*, through the modulation of macrophage recruitment into the site of infection. Other studies have proven that is possible to confirm such an anti-inflammatory effect of a probiotic *in vivo*. For instance, Zhang et al[18] studied the effect of probiotics on the response of gnotobiotic pigs to a challenge with human rotavirus. Although no differences in viral replication were found between treated and control groups, the results suggested that LAB administration down-regulates the recruitment of viral-activated monocytes/macrophages into the intestinal tract thereby limiting the inflammation induced by the viral infection.[18] The present study has also deepened our understanding of the mechanisms of immunobiotic action by demonstrating that the interaction between some lactobacilli strains and IECs can up-regulate the expression of TLR negative regulators and that this effect could help to regulate the production of inflammatory mediators during the generation of a TLR3-mediated immune response. The above findings, coupled with the immunobiotics evaluation system that we have developed, hold out the promise that basic research and development of "immunobiotic foods and feeds" will be a safer alternative to conventional antibiotic drug therapy for the prevention of intestinal disorders triggered by viral infection in humans and animals.

Conflicts of interest

All authors declare that they have no conflicts of interest relevant to this article.

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