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Immunobiotic lactobacilli reduce viral-associated pulmonary damage through the modulation of inflammation–coagulation interactions

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ABSTRACT

The exacerbated disease due to immune- and coagulative-mediated pulmonary injury during acute respiratory viruses infection results in severe morbidity and mortality. Identifying novel approaches to modulate virus-induced inflammation–coagulation interactions could be important alternatives for treating acute respiratory viruses infections. In this study we investigated the effect of the probiotic strain *Lactobacillus rhamnosus* CRL1505 on lung TLR3-mediated inflammation, and its ability to modulate inflammation–coagulation interaction during respiratory viral infection. Our findings reveal for the first time that a probiotic bacterium is able to influence lung immune-coagulative reaction triggered by TLR3 activation, by modulating the production of proinflammatory and anti-inflammatory cytokines as well as expression of tissue factor and thrombomodulin in the lung. We also demonstrated that the preventive treatment with the probiotic bacteria beneficially modulates the fine tune balance between clearing respiratory viruses (respiratory syncytial virus and influenza virus) and controlling immune-coagulative responses in the lung, allowing normal lung function to be maintained in the face of a viral attack. Our data also pinpoint a crucial role for IL-10 in the immune protection induced by *L. rhamnosus* CRL1505 during respiratory viral infections. These observations might be helpful to propose new preventive or therapeutic approaches to better control virus-inflammatory lung damage using probiotic functional foods.

1. Introduction

Influenza virus (IFV) and respiratory syncytial virus (RSV) are common causes of upper respiratory tract infection and pneumonia. Although several studies have examined the host inflammatory/immune responses to these viruses, some investigations have demonstrated an important role of the hemostatic system in the outcome of viral respiratory infections. Besides inflammatory pathways, respiratory viruses can trigger the coagulation system. They increase the expression of tissue factor (TF), the main initiator of coagulation, in endothelial cells and monocytes inducing a prothrombotic state by concurrent stimulation of coagulation and inhibition of fibrinolysis [1–4]. Although enhanced coagulation may be considered host protective in containing the infection [5], excessive procoagulant activity may result in alveolar fibrin formation and enhancement of inflammation and lung injury. Moreover, much information has accrued demonstrating the close interaction of inflammation, atherosclerosis and thrombosis. Several case–control studies have repeatedly confirmed the common clinical observation that viral respiratory tract infections often shortly precede or accompany acute ischemic strokes or acute myocardial infarctions [6,7].

Abbreviations: APTT, activated partial thromboplastin time; BAL, bronchoalveolar lavage; DAB, diaminobenzidine; DMEM, Dulbecco's modified Eagle's medium; dsRNA, double-stranded RNA; ELISA, enzyme-linked immunosorbent assay; FBS, fetal bovine serum; IFV, influenza virus; IL, interleukin; LAB, lactic acid bacteria; LDH, lactate dehydrogenase; Lr1505, *Lactobacillus rhamnosus* CRL1505; Lr1506, *Lactobacillus rhamnosus* CRL1506; MDCK, Madin–Darby canine kidney; MIP, macrophage inflammatory protein; MOI, multiplicity of infection; MPO, myeloperoxidase; MRS, Man–Rogosa–Sharpe; NAD, nicotinamide adenine dinucleotide; OD, optical density; PAI, plasminogen activator inhibitor; PBS, phosphate buffer saline; Pen/Strep, penicillin–streptomycin; PFU, plaque-forming unit; Poly(I:C), polyinosinic:polycytidylic acid; PRRs, pattern-recognition receptors; PT, prothrombin time; RIG-I, retinoic acid-inducible gene I; RSV, respiratory syncytial virus; TATc, thrombin–antithrombin complexes; TF, tissue factor; TFPI, tissue factor pathway inhibitor; TLR, Toll-like receptor; TGF, transforming growth factor; TM, thrombomodulin; TNF, tumor necrosis factor; VCAM-1, vascular cell adhesion molecule 1; vWF, von Willebrand factor.

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Inflammatory and hemostatic alterations in respiratory viral infections have been associated to double-stranded RNA (dsRNA) intermediates produced during the replication of respiratory viruses such as IFV and RSV, which are recognized by a variety of pattern-recognition receptors (PRRs) in respiratory epithelial, endothelial and immune cells, including Toll-like receptor (TLR)-3 and retinoic acid-inducible gene I (RIG-I). *In vivo* studies using mice have demonstrated that the viral-associated molecular pattern polyinosinic:polycytidylic acid (poly(I:C)), treatment results in TLR3- and CXCR2-dependent neutrophilic pulmonary inflammation, interstitial edema, bronchiolar epithelial hypertrophy, and altered lung function [8,9]. These changes were accompanied by elevated levels of proinflammatory cytokines and type I interferons in broncho-alveolar lavages (BAL) [8] and, increased airway epithelial cell TLR3 protein expression [9]. In addition, studies have reported that poly(I:C) can upregulate TF and downregulate thrombomodulin (TM) expression on endothelial cells. Moreover, *in vivo* application of poly(I:C) induces similar changes in the aortic endothelium of mice and increases D-dimer levels indicating enhanced coagulation and fibrinolysis [10].

Certain probiotic lactic acid bacteria (LAB) strains can exert their beneficial effect on the host through their immunomodulatory activity. These strains, termed immunobiotics [11], have been used for the development of functional foods with the ability to stimulate mucosal immunity. Moreover, studies have demonstrated that some immunobiotic LAB can stimulate the common mucosal immune system to provide protection in other mucosal sites distant from the gut [12]. In this regard, several lines of evidence demonstrated that oral administration of immunobiotics is able to increase resistance against respiratory viral infections. It has been described that several aspects of respiratory antiviral immunity can be beneficially modulated by immunobiotics, including the production of type I interferons, the activity of NK cells, the generation of Th1 responses as well as the production of specific antibodies and the regulation of inflammatory lung injury [11]. We recently initiated a series of studies seeking to establish the capacity of *Lactobacillus rhamnosus* CRL1505 to improve respiratory antiviral immunity. Our research work has demonstrated that mucosal (oral and nasal) administration of the CRL1505 strain is able to beneficially modulate the immune response triggered by TLR3 activation in the respiratory tract and to increase the resistance to RSV challenge [13–15]. Moreover, *L. rhamnosus* CRL1505 administration efficiently reduces inflammatory lung tissue damage produced by poly(I:C) or RSV through its capacity to beneficially modulate proinflammatory/IL-10 and Th1/Th2 balances in the respiratory tract [13–15]. On the other hand, we demonstrated that some immunobiotic strains such as *L. casei* CRL431 or *L. rhamnosus* CRL1505 are able to beneficially modulate the inflammation–coagulation interaction during respiratory infections, indicating that LAB is able to modulate the immune-coagulative response [16–19]. Much research of our group has been done on coagulation activation during severe bacterial infections, and no data on the modulation of coagulation/inflammation interaction by immunobiotics in viral infections are available.

The exacerbated disease due to immune- and coagulative-mediated pulmonary injury during acute respiratory viruses infection results in severe morbidity and mortality. Then, identifying novel approaches to modulate virus-induced inflammation–coagulation interactions could be important alternatives for treating acute respiratory viruses infections. In this sense, studying the effect of orally administered immunobiotics on the immune-coagulative response triggered by respiratory activation of TLR3 would contribute to the knowledge of the mechanism of probiotics' protective effect against respiratory viral infections. Therefore, the aim of the present study was to deepen the understanding of the mechanisms of *L. rhamnosus* CRL1505 immunoregulatory activity by evaluating a) its effects on lung TLR3-mediated inflammation; b) its ability to modulate inflammation–coagulation interaction; c) and its influence on the outcome of respiratory viruses challenges.

2. Materials and methods

2.1. Microorganisms

Lactobacillus rhamnosus CRL1505 (Lr1505) and CRL1506 (Lr1506) were obtained from the CERELA culture collection. The culture were kept freeze-dried and then rehydrated using the following medium: peptone, 15.0 g; tryptone, 10.0 g; meat extract, 5.0 g; and distilled water, 1 l, pH 7. It was cultured for 12 h at 37 °C (final log phase) in Man–Rogosa–Sharpe broth (MRS, Oxoid, Cambridge, UK). The bacteria were harvested through centrifugation at 3000 ×*g* for 10 min and washed 3 times with sterile 0.01 mol/l phosphate buffer saline (PBS), pH 7.2, and resuspended in sterile 10% non-fat milk.

2.2. Animals and feeding procedures

Male 6-week-old BALB/c mice were obtained from the closed colony kept at Tohoku University. They were housed in plastic cages in a controlled atmosphere (22 ± 2 °C temperature, 55 ± 2% humidity) with a 12 h light/dark cycle. *L. rhamnosus* CRL1505 or CRL1506 were administered to different groups of mice for 5 consecutive days at a dose of 10^8 cells/mouse/day in the drinking water, which is the optimal dose with immunoregulatory capacities [20,21]. The treated groups and the untreated control group were fed a conventional balanced diet *ad libitum*. 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 Tohoku University, Japan.

2.3. Intranasal administration of poly(I:C)

Mice were lightly anesthetized and 100 μl of PBS, containing 250 μg poly(I:C) (equivalent to 10 mg/kg body weight), was administered dropwise, via the nares. Control animals received 100 μl of PBS. Mice received three doses of poly(I:C) or PBS with 24 h rest period between each administration [13,14].

2.4. Lung tissue injury

Forty eight hours after the last poly(I:C) challenge, whole-lung 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, lungs were cut into 4 μm serial sections and stained with hematoxylin–eosin for light microscopy examination. All slides were coded and evaluated blindly. Albumin content, a measure to quantitate increased permeability of the bronchoalveolar–capillarity barrier, and lactate dehydrogenase (LDH) activity, an indicator of general cytotoxicity, were also determined in the acellular BAL fluid 48 h post-challenge [13,14]. Albumin content was determined colorimetrically based on albumin binding to bromcresol green using an albumin diagnostic kit (Wiener Lab, Buenos Aires, Argentina). LDH activity, expressed as units per liter of BAL fluid, was determined by measuring the formation of the reduced form of nicotinamide adenine dinucleotide (NAD) using the Wiener reagents and procedures (Wiener Lab) [13,14].

2.5. Total and differential leukocyte counts in blood and bronchoalveolar lavages

Blood samples were obtained by cardiac puncture from sodium pentobarbital-anesthetized animals at 48 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 [22]. A portion of the BAL

fluid was used to determine the total number of leukocytes using a hemocytometer. The remaining sample of fluid was centrifuged for 10 min at 900 ×*g*, the pellet was used to make smears, and differential cell counts were performed by counting 200 cells stained with May-Grünwald Giemsa. The supernatant fluid was frozen at −70 °C for subsequent analyses.

2.6. Activation of blood neutrophils

Measurement of myeloperoxidase (MPO) activity of blood neutrophils was carried out by use of the Washburn test at 48 h post-challenge, which is a cytochemical method that uses benzidine as an MPO chromogen [23]. Cells were graded as negative or as weak, moderate, or strongly positive according to the intensity of reaction and were used to calculate the score. The score was calculated by counting 200 neutrophils in blood smears. The score value was calculated by the addition of neutrophils with different positive grades.

2.7. Cytokine concentrations in serum and broncho-alveolar lavages

Tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6, IL-8, IL-10, transforming growth factor (TGF)-β, and macrophage inflammatory protein (MIP)-1α concentrations in serum and BAL, were measured at 48 h post-challenge with commercially available enzyme-linked immunosorbent assay (ELISA) technique kits following the manufacturer's recommendations (R&D Systems, MN, USA).

2.8. Coagulation tests

Blood samples were obtained as described before and collected in a 3.2% (w/v) solution of trisodium citrate at a ratio of 9:1. Plasma was obtained according to Agüero et al. [24]. Prothrombin time (PT) and activated partial thromboplastin time (APTT) were performed manually on fresh plasma samples. PT was determined to evaluate the extrinsic coagulation pathway; it was determined by a one-step method (Thromborel S, Behningwerke AG, Marburg, Germany). Results are expressed as percentage of prothrombin activity (%) from a calibration curve made from a pool of fresh plasma from normal mice [16,17]. APTT was determined to evaluate the intrinsic pathway of coagulation. APTT was determined by mixing plasma with calcium chloride and a partial thromboplastin reagent (STA APTT Reagent, Stago, Asnières, France), and timing initial clot formation. Results are expressed in seconds [16–19]. Thrombin–Antithrombin complexes (TATc; markers of coagulation system activation) were measured in BAL and plasma samples by the ELISA technique, according to the manufacturer's instructions (TAT Complexes Mouse ELISA Kit, Abcam Inc., UK).

2.9. Platelet counts

Blood samples were obtained as described for the leukocyte count. Manual platelet counting was performed by visual examination of diluted whole blood with 1% (w/v) aqueous ammonium oxalate. The total number of platelets was determined with a hemocytometer [16–19].

2.10. Determination of von Willebrand factor (vWF) in plasma and BAL

vWF was measured in plasma and BAL samples by ELISA. In brief, plates were coated with rabbit anti-human vWF (DakoCytomation Denmark A/S) overnight at 4 °C, and blocked with 1% bovine serum albumin. Samples and standard curve were incubated for 2 h at room temperature. Peroxidase-conjugated anti-human vWF/FVIII (DakoCytomation Denmark A/S) was added and incubated for 1 h at room temperature. The reaction was developed with ortophenylendiamine (Ortho-Diagnostic System) and was stopped with 2 N H_2SO_4. The optical density (OD) at a wavelength of 490 nm was determined. OD shown by the background controls was subtracted from the OD of each sample. Samples and each point of standard curve were performed by duplicate. Results are expressed as percentage of vWF (%) from a calibration curve [25].

2.11. Immunohistochemistry in lung histological slides

At prechosen intervals, whole-lung samples from mice were aseptically removed and processed following Sainte-Marie's technique [26]. Once fixed, the samples were dehydrated, embedded in Histowax (Leica Microsystems Nussloch GmbH, Nussloch, Germany) at 56 °C and cut into 4 μm serial sections. Lung sections were deparaffinized and endogenous peroxidase activity was quenched with a solution of methanol/0.03% H_2O_2 (Merck, Buenos Aires, Argentina). The sections were washed with PBS and then exposed to rat anti-mouse vascular cell adhesion molecule 1 (VCAM-1, CD106, BD, Biosciences Pharmingen, San Diego, CA, USA). After washing, the slides were incubated with a secondary antibody (mouse anti-rat IgG peroxidase conjugate, Sigma-Aldrich Co, Saint Louis, MO). Peroxidase activity was detected with a 3,3′-diaminobenzidine peroxidase substrate solution (DAB, Sigma-Aldrich Co, Saint Louis, MO), after which a light counterstain with hematoxylin was performed. The results of the reaction were analyzed, taking into account the intensity (classified as mild, moderate, or strongly positive) and distribution in the pulmonary endothelia.

2.12. Quantitative expression analysis by real-time PCR

Two-step real-time quantitative PCR was performed to characterize the expression of tissue factor (TF), tissue factor pathway inhibitor (TFPI), plasminogen activator inhibitor (PAI)-1, and thrombomodulin (TM) mRNAs in lung. 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 following primers were used: TF (sense: 5′-CAA TGA ATT CTC GAT TGA TGT GG-3′; antisense: 5′-GGA GGA TGA TAA AGA TGG TGG C-3′); TFPI (sense: 5′-ACT GTG TGT CTG TTG CTT AGC C-3′; antisense: 5′GTT CTC GTT CCC TTC ACA TCC C-3′); PAI-1 (sense: 5′-AGG TCA GGA TCG AGG TAA ACG AG-3′; antisense: 5′-GGA TCG GTC TAT AAC CAT CTC CGT-3′); TM (sense: 5′-AGT GTG CCA GTT CAT AAG AAT C-3′; antisense: 5′-AGT GTG CCA GTT CAT AAG AAT C-3′). 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 μl 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.

2.13. Viruses and infection

Human RSV strain A2 was grown in Vero cells as described by [27]. Briefly, Vero cells were infected with RSV at a multiplicity of infection (MOI) of 1 in 5 ml of Dulbecco's modified Eagle's medium (DMEM). Cells were infected for 2.5 h at 37 °C and 5% CO_2. After infection, 7 ml of DMEM with 10% fetal bovine serum (FBS, Sigma, Tokyo, Japan), 0.1% penicillin–streptomycin (Pen/Strep) (Sigma, Tokyo, Japan), and 0.001% ciprofloxacin (Bayer) was added to the flask. Flasks were incubated until extensive syncytium formation was observed. Then, cells were scraped from the flask and sonicated three times, 5 s per time, at 25 W on ice. Cell debris was removed by centrifugation at 700 × *g* for 10 min at 4 °C. Virus supernatant was sucrose density gradient purified and stored in 30% sucrose at −80 °C.

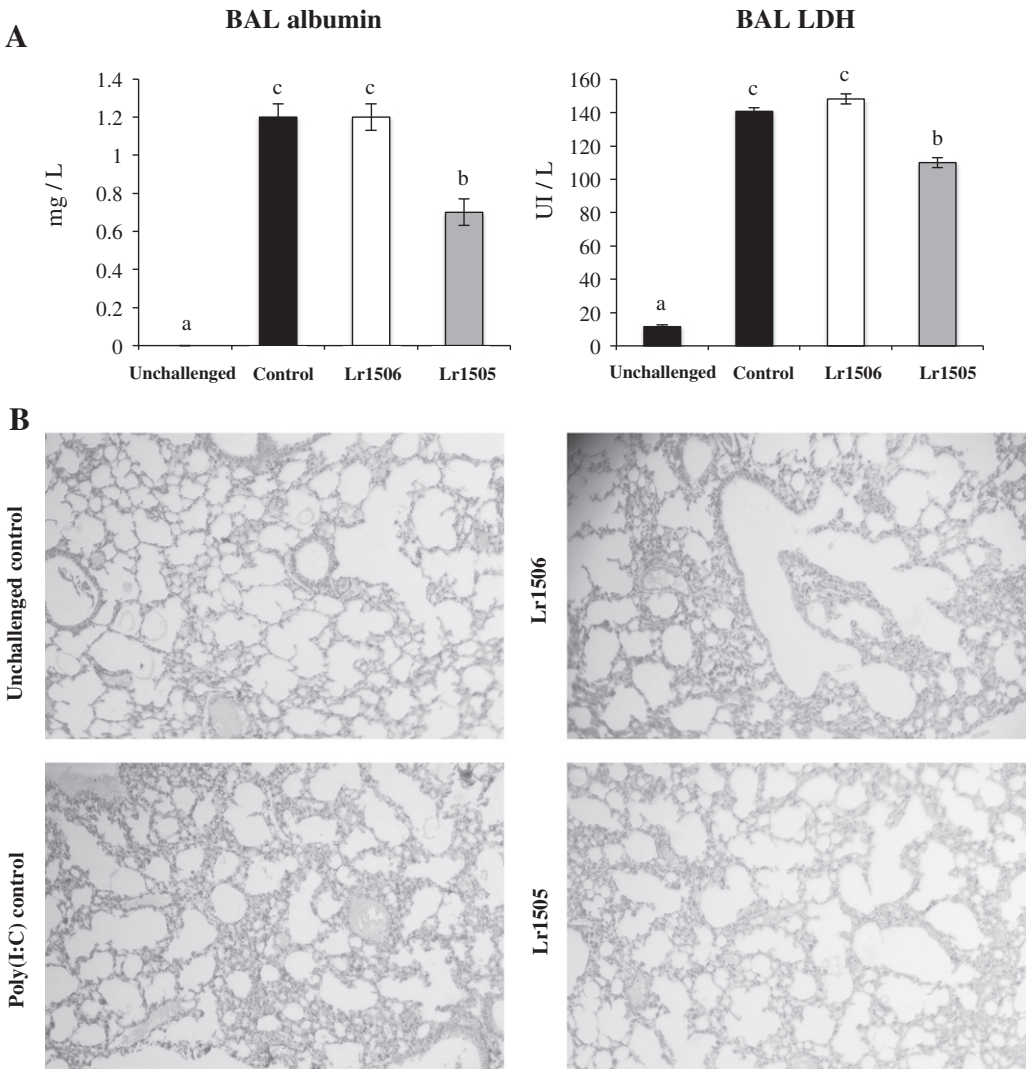

Fig. 1. Effect of lactobacilli on the lung damage induced by the nasal administration of the viral pathogen-associated molecular pattern poly(I:C). Effect of *Lactobacillus rhamnosus* CRL1505 (Lr1505) or *L. rhamnosus* CRL1506 (Lr1506) administration on the lung damage. (A) Albumin and lactate dehydrogenase (LDH) in bronchoalveolar lavages (BAL). (B) The lungs were removed, fixed, and stained with hematoxylin and eosin. Control, Lr1506, and Lr1505. Light micrographs, original magnification ×100. The results represent data from three independent experiments. Results are expressed as mean ± SD. Values for bars with different letters were significantly different ($P < 0.05$). Values for bars with shared letters do not differ significantly.

Uninfected flasks were treated identically to generate Vero cell lysate control. For *in vivo* infection, mice were lightly anesthetized with isoflurane and intranasally challenged with 2.4×10^6 plaque-forming unit (PFU) of RSV strain A2.

Influenza virus A/PR/8/34 (H1N1) was propagated in Madin–Darby canine kidney (MDCK) cells, and virus titers in the stock solution were determined by a plaque assay [28]. MDCK cells were grown and maintained in Eagle's minimum essential medium supplemented with 2% and 5% heat-inactivated fetal bovine serum, respectively. Mice were intranasally infected or mock-infected with 500 PFU of the A/PR/8/34 strain in 25 μl of PBS.

2.14. RSV immunoplaque assay

Lung tissue was removed without BAL harvest and stored in 30% sucrose for plaque assay. Lungs were homogenized using a pellet pestle and centrifuged at 2600 ×*g* for 10 min at 4 °C to clarify supernatant. Twenty-four-well tissue culture plates were seeded with 1.5×10^5 Vero cells/well in DMEM containing 10% FBS, 0.1% Pen/Strep, and 0.001% ciprofloxacin. Cells were incubated overnight at 37 °C and 5% CO_2. Medium was removed from confluent monolayers, and serial dilutions of lung tissue-clarified supernatants were absorbed to monolayers. All samples were run in triplicate wells. Plates were incubated at 37 °C and 5% CO_2 for 2.5 h for optimum infection. After incubation, supernatant was removed, and 1 ml of fresh DMEM containing 10% FBS, 0.1% Pen/Strep, and 0.001% ciprofloxacin was overlaid on monolayers. When extensive syncytia developed, the overlay was removed and monolayers were fixed with 1 ml of ice-cold acetone: methanol (60:40). Primary RSV anti-F (clones 131-2A; Chemicon) and anti-G (Mouse monoclonal [8C5 (9B6)] to RSV glycoprotein, Abcam) antibodies were added to wells for 2 h, followed by secondary horseradish peroxidase anti-mouse immunoglobulin antibody (anti-mouse IgG, HRP-linked Antibody #7076, Cell Signaling Technology) for 1 h. Plates were washed twice with PBS containing 0.5% Tween 20 (Sigma) after each antibody incubation step. Individual plaques were developed using a DAB substrate kit (ab64238, Abcam) following manufacturer's specifications. Results for immunoplaque assay were expressed as log10 PFU/g of lung.

Lungs in influenza-infected mice were treated similarly. Lung homogenates were centrifuged at 3000 rpm for 15 min, and the virus yield in the supernatant was determined by the plaque assay on MDCK cells as described by Takeda et al. [29]. Briefly, confluent monolayers of MDCK cells were incubated with the supernatant serially diluted in PBS containing 1% bovine serum albumin for 1 h at room

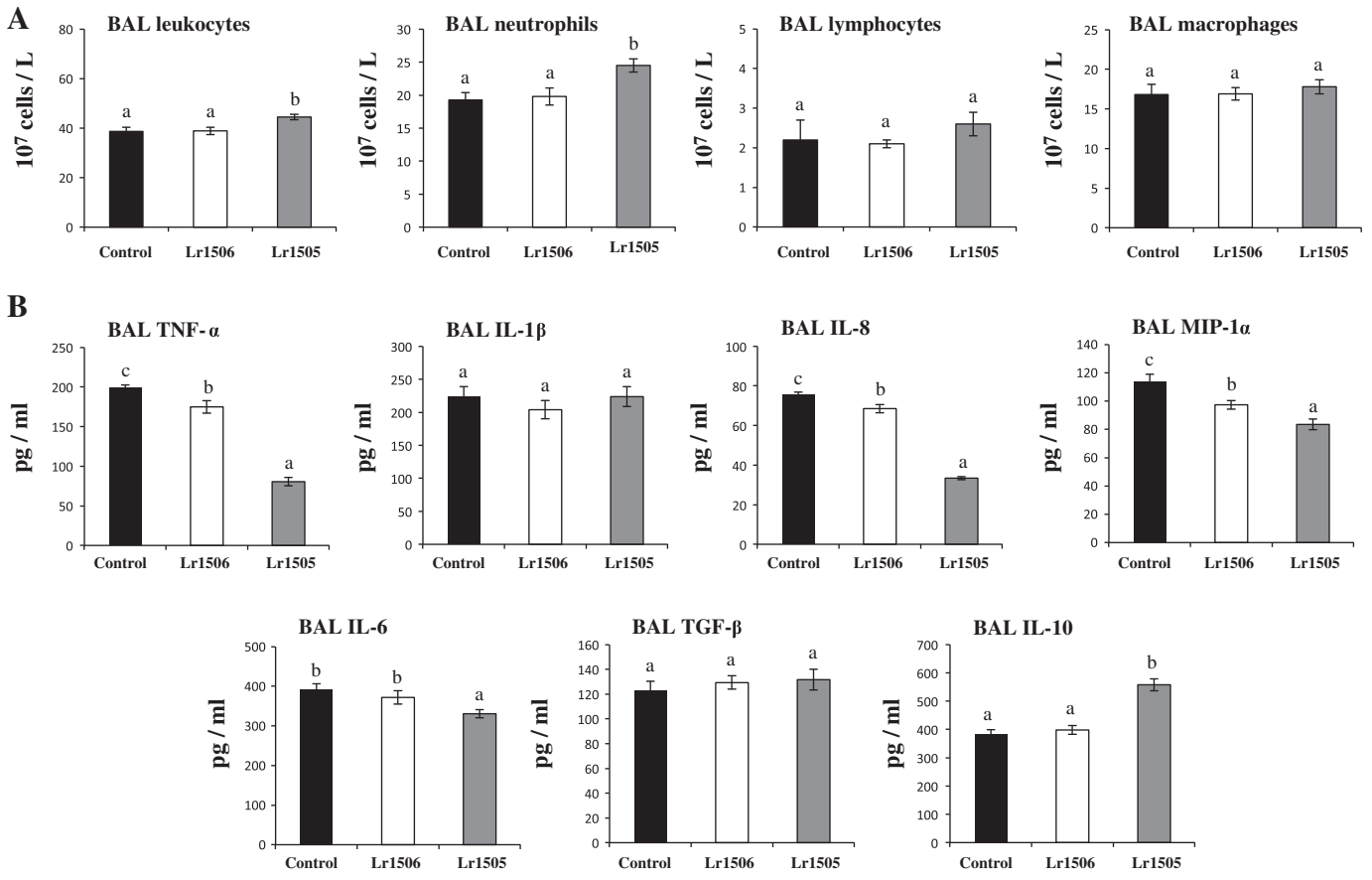

Fig. 2. Effect of lactobacilli on leukocytes and cytokines in broncho-alveolar lavages after the nasal administration of the viral pathogen-associated molecular pattern poly(I:C). Effect of *Lactobacillus rhamnosus* CRL1505 (Lr1505) or *L. rhamnosus* CRL1506 (Lr1506) administration on (A) the number of leukocytes, lymphocytes, neutrophils and macrophages, and (B) tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6, IL-8, IL-10, TGF-β, and macrophage inflammatory protein (MIP)-1α concentrations in broncho-alveolar lavages (BAL) after the challenge with poly(I:C). The results represent data from three independent experiments. Results are expressed as mean ± SD. Values for bars with different letters were significantly different ($P < 0.05$). Values for bars with shared letters do not differ significantly.

temperature. Then the cells were overlaid with nutrient agarose (0.8%) medium and cultured at 37 °C for 3 days. The cells were fixed with 5% formalin solution and stained with 0.03% methylene blue solution. Visualized plaques were counted under a dissecting microscope.

2.15. Blocking experiments

In order to evaluate the role of IL-10 in the protective effect of *L. rhamnosus* CRL1505, anti-IL-10 receptor (IL-10R) blocking antibodies were used. Different groups of mice were orally treated with *L. rhamnosus* CRL1505 for 5 consecutive days at a dose of 10^8 cells/mouse/day as described above. On day 6 the mice were injected intraperitoneally with 50 μg of purified anti-IL10R antibodies (LEAF™ Purified anti-mouse IL-10R Antibody) or 250 μg isotype control antibodies (LEAF™ Purified Rat IgG1, κ Isotype Ctrl) and 2 h later they were challenged with RSV or IFV. Virus titer, BAL TATc and TM and TF expression were determined as described previously.

2.16. 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 $P < 0.05$.

3. Results

3.1. L. rhamnosus CRL1505 reduces poly(I:C)-induced lung damage

We studied the lung tissue damage by evaluating lung histology and biochemical markers of alveolar–endothelial barrier alteration and cellular lysis. As we demonstrated previously, poly(I:C) significantly increased levels of albumin concentrations as well as LDH activity in BAL samples (Fig. 1A) [13,14]. Moreover, we showed here that challenge with poly(I:C) induced a clear tissue inflammation around alveoli and blood vessels in lung, with a significant reduction of gas exchange space in some regions of lungs (Fig. 1B). Lr1505 treatment decreased significantly the biochemical parameters that we use to evaluate pulmonary damage, whereas Lr1506-treated mice showed lung injuries similar to those observed in the control group (Fig. 1A). In addition, Lr1505 treatment significantly reduced inflammation and lung tissue alterations (Fig. 1B) while Lr1506 was not able to significantly modify lung alterations.

3.2. L. rhamnosus CRL1505 differentially modulates poly(I:C)-triggered respiratory inflammation

Total and differential leukocyte counts and the levels of cytokines in BAL were evaluated in order to study the respiratory inflammatory response (Fig. 2A). Challenge with poly(I:C) significantly increased the number of macrophages and neutrophils in the respiratory tract of all

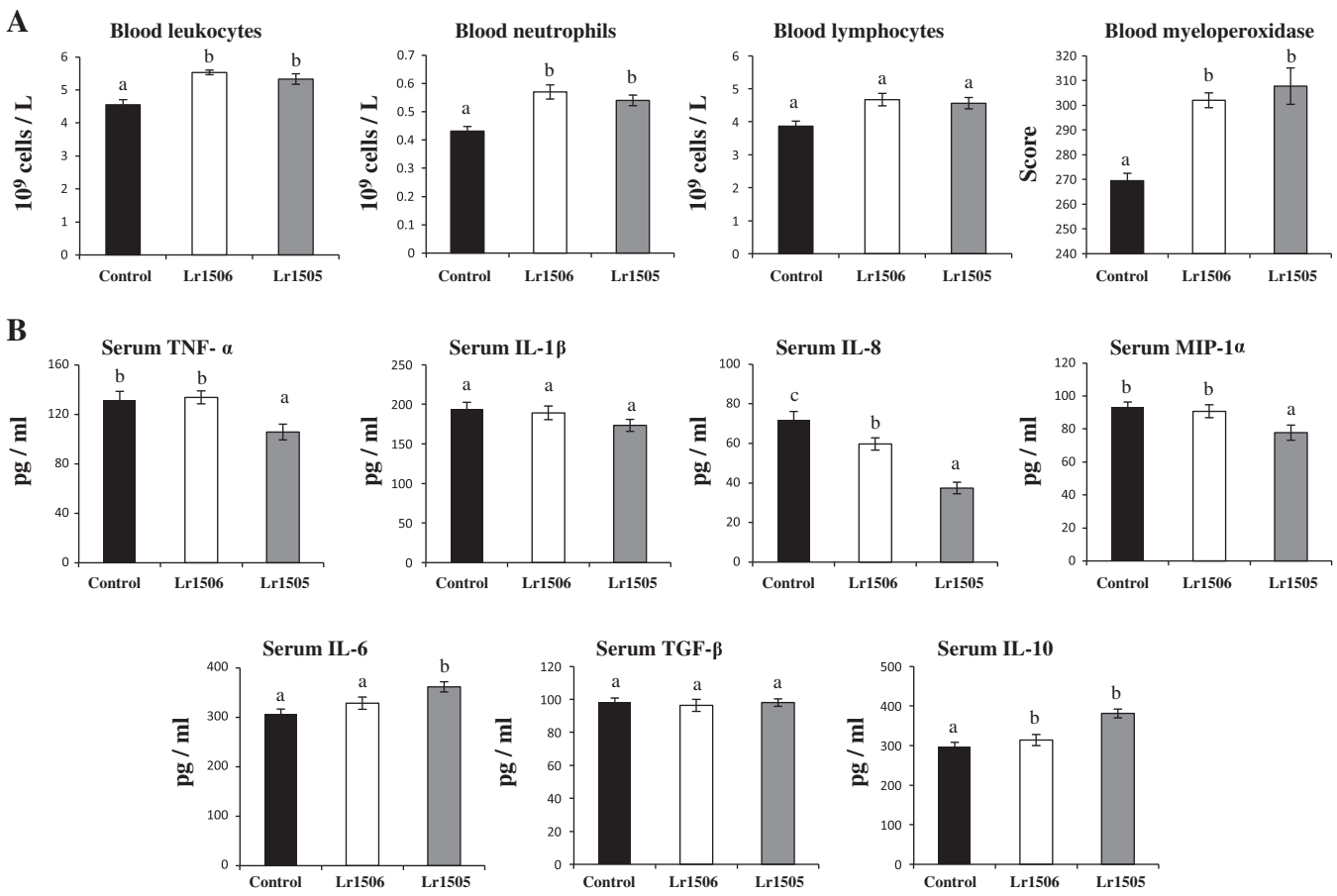

Fig. 3. Effect of lactobacilli on blood leucocytes and cytokines induced by the nasal administration of the viral pathogen-associated molecular pattern poly(I:C). Effect of *Lactobacillus rhamnosus* CRL1505 (Lr1505) or *L. rhamnosus* CRL1506 (Lr1506) administration on (A) the number of leukocytes, lymphocytes, neutrophils and peroxidase positive cells, and (B) tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6, IL-8, IL-10, TGF-β, and macrophage inflammatory protein (MIP)-1α concentrations in blood after the challenge with poly(I:C). The results represent data from three independent experiments. Results are expressed as mean ± SD. Values for bars with different letters were significantly different ($P < 0.05$). Values for bars with shared letters do not differ significantly.

the experimental groups (Fig. 2A). Lr1505 treated mice presented significantly higher values of inflammatory cells than Lr1506 and control mice (Fig. 2A). In addition, nasal administration of poly(I:C) significantly increased respiratory levels of the proinflammatory mediators IL-6, TNF-α, IL-1β, IL-8 and MIP-1, which correlated with the increased levels of inflammatory cells (Fig. 2B). No differences were observed between the lactobacilli-treated mice and controls when analyzing IL-1β levels (Fig. 2B). However, levels of IL-6, TNF-α, IL-8 and MIP-1 were significantly lower in the Lr1505 group when compared with Lr1506 and control mice (Fig. 2B). IL-10 and TGF-β in BAL were also increased after the challenge with poly(I:C) in all the experimental groups; however Lr1505 mice showed higher levels of BAL IL-10 than Lr1506 and control mice (Fig. 2B).

3.3. L. rhamnosus CRL1505 differentially modulates poly(I:C)-triggered systemic inflammation

Blood leukocyte counts were next evaluated in order to study the systemic inflammatory response. Challenge with poly(I:C) significantly increased the number of leucocytes and neutrophils in blood (Fig. 3A). The numbers of these cell populations were superior in Lr1505 and Lr1506 treated mice when compared to the control (Fig. 3A). In addition to the quantitative changes of blood neutrophils, we studied MPO as a measure of their activity. Challenge with poly(I:C) significantly increased MPO activity in blood neutrophils of all the experimental groups; however Lr1505 and Lr1506 mice showed MPO scores that were significantly higher than the control group (Fig. 3A). On the other hand, nasal challenge with poly(I:C) increased cytokines levels in serum (Fig. 3B). Lr1505 treated mice showed higher levels of IL-6, and a decreased production of TNF-α, IL-8, IL-1β and MIP-1 when compared to Lr1506 and control mice (Fig. 3B). In addition, Lr1505 group showed higher levels of serum IL-10 after the poly(I:C) challenge (Fig. 3B).

3.4. L. rhamnosus CRL1505 reduces coagulation activation after poly(I:C) challenge

Global coagulation tests in plasma were next evaluated in order to study the systemic hemostasis. Challenge with the poly(I:C) significantly decreased prothrombin activity in Lr1506 mice and the control group, while this parameter was close to 100% in Lr1505 treated mice (Fig. 4). On the contrary, no modifications in the APTT test were observed after challenge with the poly(I:C) in all the experimental groups (Fig. 4). In order to study the systemic coagulation activation state, we determined the concentration of TATc which are considered markers of *in vivo* coagulation activation. Normal values of TATc in plasma of mice are 4.5 ± 0.5 μg/l. Challenge with the poly(I:C) significantly increased plasma TATc levels; however Lr1505 treated mice showed levels of TATc that were significantly lower than the Lr1506 treated mice and the control group (Fig. 4). On the other hand, the nasal challenge with poly(I:C) slightly increased platelet counts in blood. Lr1505 and Lr1506-treated mice show significant higher numbers of platelets than the control group (Fig. 4). Finally, we determined vWF in plasma and observed that poly(I:C) challenge significantly increased this parameter,

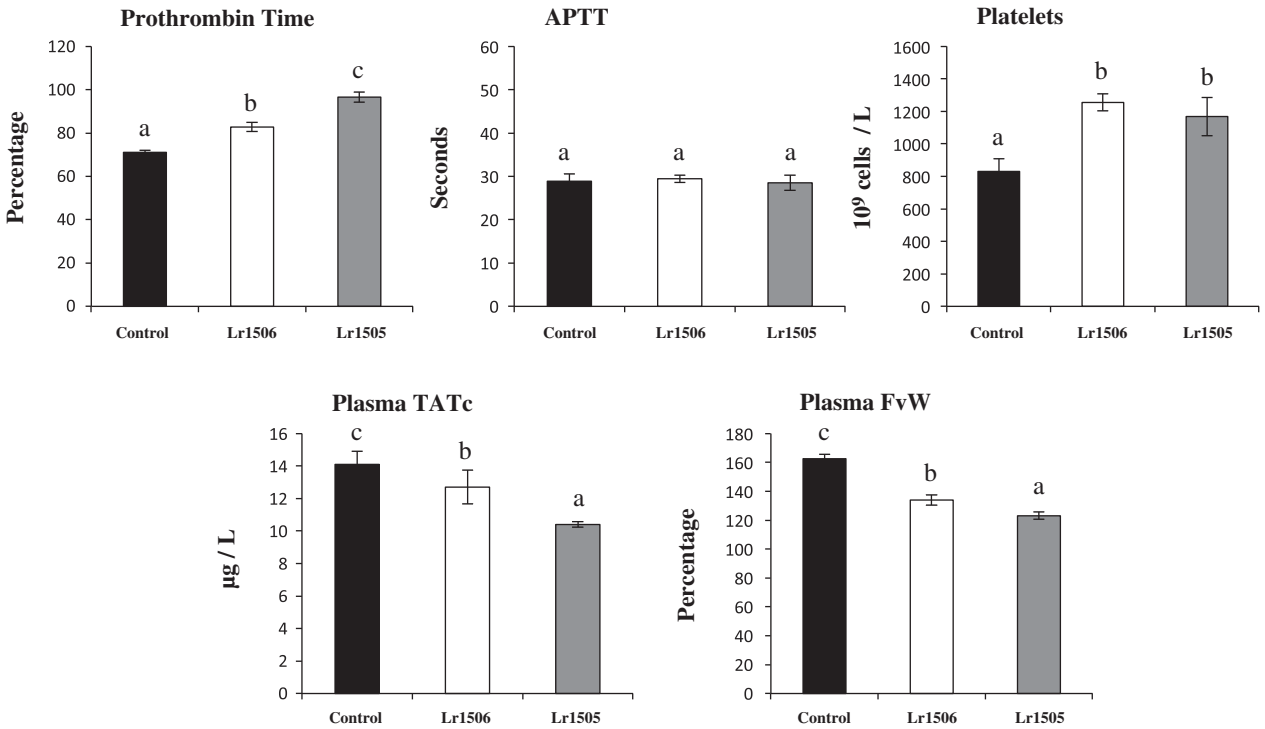

Fig. 4. Effect of lactobacilli on the hemostatic alterations at systemic level induced by the nasal administration of the viral pathogen-associated molecular pattern poly(I:C). Effect of *Lactobacillus rhamnosus* CRL1505 (Lr1505) or *L. rhamnosus* CRL1506 (Lr1506) administration on the hemostatic alterations at systemic level. Prothrombin time, activated partial thromboplastin time (APTT), thrombin–antithrombin complexes (TATc), and von Willebrand factor (vWF) concentration were evaluated in plasma. In addition, platelet counts were performed in blood. The results represent data from three independent experiments. Results are expressed as mean ± SD. Values for bars with different letters were significantly different ($P < 0.05$). Values for bars with shared letters do not differ significantly.

indicating systemic endothelial activation (Fig. 4). Treatments with Lr1505 or Lr1506 were able to significant reduce vWF values, being Lr1505 more effective than Lr1506 to achieve this effect (Fig. 4).

3.5. *L. rhamnosus CRL1505 reduces activation of coagulation in the respiratory tract*

We studied the levels of TATc in BAL in order to determine the local coagulation activation. Challenge with the poly(I:C) increased the levels of this parameter in BAL; however the Lr1505 treatment significantly reduced TATc in the respiratory tract when compared with Lr1506-treated and control mice (Fig. 5A). In addition, we observed higher levels of vWF in BAL of Lr1505 treated mice (Fig. 5A). In order to determine the pulmonary endothelial activation, we studied VCAM-1 expression by immunohistochemical techniques (Fig. 5B). Challenge with poly(I:C) significantly increased VCAM-1 expression in lung. The control group showed positive reaction of moderate intensity and focal localization in pulmonary endothelia (Fig. 5B). VCAM-1 expression in Lr1506 treated mice was not different from the control. On the contrary, Lr1505 treated mice showed an intense positive reaction with a diffuse location (Fig. 5B).

We also evaluated the changes of TF, TFPI, PAI-1 and TM lung expressions before and after the nasal challenge with poly(I:C). No changes in the expression of lung TF, TFPI, PAI-1 or TM were observed after lactobacilli treatment and before poly(I:C) challenge (data not shown). Increased expression of these factors was observed in all the experimental groups, especially in the levels of TF and TM that were increased 2.5 and 3.5 fold respectively, when compared to basal levels (Fig. 6). TF expression in Lr1505 treated mice was significantly lower than control and Lr1506 mice (Fig. 6). In addition, TM expression was superior in Lr1505 treated mice when compared to the control and Lr1506 groups (Fig. 6). TFPI and PAI-1 expressions in lungs were not different between Lr1505, Lr1506 and control mice (Fig. 6).

3.6. *L. rhamnosus CRL1505 differentially modulates the coagulative response to RSV or IFV infections*

We have demonstrated recently that Lr1505 administration to infant mice significantly improves their respiratory immune response against RSV [15]. Then, we next addressed the question of whether Lr1505 affected the outcome and the immune-coagulative response to RSV or IFV infections in adult mice. Adult mice orally treated with Lr1505 or Lr1506 were challenged with 10^6 PFU of RSV. Similarly to our previous work with infant mice [15], the virus was detected during 5 days post-infection in lungs of all the experimental groups, with a peak of viral loads on day 4 (Fig. 7A). However, Lr1505 treated mice showed significantly lower lung virus counts than the Lr1506 treated and control mice (Fig. 7A). We also observed that the challenge with RSV increased the levels of the TATc in the respiratory tract of adult mice. Lr1505 treated mice showed lower levels of BAL TATc when compared with Lr1506 and control mice (Fig. 7A). In addition, challenge with RSV significantly increased the expression of TF, TFPI, PAI-1 and TM in lung although the fold increases were lower than those observed in poly(I:C)-challenged mice (Fig. 7B). Lr1505 was able to downregulate TF and upregulate TM expression (Fig. 7B). No differences between the groups were observed in TFPI and PAI-1 expressions (Fig. 7B).

Several works have established strong connections between IFV infection and hemostasis; therefore we were interested in evaluating the effect of immunobiotic bacteria in the coagulative response to IFV. Similarly to RSV infection, IFV was detected during 5 days postinfection in lungs of all the experimental groups, with a peak of viral loads on days 3–4 (Fig. 8A). Lr1505 treated mice showed significantly lower IFV titers in lung when compared with Lr1506 and control groups. The levels of the TATc and the expression of TF, TFPI, PAI-1 and TM in the respiratory tract of adult mice infected with IFV were significantly higher than those observed in RSV-challenged mice (Fig. 8). Lr1505 treated mice showed lower concentrations of BAL TATc (Fig. 8A) and lower levels of TF (Fig. 8B) when compared with Lr1506 and control mice. In addition,

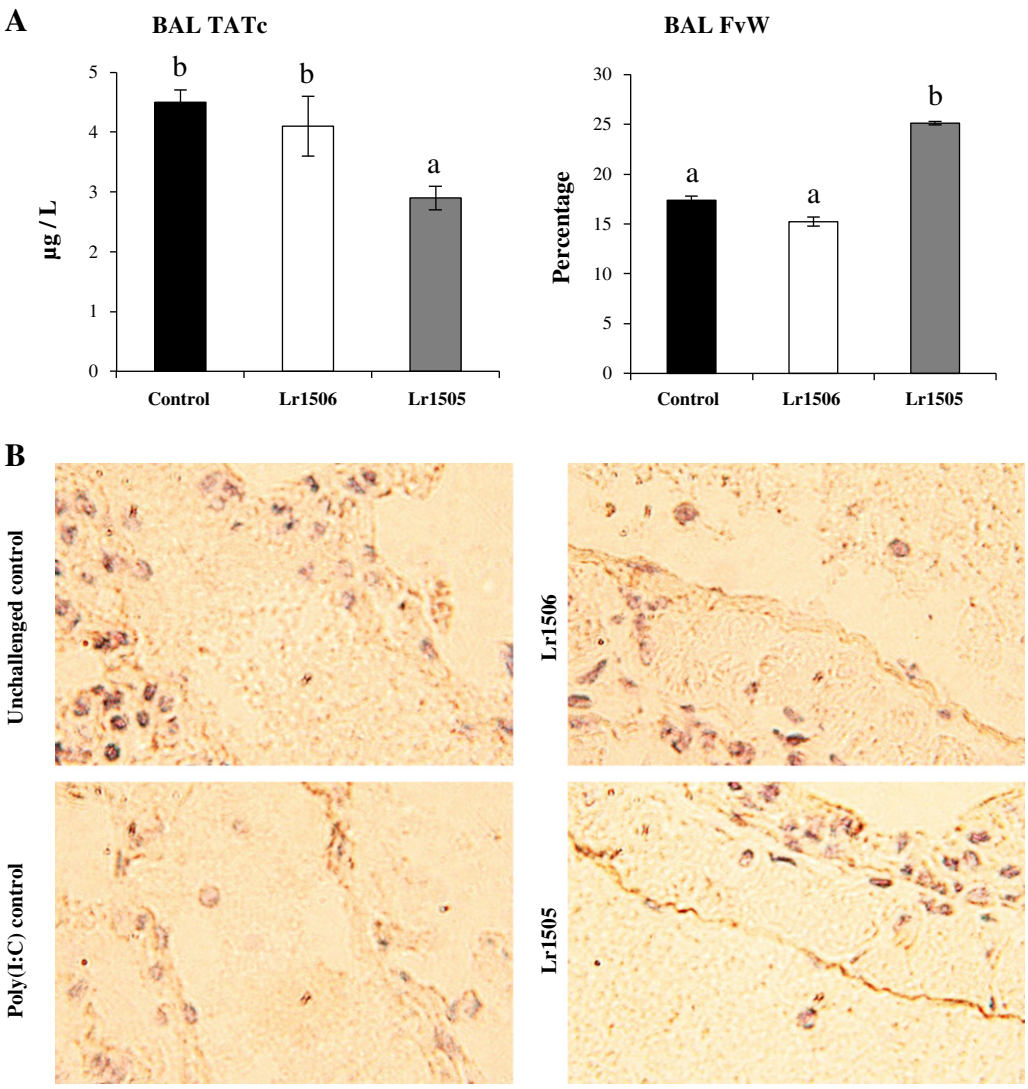

Fig. 5. Effect of lactobacilli on the hemostatic alterations in lung induced by the nasal administration of the viral pathogen-associated molecular pattern poly(I:C). Effect of *Lactobacillus rhamnosus* CRL1505 (Lr1505) or *L. rhamnosus* CRL1506 (Lr1506) administration on the hemostatic alterations in lung. (A) Thrombin–Antithrombin complexes (TATc) and von Willebrand Factor (vWF) concentrations were evaluated in broncho-alveolar lavages (BAL). The results represent data from three independent experiments. Results are expressed as mean ± SD. Values for bars with different letters were significantly different ($P < 0.05$). Values for bars with shared letters do not differ significantly. (B) VCAM-1 expression in lung was determined by immunohistochemistry. Light micrographs, original magnification ×400 and ×1000.

Lr1505 was able to upregulate TM expression while no differences between the groups were observed in TFPI and PAI-1 expressions after IFV infection (Fig. 8B).

3.7. IL-10 is involved in the capacity of L. rhamnosus CRL1505 to modulate the coagulative response during respiratory virus infection

In order to evaluate the role of IL-10 in the effect of *L. rhamnosus* CRL1505 on the coagulative response during RSV and IFV infections we used blocking anti-IL-10R antibodies. As shown in Fig. 9, anti-IL-10R antibodies did not induce significant effects in the reduction of RSV or IFV titers induced by the CRL1505 strain. However, treatment of mice with anti-IL-10R antibodies significantly abolished the capacity of the immunobiotic strain to reduce concentrations of BAL TATc and the expression of TF in lung as well as to increase the expression of TM during RSV or IFV infections (Fig. 9).

4. Discussion

In general terms, respiratory viruses tend to evoke remarkably similar innate and adaptive immune responses despite the variety of receptors they use to gain entry into host cells and their genetics composition. In this regard, although genetically dissimilar, both RSV and IFV generate dsRNA replication intermediates that act as TLR3 ligands and contribute to immune system activation. IFV, a single-stranded RNA virus has been shown to trigger type I IFN through recognition by TLR3 in myeloid DCs, fibroblasts or alveolar epithelial cells [30]. In addition, TLR3 expressed by respiratory epithelial cells and DCs contributes at recognizing RSV during infection by binding to viral RNA [31]. Challenge-infection experiments in TLR3 −/− mice demonstrated that TLR3 does not alter respiratory viruses clearance but it is important for the regulation of the pathogenic responses in the lung. It was shown that RSV does not require TLR3 for effective clearance but an important role of this PRR in the regulation of pulmonary immune microenvironment and subsequent mucus hypersecretion [32] was suggested. In addition, Le Goffic et al. [33], indicated that a potent inflammatory reaction occurs in the lung of wild-type mice after the IFV infection and that this process is critically reduced or altered in TLR3 −/− animals. Thus, in comparison with wild-type mice, TLR3 −/− animals showed clear reduced levels of inflammatory mediators in BAL, reduction of inflammatory cells recruitment into the lungs and a paradoxical longer survival. Additionally, siRNA-mediated knockdown of TLR3 indicated that this

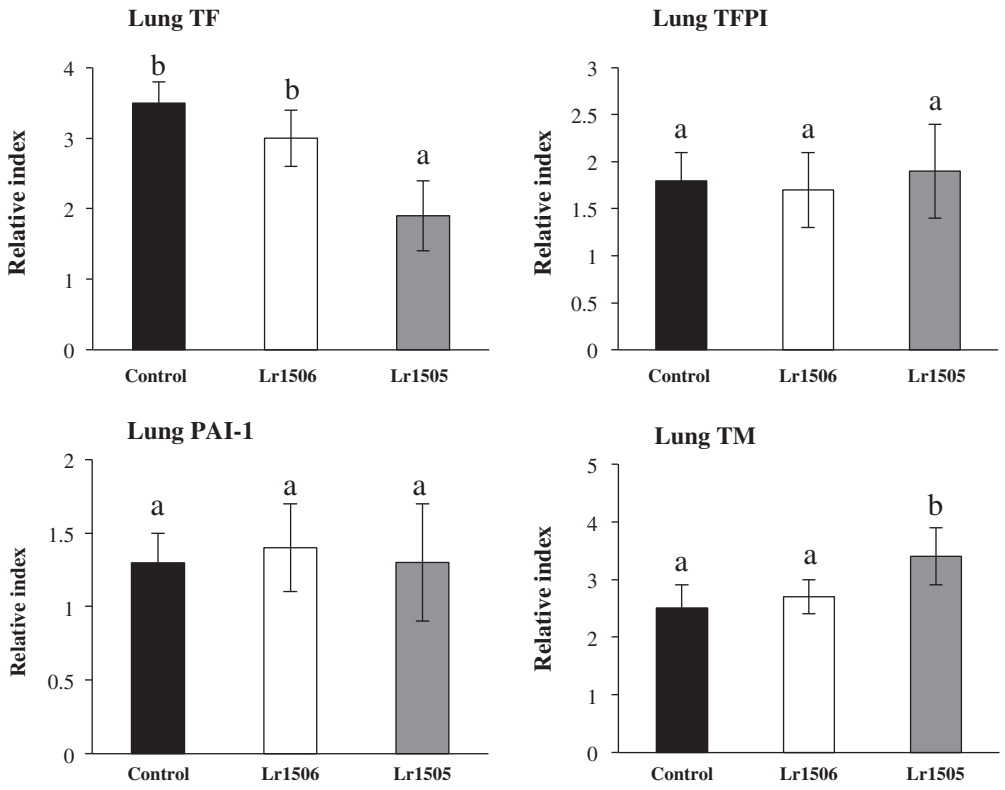

Fig. 6. Effect of lactobacilli on the expression of hemostatic factors in lung after nasal administration of the viral pathogen-associated molecular pattern poly(I:C). Effect of *Lactobacillus rhamnosus* CRL1505 (Lr1505) or *L. rhamnosus* CRL1506 (Lr1506) administration on the expression of hemostatic factors in lung. Tissue factor (TF), tissue factor pathway inhibitor (TFPI), plasminogen activator inhibitor (PAI)-1, and thrombomodulin (TM) mRNA expressions in lung were examined using RT-qPCR. The results represent data from three independent experiments. Results are expressed as mean ± SD. Values for bars with different letters were significantly different ($P < 0.05$). Values for bars with shared letters do not differ significantly.

receptor was the predominant receptor responsible for inducing a procoagulant state in endothelial cells [10]. Stimulation of endothelial cells with poly(I:C) induces a dose- and time-dependent increase in TF expression and downregulation of TM. *In vivo* studies showed that poly(I:C) could upregulate the expression of proinflammatory and antiviral cytokines [34], influence vascular permeability [35] and increase circulating D-dimer levels indicating that both coagulation and fibrinolysis are stimulated. Moreover, it was shown that D-dimer levels were smaller in TLR3 −/− mice after poly(I:C) challenge [10]. It was suggested then, that the shift to a hypercoagulation state in certain respiratory virus infections could be due to a procoagulant cytokine status and the direct stimulation of TF and repression of TM via TLR3.

Overall, these data suggest that TLR3 activation can be a critical component in the modulation of viral infection-associated inflammatory and procoagulant diseases. The results of this work are in line with these previous studies. In our experiments, administration of poly(I:C) to the lungs of mice induced a marked increase in levels of the proinflammatory mediators IL-6, TNF-α, IL-1β, IL-8 and MIP-1, as well as increases in inflammatory cells. Moreover, *in vivo* TLR3 agonism by poly(I:C) also resulted in increased TATc levels and TF expression in the respiratory tract, and reduced TM expression in lung. These inflammatory-coagulative changes were accompanied by pulmonary injury and impairment of lung function. In addition, we demonstrated here that the inflammatory-coagulative response induced by the nasal administration of poly(I:C) could be differentially modulated by the preventive administration of the probiotic bacterium *L. rhamnosus* CRL1505. We observed that the CRL1505 strain was able to significantly reduce activation of coagulation in blood and in the respiratory tract after the nasal challenge with poly(I:C). Those effects were associated to the capacity of the probiotic treatment to reduce the expression of TF and increase levels of TM in lungs after the stimulation with the TLR3 agonist.

We further investigated the effect of viral infections in pulmonary coagulation. Evaluation of coagulation activation and viral titers was performed on day 4 postinfection, the time point at which both RSV and IFV peak. Both respiratory viruses were able to induce activation of coagulation in the respiratory tract as observed by the increased levels of TATc in BAL samples, and the changes in the expression of TF and TM in lungs. However, procoagulant changes induced by IFV were markedly higher than those induced by RSV. In fact, TATc levels and lung TF expression in IFV-infected mice were 1.8 and 1.6 fold higher than in RSV-infected animals. Respiratory viruses can induce a direct procoagulant state through infection of endothelial and/or monocytes, or indirectly by the induction of proinflammatory cytokines such as IL-6 [1,2]. The infection of endothelial cells can result in the activation of these cells and, consequently, the activation of coagulation. *In vitro* and *in vivo* studies have shown that a variety of prothrombotic viruses are able to infect endothelial cells. In this regard, it has been demonstrated that IFV and other respiratory viruses can activate coagulation by increasing TF expression on endothelial cell surfaces [1,2]. Visseren et al. [1] determined the effect of infection with various respiratory viruses on the procoagulant activity of intact endothelial cell monolayers. Authors demonstrated that both RSV and IFV were able to infect human umbilical vein endothelial cells. Moreover, the study showed that both respiratory viruses induced a procoagulant activity by stimulating the expression of TF. However, procoagulant activity and TF antigen expression by endothelial cells were significantly higher in IFV-infected cells when compared to those challenged with RSV. In addition, increased numbers of intravascular thrombi and fibrin deposition in lungs were found in cases of influenza in both animal models and clinical studies [3,36,37]. However, extensive fibrin depositions have not been described in lung tissue from patients or animals infected with RSV. Then, our results are in line with these previous studies showing a higher procoagulant activity for IFV when compared to RVS.

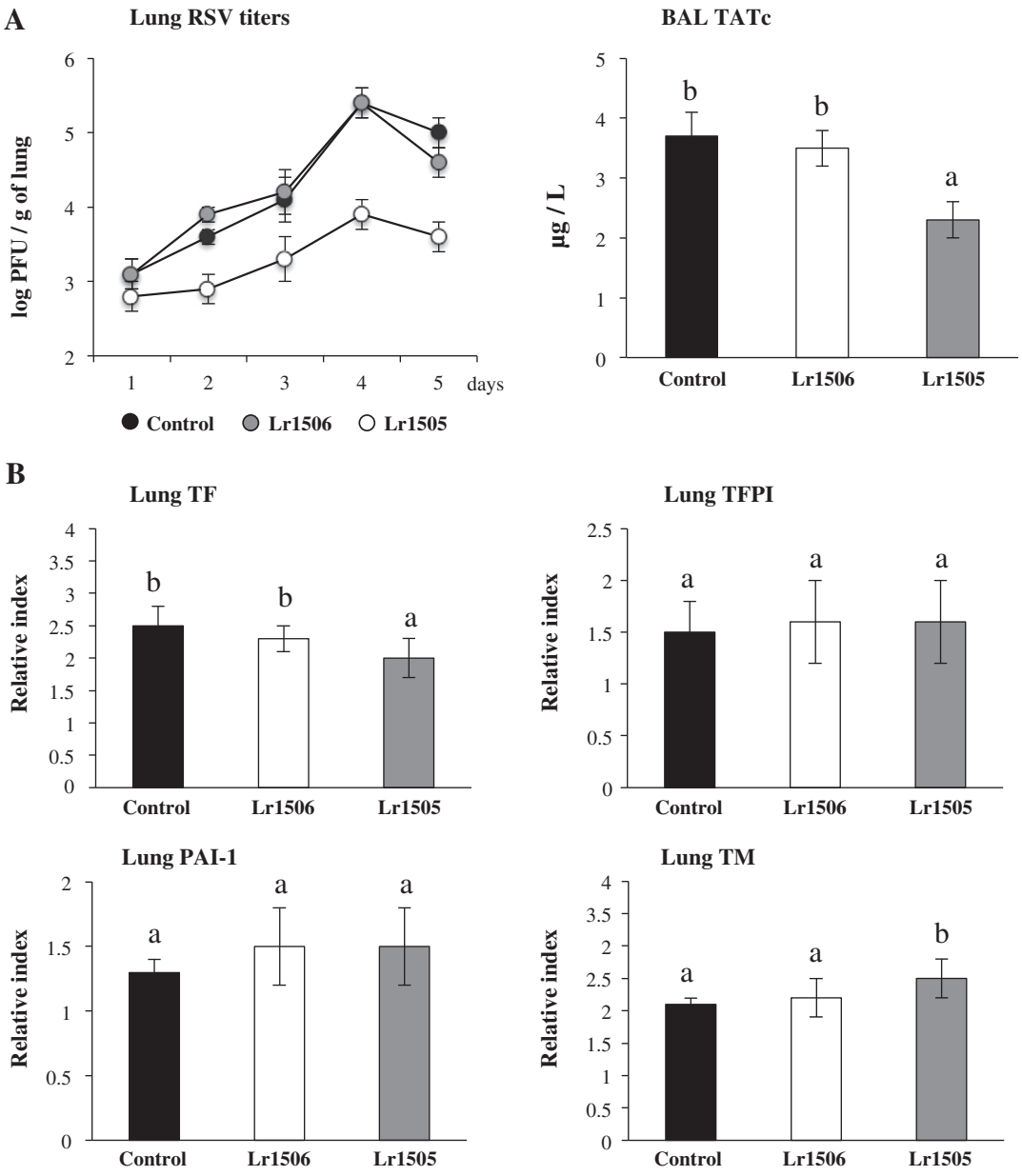

Fig. 7. Effect of lactobacilli on the resistance and coagulation activation during respiratory syncytial virus (RSV) infection. Effect of *Lactobacillus rhamnosus* CRL1505 (Lr1505) or *L. rhamnosus* CRL1506 (Lr1506) administration on (A) the resistance against respiratory syncytial virus (RSV) infection, and coagulation activation (TATc levels) in lungs. (B) Expression of hemostatic factors in lung. Tissue factor (TF), tissue factor pathway inhibitor (TFPI), plasminogen activator inhibitor (PAI)-1, and thrombomodulin (TM) mRNA expression in lung was examined using RT-qPCR. Results are expressed as mean ± SD. Values for bars with different letters were significantly different ($P < 0.05$). Values for bars with shared letters do not differ significantly.

Our data also showed that the preventive administration of *L. rhamnosus* CRL1505 significantly reduced both RSV and IFV lung viral titers. Previously it was shown that oral administration of *L. rhamnosus* CRL1505 to infant mice significantly reduces lung viral loads and tissue injuries after the challenge with RSV and that the protective effect achieved by the CRL1505 strain is related to its capacity to differentially modulate the respiratory antiviral immune response [15]. Moreover, our studies demonstrated that *L. rhamnosus* CRL1505 is able to increase the number of $CD3^+CD4^+IFN\text{-}\gamma^+$ T cells in the gut, induce a mobilization of these cells into the respiratory mucosa, and improve local production of IFN-γ and the activity of lung antigen presenting cells [13,15]. In this work we extend these findings by demonstrating that the CRL1505 strain is also able to improve resistance of adult immunocompetent mice against IFV and RSV. In addition, the results of this work showed that *L. rhamnosus* CRL1505 significantly reduced the activation of coagulation in both IFV- and RSV-infected mice. As observed in poly(I:C) challenge experiments, in *L. rhamnosus* CRL1505-treated mice infected with IFV or RSV, levels of TATc and expression of TF were significantly lower than the respective controls. Several studies have demonstrated that some orally administered immunobiotics do have the ability to stimulate respiratory immunity and increase resistance to viral infections, including IFV, RSV and pneumonia virus of mice [11]. Moreover, during the last decade scientists have significantly advanced in the knowledge of the cellular and molecular mechanisms involved in the protective effect of immunobiotics in the respiratory tract, and demonstrated that orally administered probiotics are able to increase NK cells and macrophages activities, modulate type I IFN and IFN-γ production and, antigen presenting cells functions in lungs infected with viruses, allowing an improved immune response and a higher resistance to the infection [11]. However,

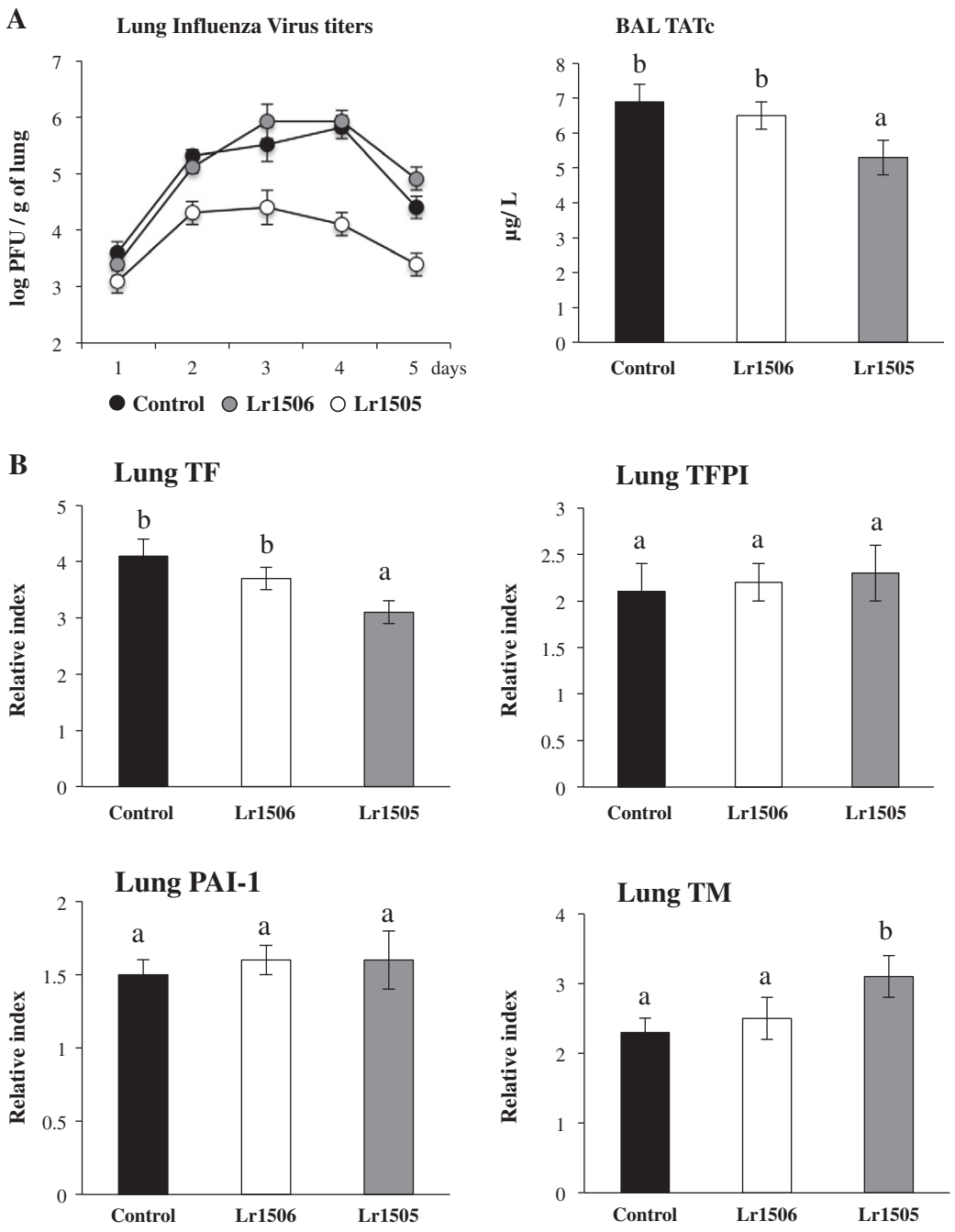

Fig. 8. Effect of lactobacilli on the resistance and coagulation activation during influenza virus infection. Effect of *Lactobacillus rhamnosus* CRL1505 (Lr1505) or *L. rhamnosus* CRL1506 (Lr1506) administration on (A) the resistance against influenza virus infection, and coagulation activation (TATc levels) in lungs. (B) Expression of hemostatic factors in lung. Tissue factor (TF), tissue factor pathway inhibitor (TFPI), plasminogen activator inhibitor (PAI)-1, and thrombomodulin (TM) mRNA expression in lung was examined using RT-qPCR. Results are expressed as mean ± SD. Values for bars with different letters were significantly different ($P < 0.05$). Values for bars with shared letters do not differ significantly.

the effect of immunobiotics on the inflammation–coagulation interaction during respiratory viral infections was not studied until now. Then, this work is the first demonstration of the beneficial modulation of the immune-coagulative response during respiratory viral infection induced by a probiotic bacterium.

Future research will concentrate in elucidating the mechanism(s) by which *L. rhamnosus* CRL1505 influences coagulation after poly(I:C) challenge or during IFV or RSV infections. From the information obtained until now, it could be speculated that the modulation of the inflammatory response induced by the probiotic strain would indirectly modulate the coagulation system. In fact, the experiments performed here using anti-IL-10R blocking antibodies clearly demonstrated that the variations in IL-10 levels are important for the regulation of coagulation induced by *L. rhamnosus* CRL1505. We have previously demonstrated that orally administered *L. rhamnosus* CRL1505 significantly increased IL-10 levels that contribute to protection against inflammatory damage in poly(I:C)- and RSV-challenged mice. Moreover, blocking IL-10R significantly reduced the capacity of the CRL1505 strain to protect against lung tissue damage although it did not affect viral load [15]. In addition, we demonstrated in this study that blocking IL-10R impaired the ability of *L. rhamnosus* CRL1505 treatment to reduce activation of coagulation in mice infected with IFV or RSV. We also observed previously that CRL1505-treated mice were able to early increase the levels of TNF and IL-6 in the respiratory tract when compared to controls while the levels of TNF were significantly lower later in RSV infection [15]. Then, the early increase of proinflammatory cytokines together with the improved

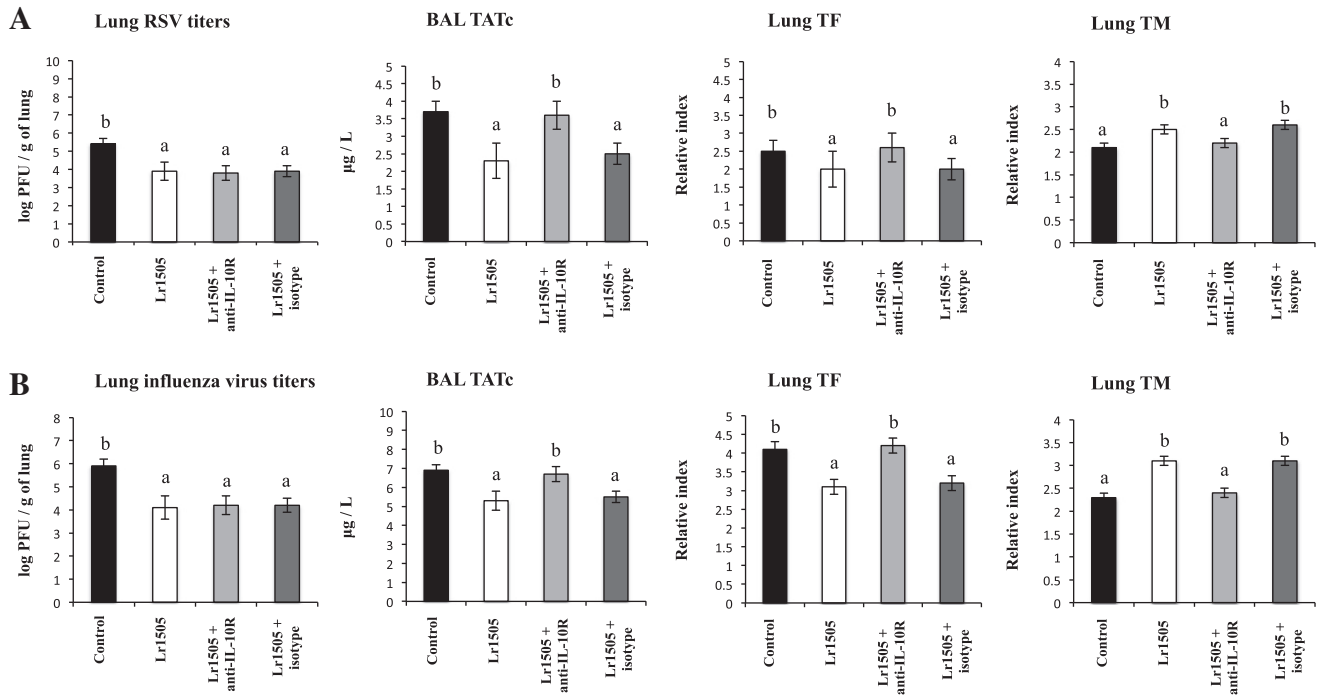

Fig. 9. Role of IL-10 in the immunoregulatory effect of *Lactobacillus rhamnosus* CRL1505 in the resistance and coagulation activation during respiratory viruses infections. Mice were orally treated with *Lactobacillus rhamnosus* CRL1505 (Lr1505), intraperitoneally injected with anti-IL-10R antibodies and then challenged with (A) respiratory syncytial virus or (B) influenza virus. Untreated and isotype-treated mice were used as controls. Virus titers, coagulation activation (TATc levels) and expression of tissue factor (TF), and thrombomodulin (TM) were examined in lungs. Results are expressed as mean ± SD. Values for bars with different letters were significantly different ($P < 0.05$). Values for bars with shared letters do not differ significantly.

levels of IFN-γ explains the higher capacity of CRL1505-treated mice to reduce viral loads while the improved levels of IL-10 induced during infection would lead to markedly reduced severity in lung damage through the modulation of inflammation and coagulation. In support of this hypothesis, several works have described beneficial effects of IL-10 during respiratory viral infections. Sun et al. [38] showed that IL-10 prevents immunopathology and lethal disease during acute IFV infection. On the other hand, IL-10 also seems to play a crucial role in controlling disease severity in RSV infection [39,40]. It was found that IL-10 deficiency during RSV challenge did not affect viral load, but led to markedly increased disease severity with enhanced weight loss, delayed recovery and a greater influx of inflammatory cells into the lung and airways and enhanced release of inflammatory mediators [41]. Additionally, IL-10 has been shown to downregulate TF expression and inhibit procoagulant activity in human and mice monocytes and monocytes/endothelial cell co-cultures in a dose-dependent manner [42,43].

In addition, our present data showed that *L. rhamnosus* CRL1506 has no ability to beneficially modulate the immune-coagulative response in the respiratory tract despite its capacity to improve coagulation at the systemic level. This fact could be explained by the ability of this strain to induce changes in cytokines profiles in blood and not in the respiratory tract as we demonstrated here and in previously published works [13]. Then, these results confirm our hypothesis that changes in cytokines profiles are responsible for the modifications in the immune-coagulative response during viral respiratory infections.

Acute respiratory tract infections are associated with an increased risk of acute ischemic heart disease, stroke and venous thromboembolism [6,44]. A transient change in local hemodynamic factors, coagulation activation, reduced generation of anticoagulant activated protein C (APC), inhibition of fibrinolysis and endothelial cell perturbation as a result of systemic inflammation might be underlying mechanisms [45]. Indeed, it has been shown that respiratory viruses are able to activate coagulation, causing a reduction in clotting time and an increase in the expression of TF and thrombin generation; the latter reduced levels of protein C are risk factors for thrombotic diseases during respiratory viral infections [1,3]. In this regard, the effect of a naturally occurring acute respiratory tract infection on hemostatic proteins in human subjects was evaluated by van Wissen et al. [46] who found that respiratory tract infections result in endothelial cell perturbation and an increased fibrinolytic state with the potential for increased coagulation activation. Because endothelial cell perturbation and increased levels of hemostatic markers, such as vWF, D-dimer, plasmin–α2–antiplasmin complexes, PAI-1 and resistance to APC have been suggested to increase the risk of ischemic heart disease and venous thromboembolism, authors suggested that the induced hemostatic changes may form a link between acute respiratory tract infections and acute atherothrombotic disease. Then, the results presented in this work suggest that probiotic bacteria could be an interesting alternative not only to prevent or reduce the severity of respiratory viral infections, but in addition to reduce the risk of atherothrombotic diseases associated to acute respiratory tract infections.

In summary, our findings reveal for the first time that a probiotic bacterium is able to modulate lung immune-coagulative reaction triggered by TLR3 activation. We demonstrated that probiotic bacteria could be of value to beneficially modulate the fine tune balance between clearing the virus and controlling immune-coagulative responses in the respiratory tract, allowing normal gas exchange to be maintained in the face of a viral attack. Our data also pinpoint a crucial role for IL-10 in the immune protection induced by *L. rhamnosus* CRL1505 during respiratory viral infections. These observations might be helpful to propose new preventive or therapeutic approaches to better control virus-inflammatory lung damage using probiotic functional foods.

Acknowledgements

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Disclosure

The authors have no conflicts of interest to disclose.

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