Inflamm. Res. (2016) 65:771–783
DOI 10.1007/s00011-016-0957-7

Inflammation Research

ORIGINAL RESEARCH PAPER

Immunobiotic *Lactobacillus* strains reduce small intestinal injury induced by intraepithelial lymphocytes after Toll-like receptor 3 activation

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Received: 13 April 2016 / Revised: 24 May 2016 / Accepted: 1 June 2016 / Published online: 9 June 2016

Abstract
Objective Intestinal intraepithelial lymphocytes (IELs) play critical roles in disrupting epithelial homeostasis after Toll-like receptor (TLR)-3 activation with genomic rotavirus dsRNA or the synthetic dsRNA analog poly(I:C). The capacity of immunobiotic *Lactobacillus rhamnosus* CRL1505 (Lr1505) or *Lactobacillus plantarum* CRL1506 (Lp1506) to beneficially modulate IELs response after TLR3 activation was investigated in vivo using a mice model.
Results Intraperitoneal administration of poly(I:C) induced inflammatory-mediated intestinal tissue damage through the increase of inflammatory cells ($CD3^+NK1.1^+$, $CD3^+CD8\alpha\alpha^+$, $CD8\alpha\alpha^+NKG2D^+$) and pro-inflammatory mediators (TNF-α, IL-1β, IFN-γ, IL-15, RAE1, IL-8). Increased expression of intestinal TLR3, MDA5, and RIG-I was also observed after poly(I:C) challenge. Treatment with Lr1505 or Lp1506 prior to TLR3 activation significantly reduced the levels of TNF-α, IL-15, RAE1, and increased serum and intestinal IL-10. Moreover, $CD3^{+-}NK1.1^+$, $CD3^+CD8\alpha\alpha^+$, and $CD8\alpha\alpha^+NKG2D^+$ cells were lower in lactobacilli-treated mice when compared to controls. The immunomodulatory capacities of lactobacilli allowed a significant reduction of intestinal tissue damage.
Conclusions This work demonstrates the reduction of TLR3-mediated intestinal tissue injury by immunobiotic lactobacilli through the modulation of intraepithelial lymphocytes response. It is a step forward in the understanding of the cellular mechanisms involved in the antiviral capabilities of immunobiotic strains.

Keywords Lactobacilli · TLR3 · Intestinal damage · Intraepithelial lymphocytes · Poly(I:C) · Immunobiotics

Abbreviations

AST	Aspartate aminotransferase
APC	Antigen presenting cells
DC	Dendritic cells
dsRNA	Double-stranded RNA
IELs	Intraepithelial lymphocytes
IECs	Intestinal epithelial cells
IFN	Interferon
IL-1	Interleukin
LAB	Lactic acid bacteria
LDH	Lactate dehydrogenase
Lp1506	*Lactobacillus plantarum* CRL1506
Lr1505	*Lactobacillus rhamnosus* CRL1505
MAMPs	Microbe-associated molecular patterns
RAE1	Retinoic acid early inducible-1
RVs	Rotavirus
TLR	Toll-like receptor

Responsible Editor: John Di Battista.

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TNF	Tumor necrosis factor
PIE	Porcine intestinal epithelial cells
PRRs	Pattern recognition receptors

Introduction

Acute diarrhea is a major cause of global death in infant and young children and, rotaviruses (RVs) are the cause of 40–50 % of this gastrointestinal alteration [1, 2]. In fact, it was reported that more than half a million young children die annually from rotaviral gastrointestinal disease especially in developing countries [1, 2]. RVs are non-enveloped viruses formed by three concentric layers of protein that enclose a genome of double-stranded RNA (dsRNA). RVs infect the host through the villi of the small intestine causing apical cell death, and necrosis of apical villi. Those intestinal mucosa alterations result in primary maladsorption, lower digestion, and acute diarrhea [3].

Intestinal epithelial cells (IECs) are involved in the initial contact between the host and microorganisms of the gut [4]. These cells are able to distinguish different microbial antigens using pattern recognition receptors (PRRs), such as Toll-like receptors (TLRs), that play a critical role in innate immunity by recognizing structurally conserved bacterial and viral components so called microbe-associated molecular patterns (MAMPs) [5, 6]. Among PRRs, TLR3 is activated by genomic dsRNA of several viruses or dsRNA that is synthesized during virus replication. Studies performed with the synthetic dsRNA analog poly(I:C) that mimic TLR3 viral activation, and TLR3 knock-out mice demonstrated the important role of this PRR in antiviral immunity [7, 8]. In this regard, it was reported that epithelial cells from different mucosa overexpress TLR3 when challenged with viruses, and overexpression of this receptor allow cells to detect virus and acquire resistance. Moreover, TLR3 has an essential function in the induction of inflammatory cytokines and chemokines to allow the recruitment and activation of immune cells [7]. However, several studies also indicate that TLR3 signaling produces dual actions contributing not only to host defenses but also to viral pathogenesis [9–12].

It is possible to imitate the local intestinal immune response triggered by enteric viral infection using an intraperitoneal administration of poly(I:C) [9, 13]. In this regard, both poly(I:C) and purified RVs dsRNA are able to induce severe mucosal damage in the gut in a TLR3-dependent manner. Zhou et al. [9] clearly demonstrated that intestinal intraepithelial lymphocytes (IELs) play critical roles in disrupting epithelial homeostasis caused by abnormal TLR3 signaling.

IELs are mostly T cells dispersed as single cells within the epithelial cell layer. Therefore, IELs are located at the interface between the inner intestinal tissue and the lumen. These specialized immune cells are important as a first line of defense against microbes as well as for their role in the maintenance of epithelial barrier homeostasis. IELs are constituted mostly by $CD8^+$ cells, and are simply classified as $CD8\alpha\alpha^+$ or $CD8\alpha\beta^+$. The $CD8\alpha\beta^+$ IELs bear the hallmarks of adaptive immune cells. In contrast, the $CD8\alpha\alpha^+$ IELs are considered as innate immune cells [14].

It was reported that IL-15 and $CD3^+NK1.1^+CD8\alpha\alpha^+$ IELs are involved in the disruption of epithelial homeostasis caused by purified genomic dsRNA from RVs or poly(I:C), after abnormal activation of TLR3 signaling [9]. Intraperitoneal injection of poly(I:C) or RVs genomic dsRNA induce severe small intestinal injury, including villous atrophy, mucosal erosion, and gut wall attenuation [9]. Moreover, it was showed that intestinal injury was produced primarily by $CD3^+NK1.1^+CD8\alpha\alpha^+$ IELs, which are activated by IL-15 derived from poly(I:C)-treated IECs. In addition, it was demonstrated that TLR3 activation in IECs induce the expression of retinoic acid early inducible-1 (RAE1), which mediate epithelial destruction and mucosal injury by interacting with NKG2D receptor expressed on IELs [10].

Probiotics are defined as live microorganisms which, when administered in adequate amounts, confer a health benefit for the host [15]. It is well known that probiotics benefits intestinal tract health by improving the local microbial balance and immune functions [16]. Certain probiotic lactic acid bacteria (LAB) with immunoregulatory properties (immunobiotics) are able to beneficially modulate antiviral immune response and provide protection against viral infections such as those produced by RVs [17–19]. In this regard, our previous in vitro studies demonstrated that *Lactobacillus rhamnosus* CRL1505 (Lr1505) and *Lactobacillus plantarum* CRL1506 (Lp1506) are capable to differentially modulate pro-inflammatory cytokines production in response to TLR3 activation and improve antiviral defense mechanisms in both IECs and intestinal antigen presenting cells (APCs) [20].

In this study, we investigated whether immunobiotic Lr1505 or Lp1506 strains are capable to beneficially modify TLR3-induced intestinal immune response in vivo using a mice model. Moreover, we evaluated whether immunobiotic administration is able to modulate IECs–IELs interactions and reduce intestinal tissue damage induced by the TLR3 agonist poly(I:C).

Materials and methods

Microorganisms

Lactobacillus plantarum CRL1506 (Lp1506) and, *Lactobacillus rhamnosus* CRL1505 (Lr1505) were obtained

from the Reference Centre for Lactobacilli (CERELA-CONICET) culture collection (Tucuman, Argentina). Cultures were kept freeze-dried and then rehydrated using the following medium: tryptone, 10.0 g; meat extract, 5.0 g; peptone, 15.0 g; and distilled water, 1 L, pH 7. Bacteria were cultured for 12 h at 37 °C (final log phase) in Man-Rogosa-Sharpe broth (MRS, Oxoid, Cambridge, UK). Lactobacilli were harvested through centrifugation at $3000 \times g$ for 10 min and washed 3 times with sterile 0.01 mol/L phosphate buffer saline (PBS), pH 7.2, and suspended in sterile 10 % non-fat milk for administration to mice [20, 21].

Animals, feeding procedures, and administration of poly(I:C)

Male 6-week-old BALB/c mice were obtained from the closed colony kept at CERELA-CONICET. Animals were housed in plastic cages in a controlled atmosphere (22 ± 2 °C temperature, 55 ± 2 % humidity) with a 12 h light/dark cycle. Immunobiotic Lr1505 or Lp1506 were administered to different groups of mice for 5 consecutive days at a dose of 10^8 cells/mouse/day in the drinking water, that is the optimal dose with immunoregulatory capacities [22, 23]. The treated groups and the untreated control mice were fed a conventional balanced diet ad libitum. Mice were injected intraperitoneally with 100 µl of PBS containing 30 µg poly(I:C) according to Zhou et al. [9, 10]. All experiments were carried out in compliance with the Guide for Care and Use of Laboratory Animals and approved by the Ethical Committee of Animal Care at CERELA, Argentina (protocol number BIOT-CRL/14).

Intestinal tissue injury

At prechosen intervals, small intestine samples from all experimental groups were excised and washed out with PBS. Then, tissues were immersed in 4 % (v/v) formalin saline solution. Once fixed, samples were dehydrated and embedded in Histowax (Leica Microsystems Nussloch GmbH, Nussloch, Germany) at 56 °C. Finally, intestines were cut into 4 µm serial sections and stained with hematoxilin–eosin for light microscopy examination. All slides were coded and evaluated blindly.

Lactate dehydrogenase (LDH) and aspartate aminotransferase (AST) activities

Lactate dehydrogenase (LDH) and aspartate aminotransferase (AST) activities were determined in the serum to evaluate general toxicity of poly(I:C). LDH and AST activities, expressed as units per liter of serum, were determined by measuring the formation of the reduced form of nicotinamide adenine dinucleotide (NAD) using the Wiener reagents and procedures (Wiener Lab, Buenos Aires, Argentina) [21].

Total and differential leukocyte counts in blood

Blood samples were obtained by cardiac puncture from sodium pentobarbital-anesthetized animals at 12 h post challenge and were collected in tubes containing EDTA as an anticoagulant. Total number of leukocytes was determined with a hemocytometer. Differential cell counts were performed by counting 200 cells in blood smears stained with May Grünwald Giemsa stain using a light microscope (1000×), and absolute cell numbers were calculated [24].

Cytokine concentrations in serum and intestinal fluid

Intestinal fluid samples were obtained as follows: the small intestine was flushed with 5 ml of PBS and the fluid was centrifuged (10,000*g*, 4 °C 10 min) to separate particulate material. The supernatant was kept frozen until use. Tumor necrosis factor (TNF)-α, interleukin (IL)-6, IL-10, IL-15, interferon (IFN)-β and IFN-γ concentrations in serum and intestinal fluid, were measured with commercially available enzyme-linked immunosorbent assay (ELISA) technique kits following the manufacturer's recommendations (R&D Systems, MN, USA).

Flow cytometry studies for Peyer's patches and peritoneal immune cells

Peritoneal fluid samples were obtained as described previously [25]. The abdominal cavity was washed with 10 ml ice-cold PBS supplemented with 10 % fetal bovine serum, injected into the abdomen. The peritoneal lavage was centrifuged at 1500 rpm for 10 min, and the cells were resuspended in 1 ml PBS supplemented with 10 % fetal bovine serum. Peyer's patches were teased gently to release cells, and debris was removed by filtering through a cell strainer (Becton, Dickinson, San Jose, CA, USA). Cells were washed twice and suspended in PBS supplemented with 2 % of fetal bovine serum. Peritoneal and Peyer's patches cells were counted using Trypan Blue exclusion and then resuspended at an appropriate concentration of 5×10^6 cells/ml for flow cytometry studies.

Cell suspensions were pre-incubated with anti-mouse CD32/CD16 monoclonal antibody (Fc block) for 15 min at 4 °C. Cells were incubated in the antibody mixes for 30 min at 4 °C and washed with FACS buffer. The following antibodies from BD PharMingen were used: anti-mouse CD3-FITC, anti-mouse CD4-biotin, anti-mouse CD8-PE, anti-mouse CD11b-biotin, anti-mouse Gr-1-PE,

anti-mouse F4/80-APC, anti-mouse MHC-II-PE, and anti-mouse CD103-FITC. Following incubation with biotinylated primary antibodies, the labeling was revealed using streptavidin–PercP. In all cases, cells were then acquired on a BD FACSCalibur^{TM} flow cytometer (BD Biosciences) and data were analyzed with FlowJo software (TreeStar). The total number of cells in each population was determined by multiplying the percentages of subsets within a series of marker negative or positive gates by the total cell number determined for each tissue.

Quantitative expression analysis by real-time PCR

Two-step real-time quantitative PCR was performed to characterize the expression of TNF-α, IL-6, IL-8, IL-1β, IL-10, IL-15, Rae1, TLR3, RIG-I and, MDA5. Total RNA was isolated from each sample using TRIzol reagent (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, United Kingdom) and the Platinum SYBR green qPCR SuperMix uracil-DNA glycosylase (UDG) with 6-carboxyl-X-rhodamine (ROX) (Invitrogen). The primers were described previously [9, 10]. The PCR cycling conditions were 2 min at 50 °C, followed by 2 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 5 ul of sample cDNA and 15 µl of master mix, which included the sense and antisense primers. Expression of β-actin was used to normalize cDNA levels for differences in total cDNA levels in the samples.

Study of IELs

IELs were isolated as described by Zhou et al. [9, 10] with minor modifications. Briefly, Peyer's patches were excised, the small intestine was opened longitudinally and cut into 5-mm long pieces. Samples were washed twice in PBS containing 150 µg/ml streptomycin and 120 U/ml penicillin. The pieces were then stirred at 37 °C in prewarmed RPMI 1640 containing 150 µg/ml streptomycin, 120 U/ml penicillin, and 5 % FCS for 30 min, followed by vigorous shaking for 40 s. This process was repeated, and the supernatants were passed through a small cotton-glass wool column to remove cell debris and were then separated on a Percoll density gradient (Amersham Biosciences). A discontinuous density gradient (40 and 70 %) was used. The cells that layered between the 40 and 70 % fractions were collected as IELs. These IELs contained >90 % $CD3^+$ cells as determined by FACS analysis.

Cellular phenotypes in IELs populations were analyzed by flow cytometry using FITC-conjugated anti-CD3, and PE-conjugated anti-NK1.1 (PK136), and anti-CD8α (CT-CD8b), (R&D Systems). Anti-NKG2D (CX5) was purchased from eBioscience (San Diego, CA, USA). To prevent nonspecific binding, respective isotype Abs were used as controls. Images of labeled cells were acquired on a BD FACSCalibur^{TM} flow cytometer (BD Biosciences) and analyzed with FlowJo software (TreeStar).

Statistical analysis

Experiments were performed in triplicate and results were expressed as mean ± standard deviation (SD). After verification of the normal distribution of data, 2-way ANOVA was used. Tukey's test (for pairwise comparisons of the means) was used to test for differences between the groups. Differences were considered significant at $p < 0.05$.

Results

Lactobacilli reduce poly(I:C)-induced damage and body weight loss

We evaluated body weight loss, intestinal histology, and the biochemical markers LDH and AST to study general health state and intestinal alterations after poly(I:C) administration. Challenge with poly(I:C) significantly increased the body weight loss, LDH and AST activities in serum samples (Fig. 1a), and induced intestinal tissue inflammation with mild mucosal erosion (Fig. 1b). Lr1505 and Lp1506 treatment significantly reduced the body weight loss and the serum biochemical parameters that we use to evaluate general damage (Fig. 1a). In addition, immunobiotic lactobacilli treatments significantly decreased intestinal inflammation (Fig. 1b).

Lactobacilli differentially modulate poly(I:C)-triggered inflammatory cells recruitment

Total and differential blood leukocyte counts were evaluated to study the systemic inflammatory response. Challenge with poly(I:C) significantly increased the number of leucocytes and neutrophils in blood (Fig. 2a). However, the numbers of neutrophils were lower in Lr1505- and Lp1506-treated mice when compared to the control group (Fig. 2a). Challenge with poly(I:C) significantly increased the number of myeloid ($CD11b^+$) cells, activated macrophages ($F4/80^+MHC\text{-}II^+$ cells) and neutrophils ($CD11b^+GR1^+$ cells) in the peritoneal lavages of

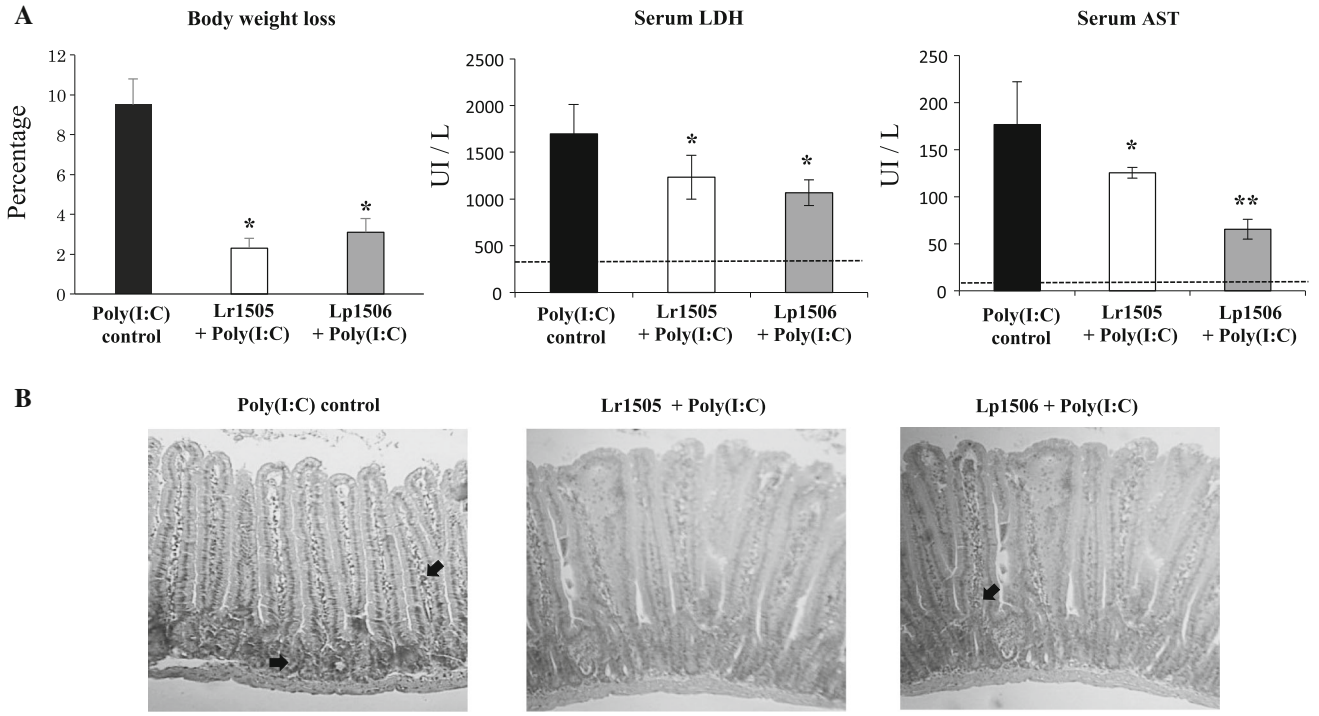

Fig. 1 Effect of lactobacilli on the body weight loss, and serum biochemical markers induced by the intraperitoneal injection of the viral pathogen-associated molecular pattern poly(I:C). **a** Effect of *Lactobacillus rhamnosus* CRL1505 (Lr1505) or *L. plantarum* CRL1506 (Lp1506) administration on body weight loss, and lactate dehydrogenase (LDH) and aspartate aminotransferase (AST) in serum after the challenge with poly(I:C). The results represent data from three independent experiments. Values of serum LDH and AST in non-lactobacilli-treated and non-poly(IC)-challenged control mice are indicated with *dot lines*. Results are expressed as mean ± SD. *Asterisk* different from Poly(I:C) control group ($p < 0.05$). **b** The intestines were removed, fixed, and stained with Hematoxylin and eosin. Poly(I:C) control, Lr1505 + Poly(I:C), Lp1506 + Poly(I:C) groups. Light micrographs, ×100. *Arrows* indicate inflammatory cellular infiltration

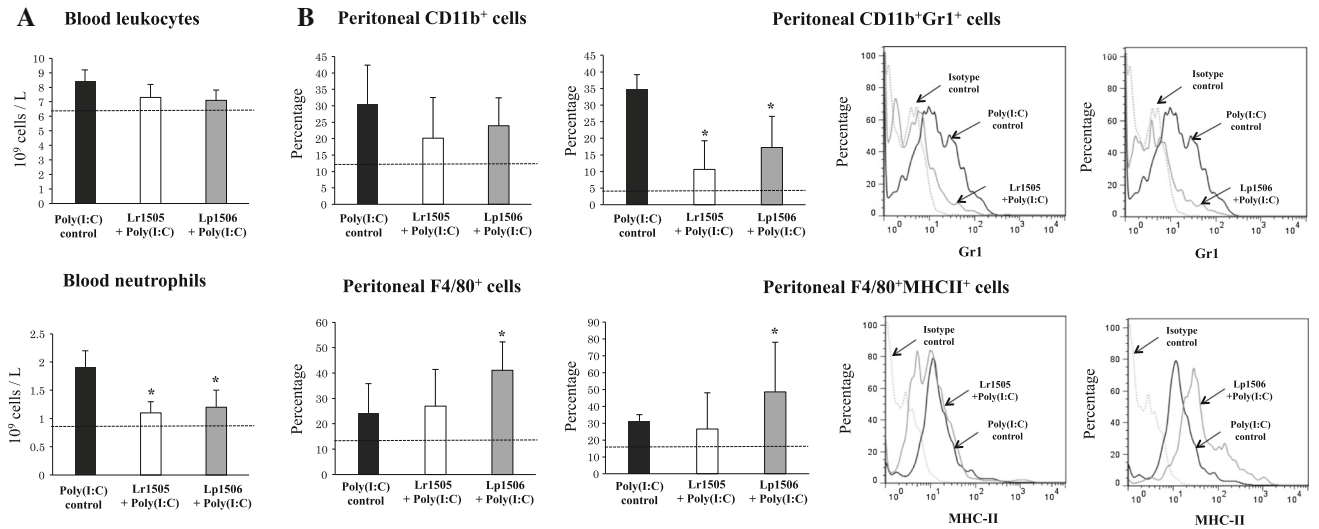

Fig. 2 Effect of lactobacilli on leucocytes after by the intraperitoneal injection of the viral pathogen-associated molecular pattern poly(I:C). Effect of *Lactobacillus rhamnosus* CRL1505 (Lr1505) or *L. plantarum* CRL1506 (Lp1506) administration on **a** the number of blood leukocytes and neutrophils, and **b** peritoneal myeloid cells ($CD11b^+$, $CD11b^+Gr1+$, $F4/80^+$, $F4/80^+MHC\text{-}II^+$ cells) after the challenge with poly(I:C). Values of cells in non-lactobacilli-treated and non-poly(IC)-challenged control mice are indicated with *dot lines*. The results represent data from three independent experiments. Results are expressed as mean ± SD. *Asterisk* different from Poly(I:C) control group ($p < 0.05$)

all the experimental groups (Fig. 2b). However, Lr1505- and Lp1506-treated mice showed significant lower numbers of $CD11b^+GR1^+$ cells when compared to controls (Fig. 2b). Besides, Lp1506 treated mice showed significant higher numbers of peritoneal $F4/80^+MHCII^+$ cells than the other experimental groups (Fig. 2b).

Lactobacilli differentially modulate immune cell populations in Peyer's patches after poly(I:C) challenge

Variations in the numbers of antigen presenting cells ($CD11b^+MHC\text{-}II^+$ and $CD103^+MHC\text{-}II^+$) and lymphocytes ($CD3^+CD4^+$ and $CD3^+CD8^+$) were next evaluated in Peyer's patches (Fig. 3). Challenge with poly(I:C) significantly increased the percentage of the four immune cells populations in Peyer's patches when compared to basal levels. Lactobacilli-treated mice showed significantly higher percentages of $CD11b^+MHC\text{-}II^+$ cells when compared to controls, while no differences were observed in $CD103^+MHC\text{-}II^+$ cells between the groups (Fig. 3b). In addition, Lr1505- and Lp1506-treated mice showed significant lower percentages of $CD3^+CD4^+$ and $CD3^+CD8^+$ lymphocytes than control mice (Fig. 3).

Lactobacilli differentially modulate cytokine response to poly(I:C)

Intraperitoneal administration of poly(I:C) significantly increased the levels of the pro-inflammatory cytokines TNF-α and IL-6 in both serum and intestinal fluid (Fig. 4). However, the levels of the both cytokines were significantly lower in Lr1505 and Lp1506 groups when compared with control mice (Fig. 4). IFN-γ, IFN-β, and IL-10 were also increased in serum and intestinal fluid after the challenge with poly(I:C) in all the experimental groups. Both lactobacilli were able to improve the production of IFNs, being Lr1505 treatment the most effective for increasing the levels of IFN-γ. In addition, both lactobacilli treatments significantly increased the levels of serum and intestinal IL-10 when compared to controls (Fig. 4).

We also evaluated the changes of intestinal mRNAs of TNF-α, IFN-β, IL-8, IL-1β, and IL-10 after the challenge with poly(I:C). Increased expression of all these cytokines was observed in all the experimental groups (Fig. 5). IL-8 and IL-1β expressions in lactobacilli-treated mice were significantly lower than control (Fig. 5). TNF-α expression was superior in Lp1506 treated mice when compared to the control and Lr1505 groups (Fig. 5). In addition, IFN-β and IL-10

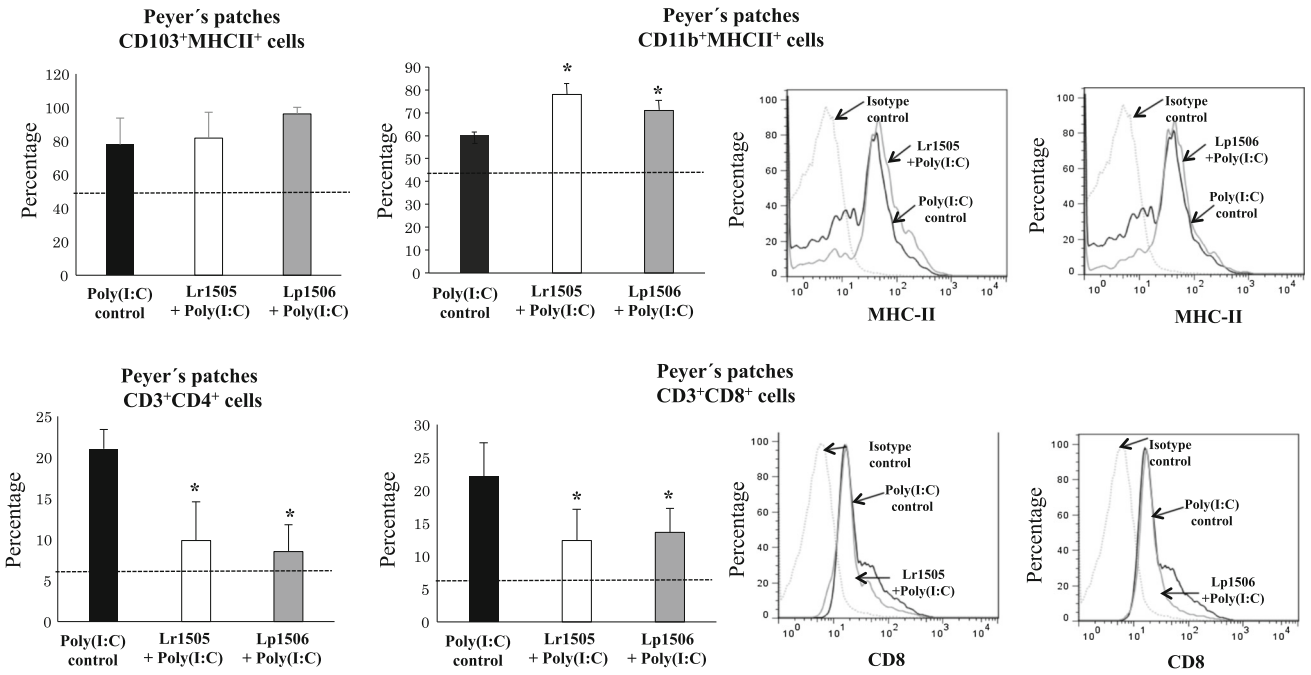

Fig. 3 Effect of lactobacilli on Peyer's patches cell populations after the intraperitoneal injection of the viral pathogen-associated molecular pattern poly(I:C). Effect of *Lactobacillus rhamnosus* CRL1505 (Lr1505) or *L. plantarum* CRL1506 (Lp1506) administration on **a** $CD103^+MHC\text{-}II^+$, **b** $CD11b^+MHC\text{-}II^+$, **c** $CD3^+CD4^+$, and **d** $CD3^+CD8^+$ cells in the Peyer's patches after the challenge with poly(I:C). Values of cells in non-lactobacilli-treated and non-poly(IC)-challenged control mice are indicated with *dot lines*. The results represent data from three independent experiments. Results are expressed as mean ± SD. *Asterisk* different from Poly(I:C) control group ($p < 0.05$)

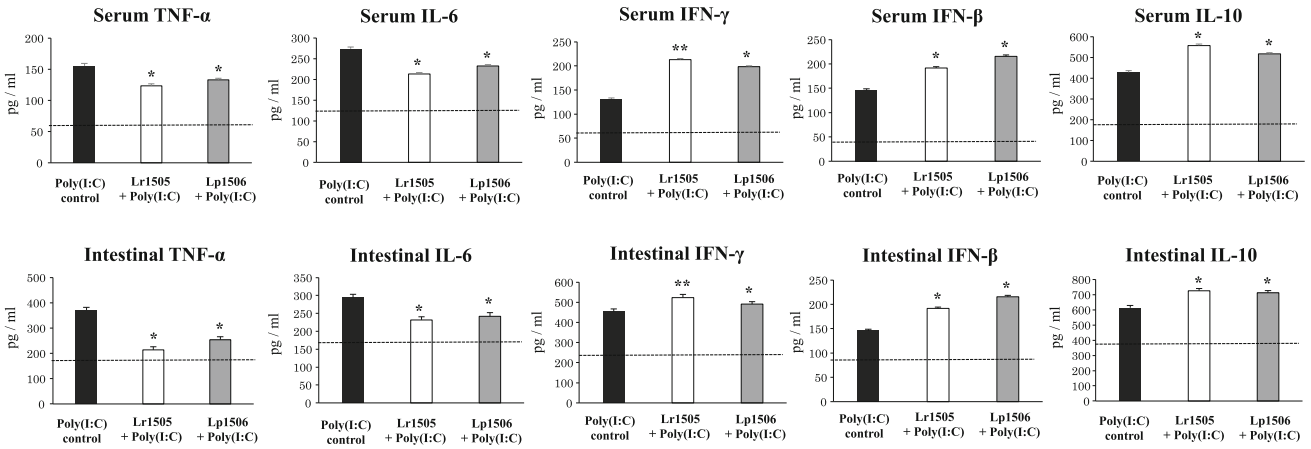

Fig. 4 Effect of lactobacilli on serum and intestinal cytokines levels after the intraperitoneal injection of the viral pathogen-associated molecular pattern poly(I:C). Effect of *Lactobacillus rhamnosus* CRL1505 (Lr1505) or *L. plantarum* CRL1506 (Lp1506) administration on tumor necrosis factor (TNF)-α, interleukin (IL)-6, interferon (IFN)-γ, IFN-β, and IL-10 concentrations in serum and intestine. Values of cytokines in non-lactobacilli-treated and non-poly(IC)-challenged control mice are indicated with *dot lines*. The results represent data from three independent experiments. Results are expressed as mean ± SD. *Asterisk* different from Poly(I:C) control group ($p < 0.05$)

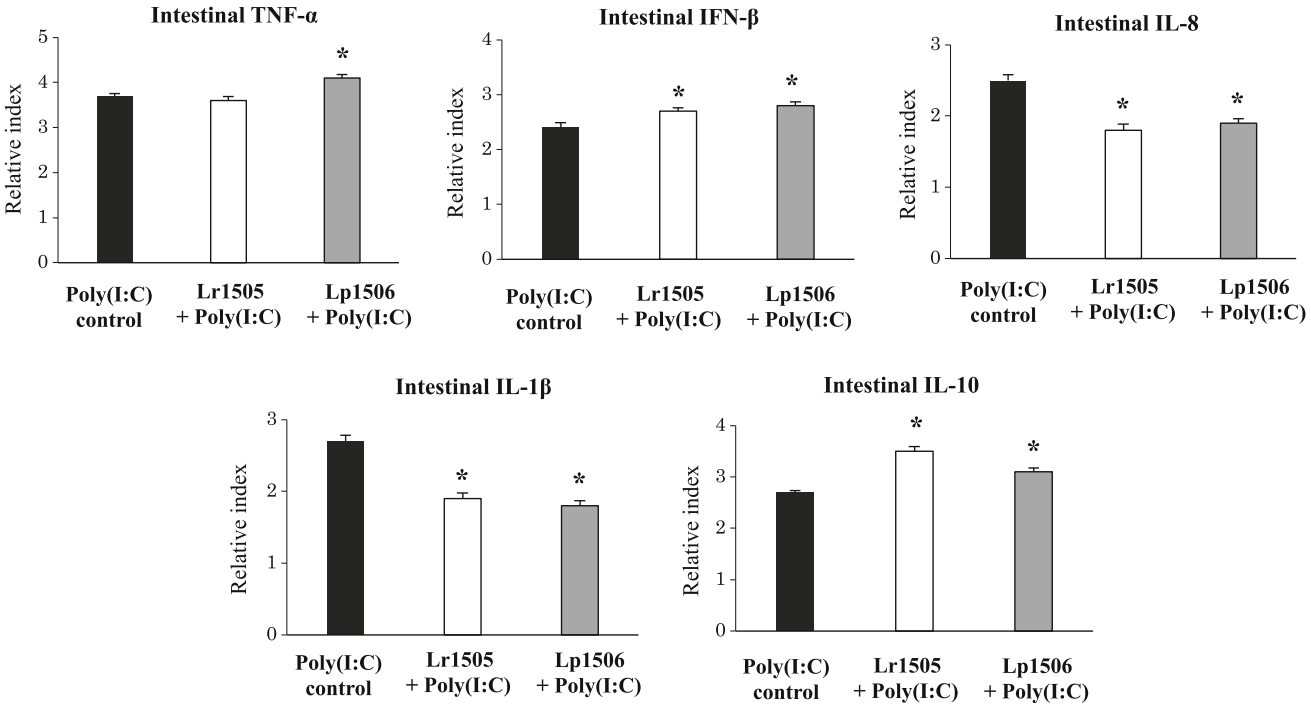

Fig. 5 Effect of lactobacilli on intestinal cytokine and chemokine expressions after the intraperitoneal injection of the viral pathogen-associated molecular pattern poly(I:C). Effect of *Lactobacillus rhamnosus* CRL1505 (Lr1505) or *L. plantarum* CRL1506 (Lp1506) administration on tumor necrosis factor (TNF)-α, interleukin (IL)-6, IL-8, IL-1β, and IL-10 expressions in intestine. Values of cytokines in non-lactobacilli-treated and non-poly(IC)-challenged control mice were set as 1. The results represent data from three independent experiments. Results are expressed as mean ± SD. *Asterisk* different from Poly(I:C) control group ($p < 0.05$)

expressions in intestine were significant higher in Lr1505 and Lp1506 groups when compared to control mice (Fig. 5).

Lactobacilli differentially modulate intestinal intraepithelial lymphocytes numbers after poly(I:C) challenge

We next assessed changes in the populations of intestinal intraepithelial lymphocytes (IELs) in mice challenged with the TLR3 agonist poly(I:C). For this purpose, we studied variations in $CD3^+NK1.1^+$ (NKT cells), $CD3^+CD8\alpha\alpha^+$ and $CD8\alpha\alpha^+NKG2D^+$ populations within IELs by flow cytometry. Poly(I:C) administration induced an increase in the number of the three populations studied: $CD3^+NK1.1^+$, $CD3^+CD8\alpha\alpha^+$ and $CD8\alpha\alpha^+NKG2D^+$ cells (Fig. 6). Lactobacilli-treated mice showed a significant decrease in the number of NKT cells, and $CD3^+CD8\alpha\alpha^+$ and $CD8\alpha\alpha^+$-$NKG2D^+$ IELs when compared to the control group (Fig. 6).

Lactobacilli differentially modulate IL-15 and Rae1 expression after poly(I:C) challenge

Taking into consideration that previous works reported that the enhanced cytotoxicity of IELs induced by poly(I:C) depends on IEC-derived IL-15 [9, 26], we investigated whether lactobacilli treatments were able to change IL-15 production. As predicted, poly(I:C) administration increased serum and intestinal IL-15 (Fig. 7). However, lactobacilli-treated mice showed lower levels of IL-15 in intestine and serum than controls. In addition, it is well known that epithelial Rae1 (retinoic acid early inducible-1) expression is a high-affinity ligand for NKG2D expressed on IELs. Here, we observed that poly(I:C) challenge induced an increase in intestinal Rae1 expression. Interestingly, Lr1505 and Lp1506 treatments were able to significantly reduce epithelial Rae1 expression when compared to control mice (Fig. 7).

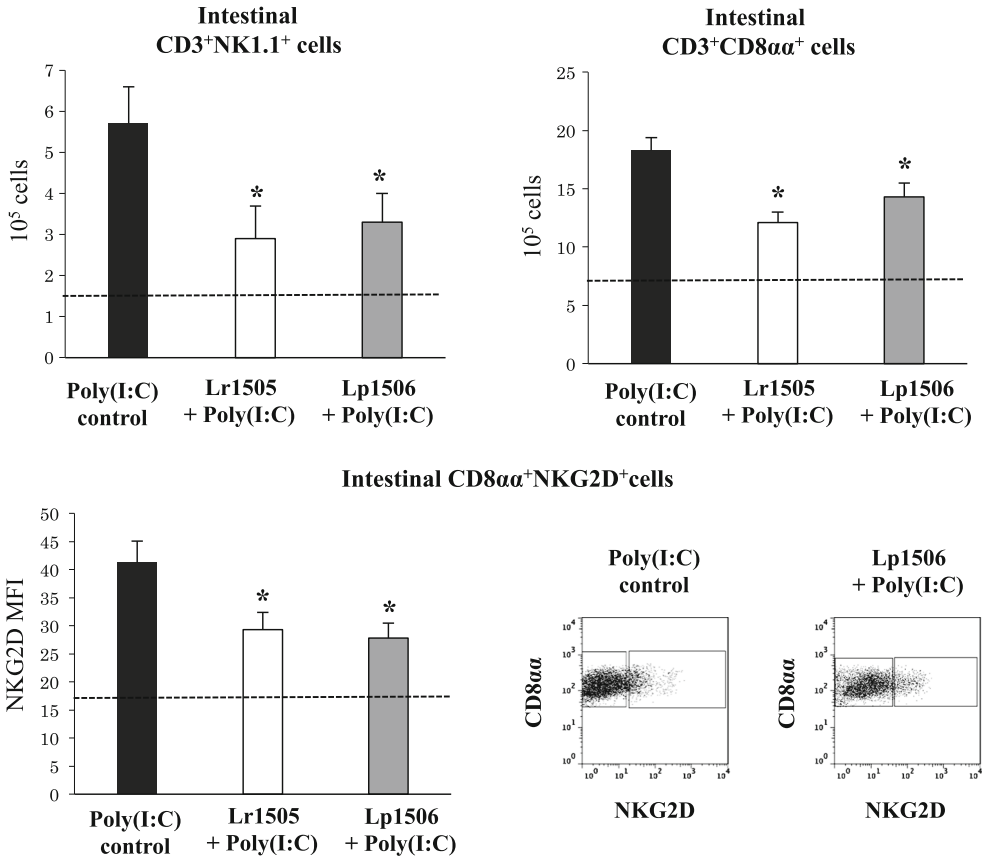

Fig. 6 Effect of lactobacilli on the intestinal intraepithelial cells after the intraperitoneal injection of the viral pathogen-associated molecular pattern poly(I:C). Effect of *Lactobacillus rhamnosus* CRL1505 (Lr1505) or *L. plantarum* CRL1506 (Lp1506) administration on intestinal $CD3^+NK1.1^+$, $CD3^+CD8\alpha\alpha^+$, and $CD8\alpha\alpha^+NKG2D^+$ cells. Values of cells in non-lactobacilli-treated and non-poly(IC)-challenged control mice are indicated with *dot lines*. The results represent data from three independent experiments. Results are expressed as mean ± SD. *Asterisk* different from Poly(I:C) control group ($p < 0.05$)

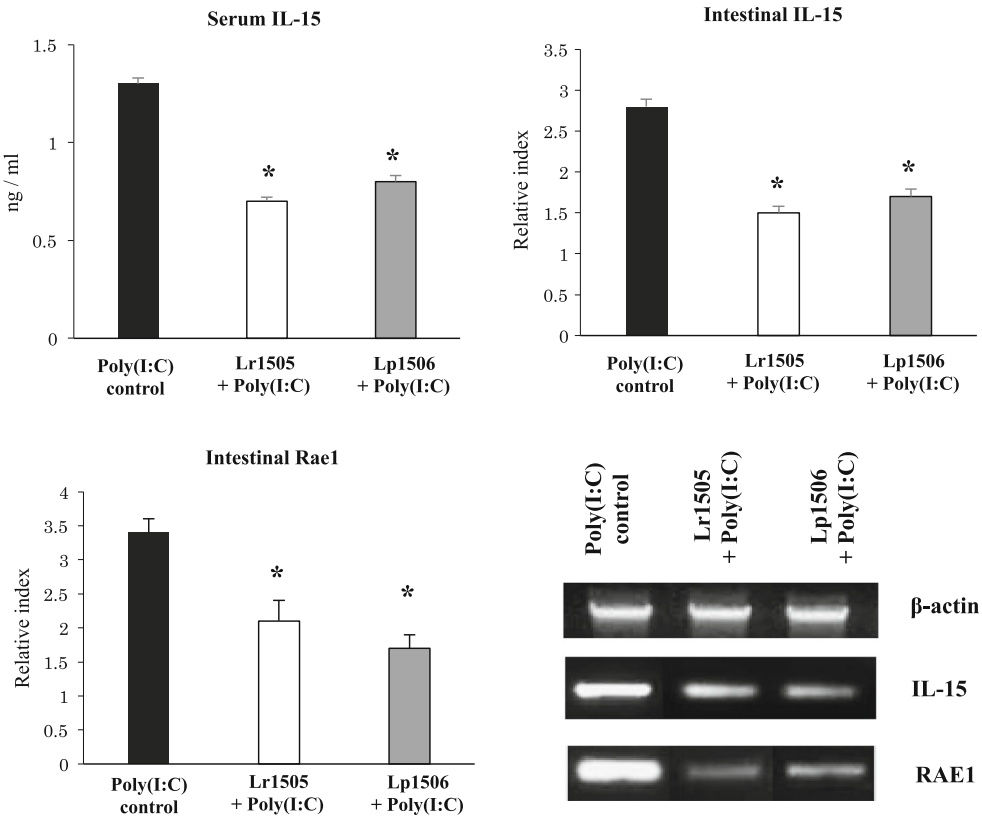

Fig. 7 Effect of lactobacilli on serum and intestinal IL-15 and RAE1 expression after the intraperitoneal injection of the viral pathogen-associated molecular pattern poly(I:C). Effect of *Lactobacillus rhamnosus* CRL1505 (Lr1505) or *L. plantarum* CRL1506 (Lp1506) administration on the IL-15 levels in serum and intestine, and intestinal RAE1 expression. IL-15 in non-lactobacilli-treated and non-poly(IC)-challenged control mice was under the detection limit. Values of intestinal IL-15 and Rae1 in non-lactobacilli-treated and non-poly(IC)-challenged control mice were set as 1. The results represent data from three independent experiments. Results are expressed as mean ± SD. *Asterisk* different from Poly(I:C) control group ($p < 0.05$)

Lactobacilli differentially modulate expression of intestinal pattern recognition receptors after poly(I:C)challenge

Finally, we studied TLR3, RIG-I, and MDA-5 expressions in intestine after poly(I:C) administration. The three receptors were increased in the intestinal tissue after poly(I:C) administration (Fig. 8). Lactobacilli-treated mice showed similar levels of MDA-5 expression when compared to control mice. However, intestinal expressions of TLR3 and RIG-I in lactobacilli-treated mice were significantly lower and higher, respectively, when compared to controls (Fig. 8).

Discussion

TLR3 is involved in both protective immunity and inflammatory tissue damage during viral infections. Some studies indicate that TLR3 knock-out mice are more vulnerable to Coxsackie virus group B infection with increase severity and mortality [27], while other studies indicate that TLR3$^{-/-}$ mice are more resistant to West Nile virus infection [28]. Furthermore, several reports indicated that TLR3 has detrimental and beneficial effects in mice challenged with respiratory viruses [8, 29, 30]. Then, TLR3 has a complex role in viral infections.

We previously investigated the capacity of immunobiotic bacteria to beneficially modulate intestinal TLR3-triggered immune response in vitro. We used a porcine intestinal epithelial cell line (PIE cells) and APCs from porcine Peyer's patches to evaluate TLR3-mediated immune response, and for the selection of LAB strains with antiviral capacities considering that the improvement of IFNs production may provide a valuable tool to increase viral defense mechanisms [20, 31]. Among the lactobacilli strains evaluated by our group, *L. casei* MEP221106, *L. rhamnosus* CRL1505, and *L. plantarum* CRL1506 were the strains with the highest capacity to increase IFN-β and IFN-γ production in poly(I:C)-challenged PIE cells and APCs, respectively [20, 31]. In addition, we found that those lactobacilli strains upregulated the expression of the immunoregulatory cytokine IL-10 after poly(I:C) challenge of APCs or PIE–APCs co-cultures in vitro, suggesting that the inflammatory conditions may be held under control with immunobiotics influence [20, 31]. Therefore, we speculated that Lr1505 and Lp1506 would induce a dual effect when administered in vivo: improvement of antiviral defenses and regulation of inflammatory tissue damage.

In this work, we confirmed that immunobiotic lactobacilli stimulated antiviral innate immunity in vivo. As mentioned before, our previous studies of Lr1505 and Lp1506 immunomodulatory activities showed that both strains were able to induce IFN-β and IFN-α expression in

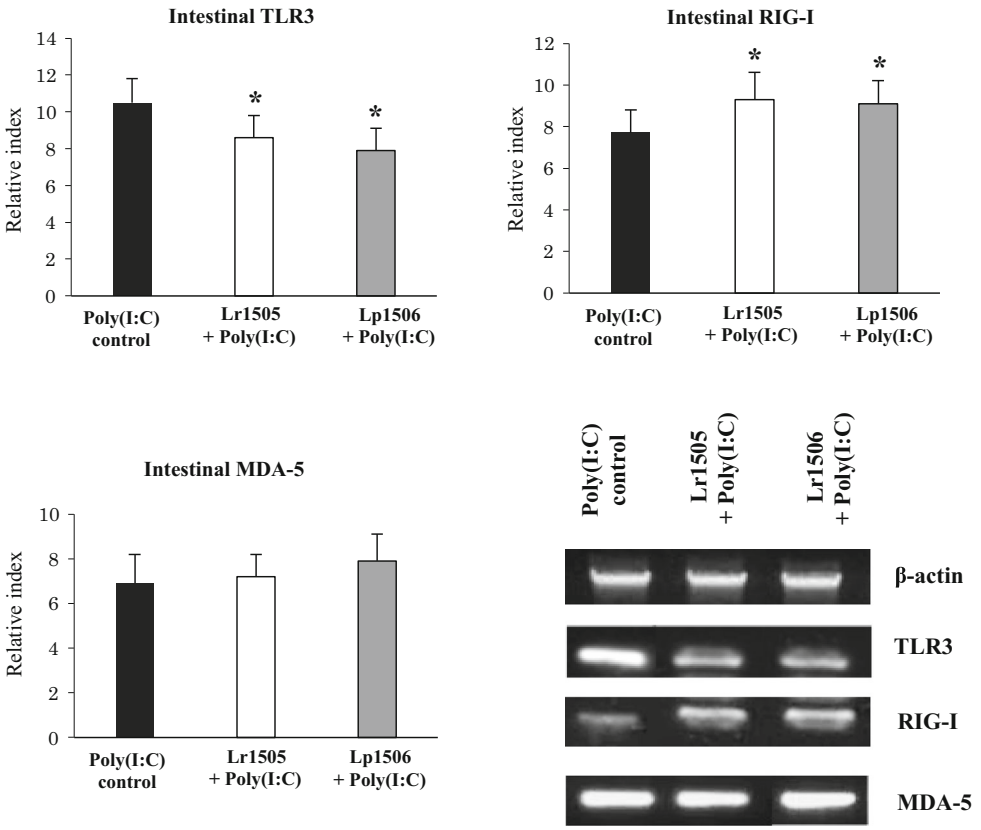

Fig. 8 Effect of lactobacilli on intestinal antiviral pattern recognition receptors expressions after the intraperitoneal injection of the viral pathogen-associated molecular pattern poly(I:C). Effect of *Lactobacillus rhamnosus* CRL1505 (Lr1505) or *L. plantarum* CRL1506 (Lp1506) administration on TLR3, RIG-I, and MDA-5 expressions in intestine. Values of pattern recognition receptors in non-lactobacilli-treated and non-poly(IC)-challenged control mice were set as 1. The results represent data from three independent experiments. Results are expressed as mean ± SD. *Asterisk* different from Poly(I:C) control group ($p < 0.05$)

PIE cells, and improve the production of type I IFNs in response to poly(I:C) challenge in a TLR2 and TLR9-independent manner [20]. Considering that type I IFNs upregulate several antiviral genes and genes of major importance for the development of an effective and coordinated cellular response, we hypothesize that both immunobiotic lactobacilli may play an important role in the improvement of innate immune responses against intestinal virus.

On the other hand, our in vitro studies showed both Lr1505 and Lp1506 significantly augmented surface molecules expression and cytokine production in intestinal DCs. However, Lp1505 had a stronger effect both when applied alone or combined with a posterior poly(I:C) challenge. The improved Th1 response induced by immunobiotic lactobacilli was triggered through TLR2 activation and included augmented expression of MHC-II, IL-1β, IL-6, and IFN-γ in DCs [20]. Here, we confirmed in vivo these differential antiviral immunomodulatory activities triggered by Lr1505 and Lp1506. Both strains increased the production of IFNs, being Lr1505 treatment the most effective for increasing the levels of IFN-γ. Then, our results suggest that these two lactobacilli strains have potential to be used as antiviral substitutes to reduce severity of gastrointestinal viruses such as RVs. In this regard, we previously conducted a human clinical trial to evaluate the effect of Lr1505 in the immune health of children and the incidence and severity of mucosal infections [32]. Our work demonstrated that administration of Lr1505 to young children reduced the incidence, the severity and the duration of intestinal infections including diarrhea. Although we did not evaluate etiology of diarrhea in that study, epidemiological evaluations have shown RVs and adenovirus to be responsible for most cases of diarrhea in children in our region. It would be interesting to evaluate in the future whether these two strains, Lr1505 and Lp1506, are able to protect against RVs infection in vitro and in vivo and if both strains are capable of protecting against infection in the same extent or whether there are differences between them. We also demonstrated here that immunobiotic *Lactobacillus* strains reduce TLR3-induced small intestinal injury by regulation of pro-inflammatory cytokines production and IECs-IELs interaction.

IECs and IELs provide the first line of defense against pathogens including viruses. Furthermore, cell–cell interaction between IECs and IELs is essential for the maintenance of an appropriate immunological homeostasis. IECs produce a variety of cytokines and chemokines, including IL-6, IL-7, IL-8, IL-15, TNF-α, TGF-β, and GM-CSF, which act as communication factors for the intestinal immune system [33]. It was shown that IECs cell death program is preferentially regulated by the self-production

of IL-15, which activates perforin-mediated killing provided by $CD3^+NK1.1^+$ IELs [33]. Moreover, IL-15 is able to enhance the cytotoxic activity of human IELs and make them more potent killers of the human epithelial cell line HT-29 [34].

IL-15 is an important mediator of TLR3-induced small intestinal injury. It was reported that abnormal TLR3 signaling induces IECs to produce elevated levels of IL-15. Upregulated IL-15 production induced by abnormal TLR3 signaling subsequently breaks down mucosal homeostasis [9]. The work showed that blocking the receptor α of IL-15 partially prevented mice from poly(I:C)-induced small intestinal injury, including villous atrophy, and mucosal erosion [9]. On the other hand, NKG2D is a stimulatory NK receptor that recognizes autologous ligands that are upregulated by cell stress, or infection [35]. In mice, the expression of Rae1 that is a high-affinity ligand for NKG2D, is strictly regulated in normal cells and minimally detected on healthy tissues [36]. It was shown that TLR3 activation increase the expression of Rae1 in IECs allowing their destruction by interacting with NKG2D expressed on IELs [10]. In fact, blockade of NKG2D–Rae1 interaction avoids the cytotoxic effect of IELs on IECs and prevents acute small intestinal injury in mice challenged with dsRNA [10]. Therefore, TLR3 signaling stimulates IECs to express IL-15 and Rae1, and induces $CD3^+NK1.1^{+-}$ $CD8\alpha\alpha^+$ IELs to express NKG2D through IEC-derived IL-15.

Our findings agrees with these previous studies since they revealed that treatment of mice with poly(I:C) increased intestinal injury in a IL-15- and $CD8\alpha\alpha^{+-}$ $NKG2D^+$-dependent manner. Poly(I:C) induced inflammatory-mediated intestinal tissue damage through the increase of $CD3^+NK1.1^+$, and $CD8\alpha\alpha^+NKG2D^+$ cells as well as pro-inflammatory mediators (TNF-α, IL-1β, IFN-γ, IL-15, RAE1, IL-8). Of note, treatment with immunobiotic lactobacilli prior to TLR3 activation significantly reduced the levels of TNF-α, IL-15, RAE1, and $CD3^+NK1.1^+$, $CD3^+CD8\alpha\alpha^+$, and $CD8\alpha\alpha^+NKG2D^+$ cells. Moreover, the immunomodulatory capacities of lactobacilli allowed a significant reduction of body weight loss and intestinal tissue damage in poly(I:C)-treated mice.

Several works have demonstrated that commensal bacteria in the gut are able to modulate IELs function. Early studies showed that IELs are significantly reduced in germ-free mice [37, 38], suggesting the important role of gut microbiota in the maintenance of IELs. It was reported that IEL provide a rapid first line of mucosal defense that promotes homeostasis with the microbiota by detecting and limiting bacterial penetration of intestinal tissue. Then, the mucosal protection afforded by IEL is a key factor during the first hours after bacterial exposure, suggesting that IEL occupy a unique temporal niche among intestinal immune responses [39]. More recent studies have gain insight into the molecular mechanisms through which gut microbiota controls the homeostasis of IELs. In this regard, Ismail et al. [39] reported that IEL antibacterial response depends on bacterial stimulation in a MyD88-dependent signaling. Later, Jiang et al. [40] investigated the role of NOD2 signaling in the maintenance of IELs and found that NOD2 maintained IELs via recognition of gut microbiota. In addition, it was found that bacterial stimulation of IEL is not direct, requiring activation of PRRs signaling in neighboring IECs [39, 40]. On the other hand, it was demonstrated that the gut microbiota strongly influences the expression of NKG2D ligands on IECs. Germ-free mice lacking a commensal microbiota had an increased surface expression of NKG2D ligands, and a similar result was seen during ampicillin treatment that depleted most of the murine commensal bacteria [41]. Moreover, it was suggested that the commensal bacteria may establish a regulatory milieu in the intestine, with increased expression of immuno-inhibitory cytokines such as TGF-β and IL-10 that have been shown to downregulate NKG2D ligand surface expression [42, 43]. These previous findings are in line with our results, since reduced expression of Rae1 and increased levels of intestinal IL-10 were found in mice treated preventively with Lr1505 or Lp1506. It would be interesting to investigate whether the immunomodulatory effects of Lr1505 or Lp1506 are induced by direct action on the IECs and indirectly on IELs, similar to commensal bacteria, or whether the immunobiotic bacteria are also able to exert direct action on IELs.

In conclusion, our in vivo studies allowed us to speculate that immunobiotic treatments are able to induce two beneficial effects in the intestinal immune response triggered by TLR3 activation: (a) improvement of antiviral innate immunity and, (b) protection against inflammatory damage. This work demonstrates the reduction of TLR3-mediated intestinal tissue injury by immunobiotic *L. rhamnosus* CRL1505 and *L. plantarum* CRL1506 through the modulation of IECs–IELs interaction. It is a step forward in the understanding of the cellular mechanisms involved in the antiviral capabilities of immunobiotic CRL1505 and CRL1506 strains.

Acknowledgments This study was supported by a ANPCyT—FONCyT Grant PICT-2013 (No. 3219) to Dr. J. Villena and Grant-in-Aid for Scientific Research (B)(2) (No. 24380146, 16H05019) and Challenging Exploratory Research (No. 23658216, 26660216, 16K15028) from the Japan Society for the Promotion of Science (JSPS) to Dr. H. Kitazawa.

Compliance with ethical standards

Ethics All experiments were carried out in compliance with the Guide for Care and Use of Laboratory Animals and approved by the

Ethical Committee of Animal Care at CERELA, Argentina (Protocol Number BIOT-CRL/14).

Conflict of interest The authors declare that they have no competing interests.

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