Microbiology and Immunology

Microbiol Immunol 2014; 58: 416–426
doi: 10.1111/1348-0421.12163

ORIGINAL ARTICLE

Modulation of the inflammation–coagulation interaction during pneumococcal pneumonia by immunobiotic *Lactobacillus rhamnosus* CRL1505: Role of Toll-like receptor 2

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ABSTRACT

The present study evaluated the effect of nasally given *Lactobacillus rhamnosus* CRL1505 on the immunocoagulative response during pneumococcal infection in immunocompetent mice. In addition, we aimed to gain insight into the mechanism involved in the immunomodulatory effect of the *L. rhamnosus* CRL1505 strain by evaluating the role of TLR2. Results showed that nasally given *L. rhamnosus* CRL1505 effectively regulates inflammation and hemostatic alterations during the pneumococcal infection. Immunobiotic treatment significantly reduced permeability of the bronchoalveolar–capillary barrier, and general cytotoxicity, decreasing lung tissue damage. The CRL1505 strain improved the production of TNF-α, IFN-γ, and IL-10 after pneumococcal challenge. In addition, increased TM and TF expressions were found in lungs of *L. rhamnosus* CRL1505-treated mice. Moreover, we demonstrated, for the first time, that the TLR2 signaling pathway has a role in the induction of IFN-γ and IL-10 and in the reduction of TF. The results also allow us to speculate that a PRR, other than TLR2, may mediate the immunobiotic activity of *L. rhamnosus* CRL1505 and could explain changes in TNF-α and TM.

Key words hemostasis–inflammation, *Lactobacillus rhamnosus* CRL1505, lung injury, Toll-like receptor 2.

Acute respiratory tract infections are associated with an increased risk of acute ischemic heart disease, stroke, and venous thromboembolism (1, 2). A transient change in local hemodynamic factors, coagulation activation, reduced generation of anticoagulant proteins, inhibition of fibrinolysis, and endothelial cell perturbation as a result of systemic inflammation might be underlying mechanisms (3). Indeed, it has been shown that respiratory pathogens are able to activate coagulation, causing a reduction in clotting time and increasing the expression of TF. Moreover, reduced levels of protein C and thrombin generation are risk factors for thrombotic diseases during respiratory infections (4, 5). Therefore, both inflammation and coagulation play key roles in host defenses against respiratory pathogens. The coagulation process as part of the innate immune response leads to local restriction and trapping of the infectious agent and protects the rest of the organism (6, 7). Moreover, the

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Received 8 May 2014; revised 26 May 2014; accepted 30 May 2014.

List of Abbreviations: APC, antigen-presenting cells; APTT, activated partial thromboplastin time; BAL, bronchoalveolar lavage; IEC, intestinal epithelial cells; LAB, lactic acid bacteria; LDH, lactate dehydrogenase; MAMP, microorganism-associated molecular patterns; MCP, monocyte chemotactic protein; MPO, myeloperoxidase activity; PRR, pattern recognition receptors; PT, prothrombin time; TF, tissue factor; TM, thrombomodulin; vWF, von Willebrand factor.

inflammatory response triggered by infection activates the hemostatic systems and their products in order to perpetuate and strengthen inflammatory reactions, indicating that both systems are tightly connected. The delicate balance between the beneficial and the negative effects of this immunocoagulative response is of great importance in the outcome of respiratory infections (8, 9).

Several studies have demonstrated that certain probiotic LAB strains can exert a beneficial effect on the host through their immunomudulatory activity. Although most research concerning LAB-mediated enhanced immune protection is focused on gastrointestinal tract pathogens, a few recent studies have clearly demonstrated that immunomodulatory probiotic LAB (immunobiotics) are capable of stimulating the common mucosal immune system to provide protection to other mucosal sites as well (10, 11). In this regard, our laboratory demonstrated that certain probiotic LAB can exert a beneficial effect on the host during *Streptococcus pneumoniae*, respiratory syncytial virus, and influenza virus infection through their capacity to beneficially modulate the immunocoagulative response (12–14). We demonstrated that orally given *Lactobacillus casei* CRL431 is able to effectively regulate coagulation activation and fibrinolysis inhibition during pneumococcal infection in immunocompetent mice, which led to decreased fibrin deposits in lung and reduced tissue damage (12). In addition, repletion of malnourished mice with supplemental *L. casei* CRL431 given by oral (13) or nasal routes (14) was able to beneficially modulate the inflammation–coagulation relationship during the pneumococcal infection, indicating that LAB are able to modulate the immunocoagulative response in immunocompromised hosts. More recently, we demonstrated that another immunobiotic strain, *Lactobacillus rhamnosus* CRL1505, is able to improve the respiratory innate immune response and reduce activation of coagulation after challenge with bacterial or viral pathogens, reducing lung tissue damage and improving survival (15, 16).

Studies of the mechanisms of probiotic action have shown that the immunomodulatory mechanisms behind the positive effects of immunobiotics are related to a PRR-mediated signaling pathway in the innate immunity (17). One member of the TLR family, TLR2, is critical for the recognition of peptidoglycan, lipopeptides, and lipoproteins of Gram-positive bacteria, mycoplasma lipopeptides or fungal zymosan (18). Results from several studies showed that TLR2 is required for some probiotics to exert their immunomodulatory effects. Studies in a primary culture of IEC from conventional mice showed that immunostimulatory lactobacilli induce the release of cytokines by these cells via TLR2 (19). In addition, lactobacilli are able to increase the expression of TLR2 in intestinal DC and macrophages (20, 21). Moreover, it was suggested that the strong induction of Th1-polarizing DC by lactobacillus strains is dependent on lipoteichoic acid interaction with TLR2 (22). In contrast, it has been demonstrated that TLR2 seems to play an important regulatory role in the recognition of lactobacilli that possess an immunoinhibitory effect. It was demonstrated that stimulation of IEC with *Lactobacillus jensenii* TL2937 is able to downregulate the levels of IL-6, IL-8, and MCP-1 produced in response to LPS challenge and that TLR2 is partially involved in this immunoregulatory effect (23). More recently, it was reported that direct exposure of APC to *L. jensenii* TL2937 activated these cells and caused them to become phenotypically and functionally mature and to display tolerogenic properties. Again, these effects were partially dependent on TLR2 activation (21).

Considering this background, the current study aimed to evaluate the effect of nasally given *L. rhamnosus* CRL1505 on the immunocoagulative response during pneumococcal infection in immunocompetent mice. In addition, we aimed to gain insight into the mechanism involved in the immunomodulatory effect of the *L. rhamnosus* CRL1505 strain by evaluating the role of TLR2.

MATERIALS AND METHODS

Animals

Male 6-week-old Swiss albino mice were obtained from the closed colony kept at CERELA (Chacabuco 145, San Miguel de Tucumán, Argentina). They were housed in plastic cages in a controlled atmosphere (22 ± 2 °C, 55 ± 2% humidity) with 12 hr light/dark cycles.

Microorganisms

Lactobacillus rhamnosus CRL1505 was obtained from the CERELA culture collection. The culture was 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 hr 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 three times with sterile 0.01 mol/L PBS, pH 7.2.

Streptococcus pneumoniae was purchased from Administración Nacional de Laboratorios e Institutos de Salud-ANLIS, Buenos Aires, Argentina. The pathogen strain belongs to the 14 serotype, one of the 10 most

frequent serotypes isolated in pneumococcal infections in Argentina (24).

Lactobacillus rhamnosus CRL1505 treatment and TLR2 blocking

The probiotic strain *L. rhamnosus* CRL1505 was isolated from goat milk. The strain was given by the nasal route for 2 consecutive days at a dose of 10^8 cells/mouse per day, which is the optimal dose with an immunomodulatory effect (25–28). In order to evaluate the role of TLR2 in the protective effect of *L. rhamnosus* CRL1505, 60 μg purified anti-TLR2 blocking antibody (Abcam Inc., Cambridge, UK; anti-mouse TLR2 antibody) was used. Therefore, four experimental groups were used (Fig. 1): (i) mice treated with *L. rhamnosus* CRL1505 (Lr05 group); (ii) mice that received by the nasal route the anti-TLR2 1 hr before *L. rhamnosus* CRL1505 treatment (anti-TLR2(n) + Lr05 group); (iii) mice that received i.p. the anti-TLR2 3 hr before *L. rhamnosus* CRL1505 treatment (anti-TLR2(p) + Lr05 group); and (iv) mice that received i.p. PBS without *L. rhamnosus* CRL1505 treatment (control group).

Experimental infection

Streptococcus pneumoniae was grown according to previous studies (29). At the end of the *L. rhamnosus* treatment (on the 3rd day) the animals were challenged with the pathogen (Fig. 1). Animals with (Lr05, anti-

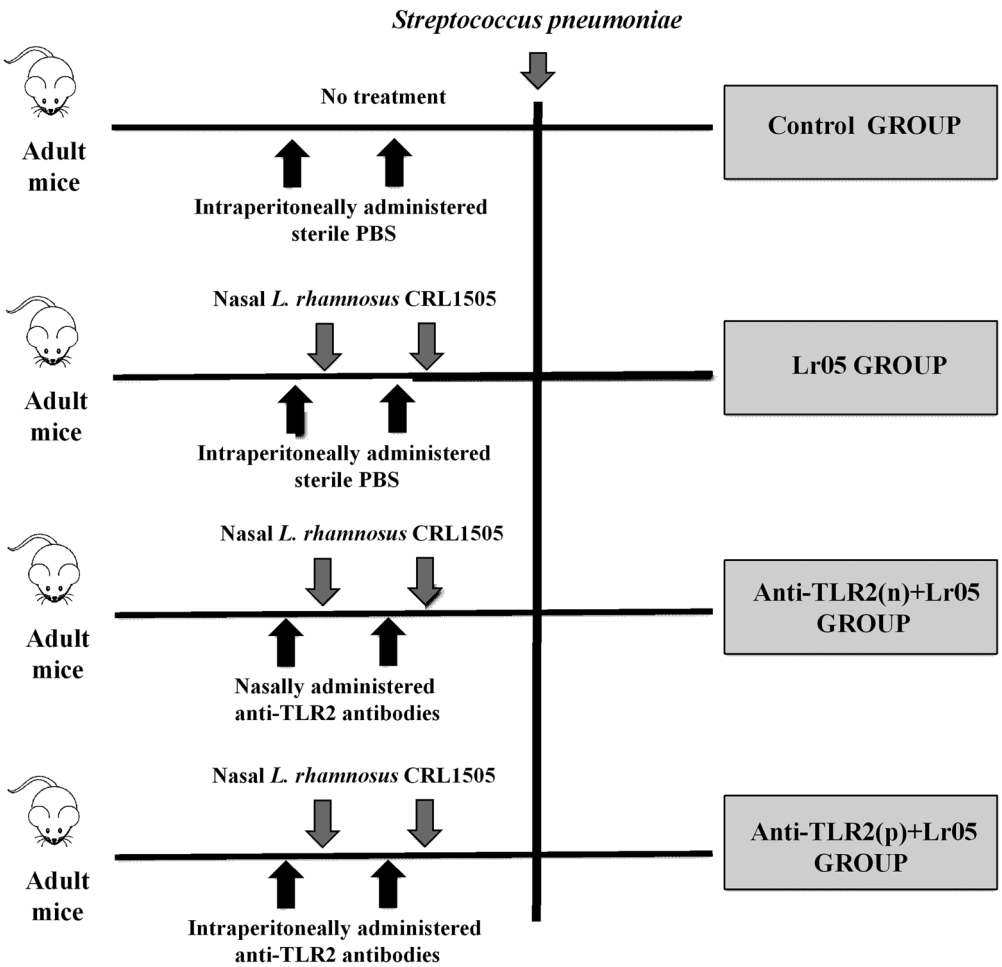

Fig. 1. Experimental design of the present study. The probiotic strain *Lactobacillus rhamnosus* CRL1505 strain was given by nasal route for 2 consecutive days at a dose of 10^8 cells/mouse per day. In order to evaluate the role of TLR2 in the protective effect of *L. rhamnosus* CRL1505, 60 μg purified anti-TLR2 blocking antibody was used. Four experimental groups were used: mice treated with *L. rhamnosus* CRL1505 (Lr05 group), mice that received by nasal route the anti-TLR2 1 hr before *L. rhamnosus* CRL1505 treatment (anti-TLR2(n) + Lr05 group), mice that received i.p. the anti-TLR2 3 hr before *L. rhamnosus* CRL1505 treatment (anti-TLR2(p) + Lr05 group), and mice that received i.p. PBS without *L. rhamnosus* CRL1505 treatment (control group).

TLR2(n) + Lr05, anti-TLR2(p) + Lr05 groups) and without (control group) lactobacillus treatment were infected by dropping 25 μL of the inoculum containing 10^7 log-phase CFU of *S. pneumoniae* in PBS into each nostril and allowing it to be inhaled (29). To facilitate migration of the inoculum to the alveoli, mice were held in a head-up vertical position for 2 min. Animals were killed on day 0 (before infection) and at different times post-infection. All experiments were approved by the Ethical Committee for Animal Care of CERELA or the Universidad Nacional de Tucumán, Argentina.

Biochemical analyses in bronchoalveolar lavage fluid

BAL samples were obtained according to the technique described previously (30). Briefly, the trachea was exposed and intubated with a catheter and two sequential lavages were done in each mouse by injecting 0.5 mL of sterile PBS. The sample of fluid was centrifuged for 10 min at 900 *g* and the supernatant fluid was frozen at −70 °C for subsequent biochemical analyses. Albumin content was determined to measure the increase in bronchoalveolar–capillary barrier permeability. It was determined colorimetrically based on albumin binding to bromocresol green (Wiener Lab., Rosario, Argentina). The results were expressed as mg/L. LDH activity was determined as an indicator of general cytotoxicity. It was determined by measuring the formation of a reduced form of nicotinamide adenine dinucleotide using Wiener Lab reagents and procedures (Wiener Lab). The results were expressed as U/L of BAL fluid.

Total and differential blood leukocyte counts

Blood samples were obtained by cardiac puncture from sodium pentobarbital-anesthetized animals and were collected in tubes containing EDTA as an anticoagulant. Total number of leukocytes was determined with a hemocytometer. Differential cell counts were done by counting 200 cells in blood smears stained with May–Grünwald–Giemsa stain using a light microscope (1000×), and absolute cell numbers were calculated (31).

Activation of blood neutrophils

Measurement of MPO activity of blood neutrophils was carried out by using the Washburn test, which is a cytochemical method that uses benzidine as an MPO chromogen (32). 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.

Cytokine concentrations in serum and bronchoalveolar lavages

TNF-α, IFN-γ, and IL-10 concentrations in serum and BAL were measured with commercially available ELISA technique kits following the manufacturer's recommendations (R&D Systems, Minneapolis, MN, USA).

Coagulation tests

Blood samples were obtained as described previously and collected in a 3.2% (w/v) solution of trisodium citrate at a ratio of 9:1. Plasma was obtained according to a previous study (33). PT and APTT were carried out 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 (34). APTT was determined to evaluate the intrinsic pathway of coagulation; it was determined by mixing plasma with calcium chloride and a partial thromboplastin reagent (Dade Actin FSL Activated PTT Reagent; Dade Behring, Marburg, Germany) and timing initial clot formation. Results are expressed in seconds (34).

Platelet counts

Blood samples were obtained as described for the leukocyte count. Manual platelet counting was done by visual examination of diluted whole blood with 1% (w/v) aqueous ammonium oxalate. The total number of platelets was determined with a hemocytometer (31).

Determination of vWF in plasma

vWF was measured in plasma samples by ELISA according to a previous study (15). In brief, plates were coated with rabbit anti-human vWF (DakoCytomation Denmark A/S, Glostrup, Denmark) overnight at 4 °C, and blocked with 1% BSA. Samples and standard curve were incubated for 2 hr at room temperature. Peroxidase-conjugated anti-human vWF/FVIII (DakoCytomation Denmark A/S) was added and incubated for 1 hr at room temperature. The reaction was developed with ortho-phenylenediamine (Ortho Diagnostic Systems Inc., Raritan, NJ, USA) and was stopped with 2N H_2SO_4. OD at a wavelength of 490 nm was determined. OD shown by background controls was subtracted from the OD of each sample.

Quantitative expression analysis by real-time PCR

Two-step real-time quantitative PCR was carried out to characterize the expression of TF and TM mRNAs in lung. Total RNA was isolated from each sample using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). All cDNAs were synthesized using a Quantitect reverse transcription (RT) kit (Qiagen, Tokyo, Japan) according to the manufacturer's recommendations. Real-time quantitative PCR was carried out using a 7300 real-time PCR system (Applied Biosystems, Warrington, UK) and 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′) and TM (sense: 5′-AGT GTG CCA GTT CAT AAG AAT C-3′; antisense: 5′-AGT GTG CCA GTT CAT AAG AAT C-3′). 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. Reaction mixtures contained 5 μL sample cDNA and 15 μL 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.

Statistical analysis

Each experiment was done in triplicate in groups consisting of 30 mice per group (six animals for each time point). Results are expressed as means ± SD. After verification of the normal distribution of data, two-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

Lactobacillus rhamnosus CRL1505 reduces lung tissue injuries induced by pneumococcal infection

LDH activity, as well as albumin concentration in BAL, were used to evaluate lung injury. As expected, challenge with *S. pneumoniae* significantly increased LDH activity and albumin content in BAL in control mice, indicating increased permeability of the bronchoalveolar–capillary barrier, and general cytotoxicity, respectively (Fig. 2). Both parameters showed significantly lower levels in pneumococci-infected mice fed with *L. rhamnosus* CRL1505 (Lr05 group), reaching levels close to unchallenged mice (Fig. 2). In addition, reduced levels of BAL LDH and albumin were observed in mice treated with blocking anti-TLR2 antibodies by nasal (anti-TLR2 (n) + Lr05 group) or peritoneal (anti-TLR2(p) + Lr05 group) routes before priming with *L. rhamnosus* CRL1505. However, the levels of these parameters did not reach the values of the Lr05 mice (Fig. 2).

Lactobacillus rhamnosus CRL1505 beneficially modulates immune response during pneumococcal infection

In order to evaluate the effect of nasally given *L. rhamnosus* CRL1505 on the immune response to

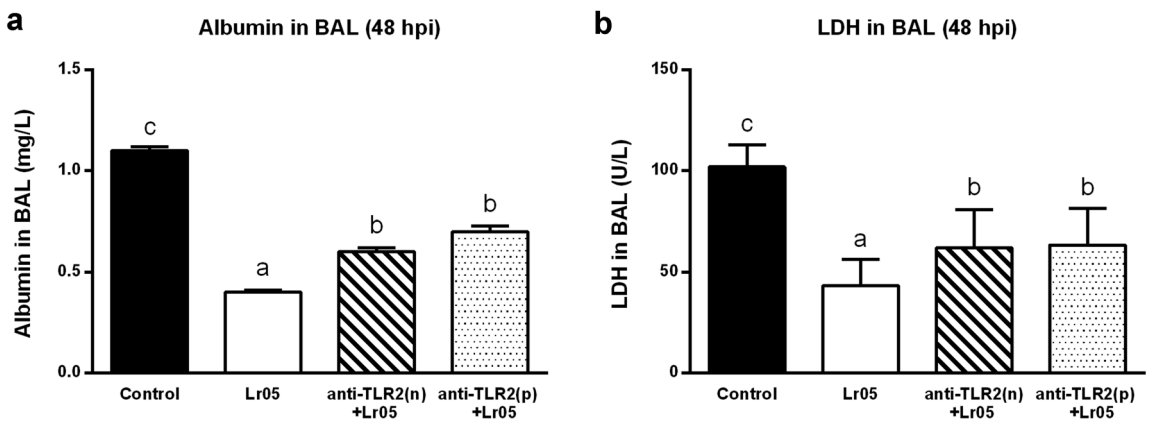

Fig. 2. Evaluation of lung tissue injury. (a) Albumin and (b) LDH in BAL. Mice were treated nasally with *L. rhamnosus* CRL1505 (Lr05 group), anti-TLR2 antibodies by the nasal route 1 hr before *L. rhamnosus* CRL1505 treatment (anti-TLR2(n) + Lr05 group), anti-TLR2 antibodies i.p. 3 hr before *L. rhamnosus* CRL1505 treatment (anti-TLR2(p) + Lr05 group), or PBS i.p. and no *L. rhamnosus* CRL1505 treatment (control group). After the treatments, mice in all groups were infected with *Streptococcus pneumoniae*. Results represent data from three independent experiments. Results are expressed as mean ± SD. Values for bars with different letters are significantly different ($P < 0.05$).

S. pneumoniae, we analyzed cytokine profiles and leukocytes in blood and BAL. Challenge of adult mice with pneumococci significantly increased the levels of blood and BAL leukocytes as well as the peroxidase activity of blood neutrophils (Fig. 3). Leukocytes were augmented in blood and BAL from 12 hr post-infection and remained elevated until 48 hr. Treatment with Lr05 did not induce significant changes in blood and BAL leukocytes when compared to the control group (Fig. 3). Additionally, no differences were observed in the levels of leukocytes in anti-TLR2(n) + Lr05 and anti-TLR2 (p) + Lr05 groups when compared to Lr05 mice (Fig. 3). MPO in blood neutrophils was also augmented with *S. pneumoniae* infection. Lr05 mice showed significantly higher values of blood peroxidase than control mice at 48 hr post-infection. Blocking anti-TLR2 antibodies did not induce changes in blood peroxidase, as the levels of this parameter were equal in Lr05, anti-TLR2(n) + Lr05, and anti-TLR2(p) + Lr05 groups (Fig. 3).

Pneumococcal infection significantly increased the levels of TNF-α, IFN-γ, and IL-10 in blood and BAL samples (Fig. 4). The strain CRL1505 significantly modified the cytokine expression patterns of all cytokines tested. Lr05 mice showed higher levels of TNF-α, IFN-γ, and IL-10 than control mice in both blood and BAL (Fig. 4). In addition, improved levels of TNF-α and IFN-γ were observed in mice treated with blocking anti-TLR2 antibodies. However, the levels of blood and BAL IFN-γ in anti-TLR2(n) + Lr05 and anti-TLR2(p) + Lr05 groups did not reach the values of the Lr05 mice (Fig. 4). In addition, anti-TLR2(n) + Lr05 and anti-TLR2(p) + Lr05 mice showed increased levels of blood IL-10 that did not reach the values observed in the Lr05 group. Interestingly, the improvement of BAL IL-10 induced by the CRL1505 strain was completely abolished with blocking anti-TLR2 antibodies (Fig. 4).

Lactobacillus rhamnosus CRL1505 differentially modulates the coagulative response to pneumococcal infection

Global coagulation tests in plasma were next evaluated in order to study systemic hemostasis. Challenge with *S. pneumoniae* significantly decreased prothrombin activity in Lr05 mice and in the control group at 12 hr post-infection. However, values of prothrombin activity in Lr05 mice were higher than in the controls. This parameter returned to normal levels at 48 hr in Lr05 mice while prothrombin activity remained decreased in controls (Fig. 5). In addition, we observed that APTT was increased from 12 hr post-infection, and no differences were observed between Lr05 and control mice. Moreover, treatment with blocking anti-TLR2 antibodies did not induce modifications in prothrombin activity or in APTT. In fact, values of these parameters in anti-TLR2(n) + Lr05 and anti-TLR2(p) + Lr05 mice were not different from the Lr05 group (Fig. 5). No modifications in platelet counts were observed after challenge with the respiratory pathogen in all the experimental groups (Fig. 5). Challenge with *S. pneumoniae* also increased vWF in all groups; however, mice in the Lr05 group showed higher values than in controls (Fig. 5). In addition, anti-TLR2(n) + Lr05 and anti-TLR2 (p) + Lr05 mice showed increased levels of vWF that did not reach the values observed in the Lr05 group.

Finally, we evaluated the expression of TF and TM in lungs of infected mice. Increased expression of these factors was observed in control mice. TF and TM were increased 3.7- and 2.4-fold, respectively, when compared

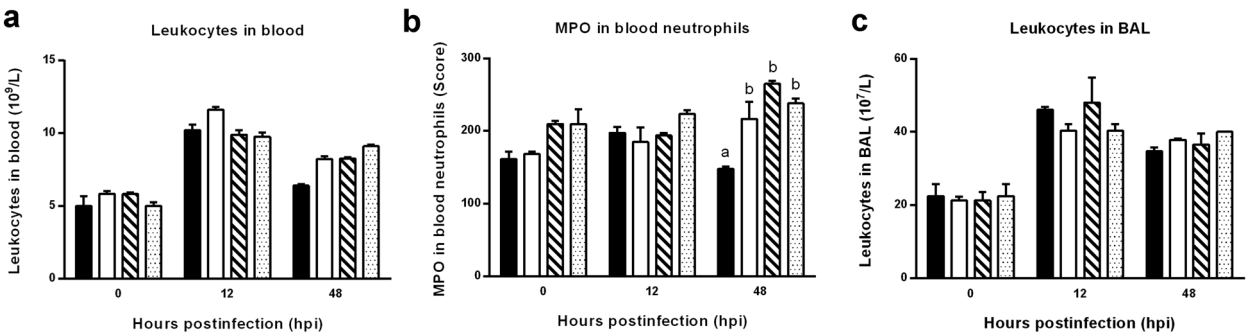

Fig. 3. Blood and BAL leukocyte counts. (a) Blood leukocyte counts, (b) MPO activity in blood neutrophils, and (c) BAL leukocyte counts. Mice were treated nasally with *L. rhamnosus* CRL1505 (□, Lr05 group), anti-TLR2 antibodies by the nasal route 1 hr before *L. rhamnosus* CRL1505 treatment (▭, anti-TLR2(n) + Lr05 group), anti-TLR2 antibodies i.p. 3 hr before *L. rhamnosus* CRL1505 treatment (▭, anti-TLR2(p) + Lr05 group), or PBS i.p. and no *L. rhamnosus* CRL1505 treatment (■, control group). After the treatments, mice in all groups were infected with *Streptococcus pneumoniae*. Results represent data from three independent experiments. Results are expressed as mean ± SD. Values for bars with different letters are significantly different ($P < 0.05$).

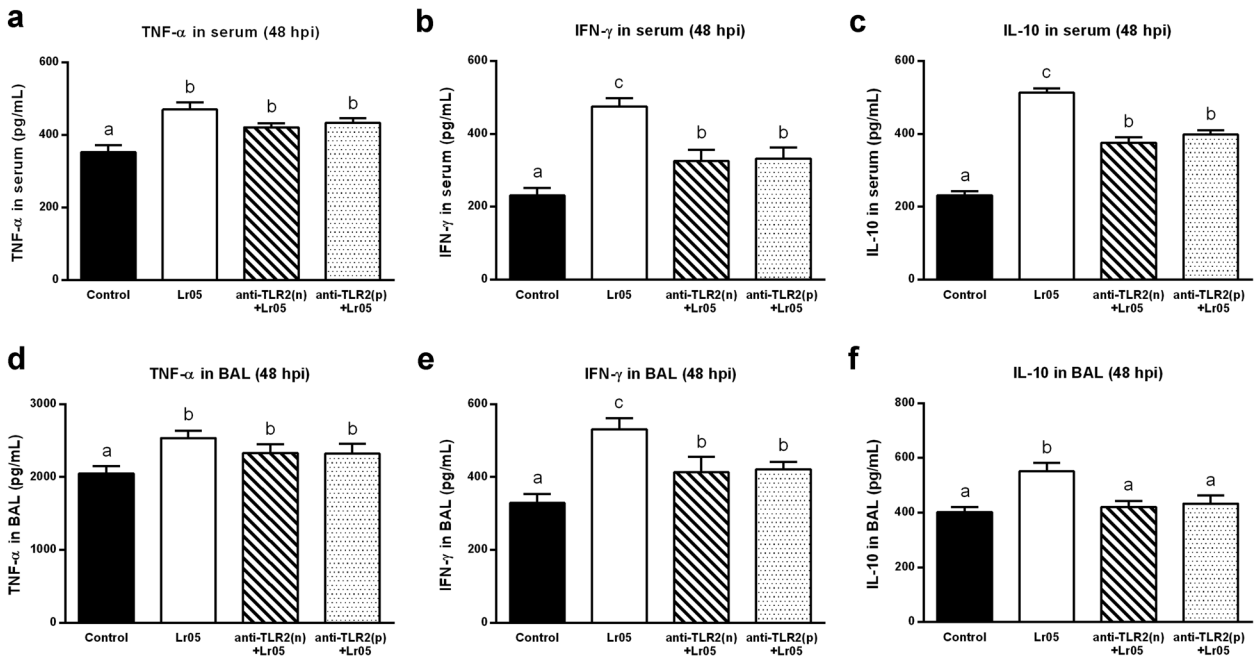

Fig. 4. Blood and BAL cytokine concentrations. (a,d) TNF-α, (b,e) IFN-γ, and (c,f) IL-10 concentrations. Mice were treated nasally with *L. rhamnosus* CRL1505 (Lr05 group), anti-TLR2 antibodies by the nasal route 1 hr before *L. rhamnosus* CRL1505 treatment (anti-TLR2(n) + Lr05 group), anti-TLR2 antibodies i.p. 3 hr before *L. rhamnosus* CRL1505 treatment (anti-TLR2(p) + Lr05 group), or PBS i.p. and no *L. rhamnosus* CRL1505 treatment (control group). After the treatments, mice in all groups were infected with *Streptococcus pneumoniae*. Results represent data from three independent experiments. Results are expressed as mean ± SD. Values for bars with different letters are significantly different ($P < 0.05$).

to basal levels (Fig. 6). Lr05 treatment significantly modified the expression of both factors. Levels of TF were significantly reduced whereas TM was augmented in Lr05 mice when compared to controls (Fig. 6). Anti-TLR2(n) + Lr05 and anti-TLR2(p) + Lr05 mice showed values of TM that were not different from Lr05 mice. On the contrary, treatments with blocking anti-TLR2 antibodies partially abolished the capacity of the CRL1505 strain to modulate TF expression. Anti-TLR2(n) + Lr05 and anti-TLR2(p) + Lr05 mice showed decreased levels of lung TF that did not reach the values observed in the Lr05 group (Fig. 6).

DISCUSSION

During respiratory infection, the release of inflammatory mediators and cytokines from cells influences a local inflammatory response in the lung. Inflammatory mediators activate circulating neutrophil and vascular endothelium, and change neutrophil–endothelial interactions, all factors that may contribute to recruitment of neutrophils into the lung (35). In addition, when acute lung injury is initiated by direct damage to the lung or as a result of systemic inflammation, a series of hemostatic pathways is triggered with the subsequent formation of fibrin that is deposited in the lung (36). The intensity and extension of inflammation and coagulation in the lung significantly influence the outcome of respiratory infections.

During the last few years, we have provided a large body of evidence demonstrating the beneficial effects of probiotic lactobacilli on the immune-coagulative response during respiratory infections (37). Among the immunobiotic strains able to modulate the inflammation–coagulation interaction, *L. rhamnosus* CRL1505 stands out as a result of its capacity to beneficially influence the outcome of viral and bacterial respiratory infections (14, 15, 38). Our studies demonstrated that orally given *L. rhamnosus* CRL1505 is able to increase the number of $CD3^+CD4^+IFN\text{-}\gamma^+$ T cells in the gut, induce mobilization of these cells into the respiratory mucosa, and improve local production of IFN-γ and the activity of lung APC. These changes induced by the CRL1505 strain increase the resistance of adult and infant immunocompetent mice against *S. pneumoniae*, respiratory syncytial virus, and influenza virus infections (15, 25, 38). In addition, *L. rhamnosus* CRL1505 significantly reduced the activation of coagulation in virus-infected mice by decreasing the expression of TF and increasing TM in the lungs (15). We also demonstrated recently that repletion of immunocompromised malnourished mice with supplemental *L. rhamnosus* CRL1505 was effective

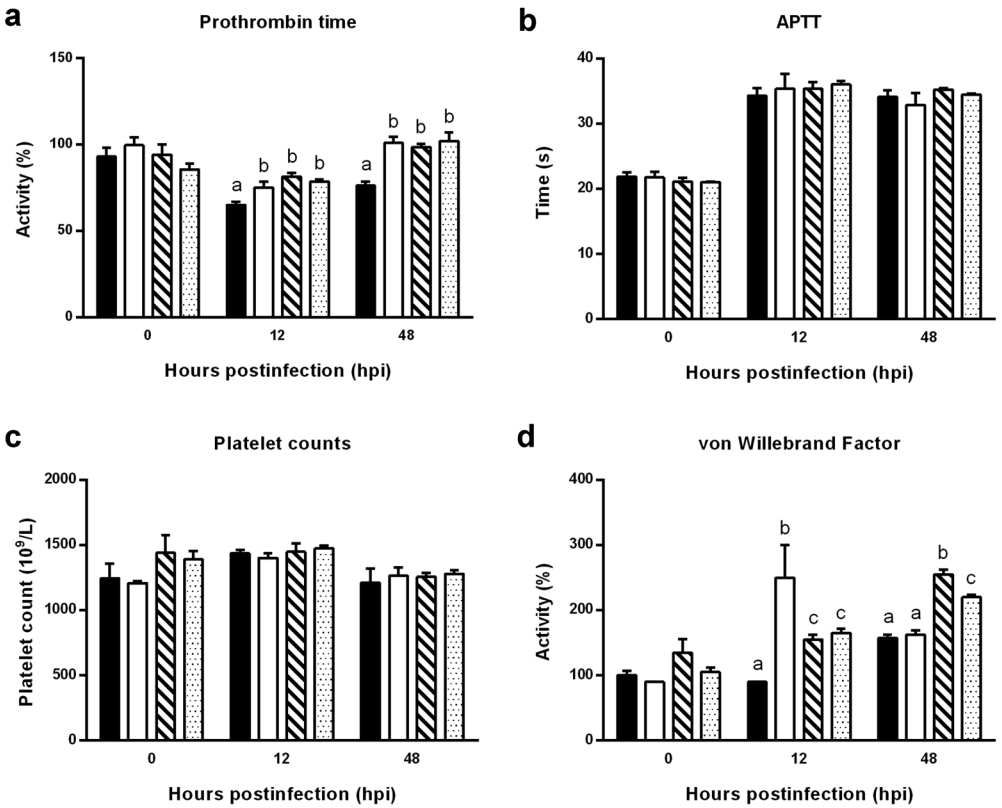

Fig. 5. Hemostatic parameters in blood. (a) Prothrombin time, (b) APTT, (c) platelet count, and (d) vWF. Mice were treated nasally with *L. rhamnosus* CRL1505 (□, Lr05 group), anti-TLR2 antibodies by the nasal route 1 hr before *L. rhamnosus* CRL1505 treatment (▭, anti-TLR2 (n) + Lr05 group), anti-TLR2 antibodies i.p. 3 hr before *L. rhamnosus* CRL1505 treatment (▭, anti-TLR2(p) + Lr05 group), or PBS i.p. and no *L. rhamnosus* CRL1505 treatment (■, control group). After the treatments, mice in all groups were infected with *Streptococcus pneumoniae*. Results represent data from three independent experiments. Results are expressed as mean ± SD. Values for bars with different letters are significantly different ($P < 0.05$).

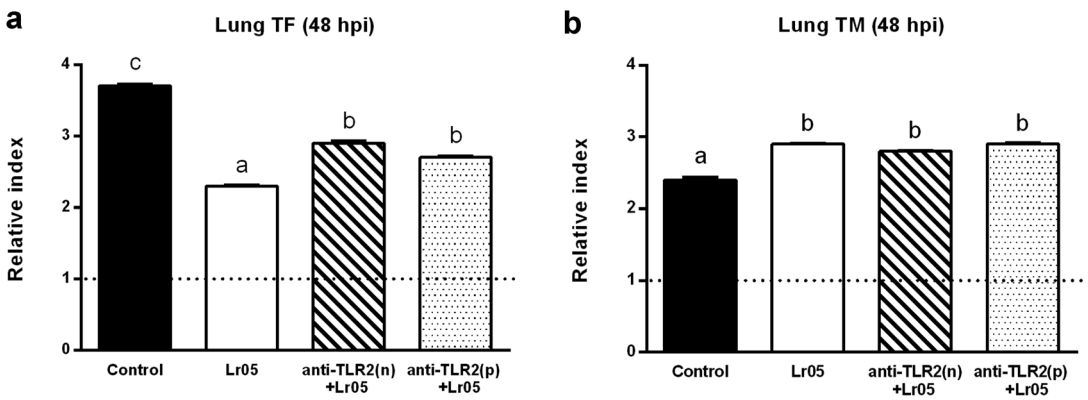

Fig. 6. Hemostatic parameters in the respiratory tract. (a) TF and TM mRNA expressions in lung were examined using RT-qPCR. Mice were treated nasally with *L. rhamnosus* CRL1505 (Lr05 group), anti-TLR2 antibodies by the nasal route 1 hr before *L. rhamnosus* CRL1505 treatment (anti-TLR2(n) + Lr05 group), anti-TLR2 antibodies i.p. 3 hr before *L. rhamnosus* CRL1505 treatment (anti-TLR2(p) + Lr05 group), or PBS i.p. and no *L. rhamnosus* CRL1505 treatment (control group). After the treatments, mice in all groups were infected with *Streptococcus pneumoniae*. Dotted lines indicate the basal levels of TF or TM that were set at 1. Results represent data from three independent experiments. Results are expressed as mean ± SD. Values for bars with different letters are significantly different ($P < 0.05$). Values for bars with shared letters do not differ significantly.

for reducing hemostatic alterations induced by both malnutrition and pneumococcal infection (16). Our study showed that oral treatment with the CRL1505 strain was able to beneficially modulate the immune-coagulative response and diminished lung tissue damage in infected malnourished mice (16). Similarly, in the present study, we showed that giving the CRL1505 strain nasally to adult immunocompetent mice improved local production of TNF-α and IFN-γ, beneficially modulated the immune-coagulative response, and reduced lung tissue injuries induced by the pneumococcal infection.

We also showed here that nasally given *L. rhamnosus* CRL1505 increased IL-10 levels in both lungs and blood. This is in line with our previous results demonstrating that orally given CRL1505 modulates the balance between pro-inflammatory cytokines and IL-10 in the respiratory tract after nasal challenge with respiratory pathogens (25, 38). We showed previously that IL-10 induced by *L. rhamnosus* CRL1505 has a significant role in its functional effect. Increased IL-10 levels contributed to protection against inflammatory damage in poly(I:C)- and respiratory virus-challenged mice. Moreover, blocking IL-10R antibodies significantly reduced the capacity of the CRL1505 strain to protect against lung tissue damage (15). Therefore, modulation of the inflammatory response induced by the probiotic strain would also indirectly modulate the coagulation system during pneumococcal infection. In fact, the improvement of IL-10 would contribute to regulation of the procoagulant and anti-fibrinolytic effects of pro-inflammatory mediators induced by the infectious challenge. In support of this hypothesis, IL-10 has been shown to down-regulate TF expression and inhibits procoagulant activity in human and mice monocytes and monocytes/endothelial cell cocultures in a dose-dependent manner (39, 40).

The results of the present study and our previous publications clearly demonstrate that the protective effect of *L. rhamnosus* CRL1505 is mediated by its capacity to increase the levels of both IFN-γ and IL-10. In addition, we demonstrated here for the first time that TLR2 has a role in the immunomodulatory activities of the CRL1505 strain.

We showed that local and systemic blocking with anti-TLR2 antibodies partially abolished the capacity of the CRL1505 strain to reduce lung tissue injuries during pneumococcal infection. In anti-TLR2-treated mice, levels of BAL albumin content, a measure of increased permeability of the bronchoalveolar–capillary barrier, and LDH activity, an indicator of general cytotoxicity, were diminished when compared to control mice. However, these parameters did not reach the levels observed in mice receiving *L. rhamnosus* CRL1505 and no anti-TLR2 antibodies. A similar behavior was observed when analyzing levels of blood and BAL IFN-γ and IL-10 and the expression of lung TF, although we did not observe significant changes in global coagulation tests in blocking experiments. This is probably related to the sensitivity of the methods used to evaluate each parameter. Therefore, these results allow us to speculate that TLR2 is partially involved in the induction of IFN-γ and IL-10 production and in the reduction of TF expression induced by the CRL1505 strain.

During infection, inflammatory mediators play a central role in triggering disturbance of endothelial cells. Inflammation reduces TM expression in endothelial cells and increases their expression of vWF. TM binding to thrombin counteracts its pro-inflammatory and procoagulant activities. TM also suppresses leukocyte adhesion and activation, and interferes with complement activation (3, 4, 6). Therefore, changes in TM in endothelial cells promote their pro-inflammatory and procoagulant activities. Our experiments showed that treatment with *L. rhamnosus* CRL1505 improved the expression of TM in lungs of infected mice, indicating that the probiotic treatment is able to attenuate pro-inflammatory and procoagulant activities of endothelial cells. Moreover, we demonstrated here that TLR2 is not involved in this effect achieved by the CRL1505 strain, as no modifications were observed with anti-TLR2 blocking antibodies. In contrast, normal hemostasis requires vWF to support platelet adhesion and aggregation at sites of vascular injury. This mechanism is crucial early after injury to avoid bleeding and spread of pathogens. Results showed that *L. rhamnosus* CRL1505-treated mice significantly augmented the production of vWF in response to pneumococcal challenge. Moreover, anti-TLR2 antibodies abolished the capacity of the immunobiotic strain to improve vWF. This is in line with studies reporting that TLR2 is involved in several functions of endothelial cells. It has been shown that treatment with TLR2 agonists has broad effects on the endothelium *in vitro* and *in vivo*, including up-regulation of endothelial inflammatory responses, increased neutrophil trafficking to the endothelium, changes in the expression of coagulation pathway factors, and increased endothelial permeability (41). Moreover, it was suggested that treatments able to modulate TRL2 signaling in lung endothelium would be beneficial to improve the outcome of respiratory infections and sepsis (42). Further studies are necessary to evaluate the effect of immunobiotic treatment on lung endothelium and the role of TLR2.

In conclusion, the present study demonstrates that nasally given *L. rhamnosus* CRL1505 effectively regulates inflammation and hemostatic alterations during pneumococcal infection in adult immunocompetent mice and decreases lung tissue damage. Induction of IFN-γ,

IL-10, and vWF through the TLR2 signaling pathway seems to have an important role in the immunobiotic effect of the CRL1505 strain. However, TLR2 is not the only receptor involved in the beneficial effect, as modifications in TNF-α and TM induced by the immunobiotic strains were not modified with blocking antibodies. Lebeer *et al.* (17) suggested that the final outcome of a host response against immunobiotic lactobacilli depends on the combination of the distinct MAMP that can interact with the various PRR and associated co-receptors that fine-tune signaling, as well as on the concentration of these MAMP. In addition, two important factors that determine the responsiveness of host cells are the accessibility of the PRR for the MAMP (i.e. the subcellular distribution, compartmentalization, and expression levels of the PRR in various host tissues) and host-derived direct or indirect negative regulators of PRR signaling (43). Therefore, it is tempting to speculate that a PRR(s) other than TLR2 may mediate the immunobiotic activity of *L. rhamnosus* CRL1505. Activation of these unknown PRR(s) would explain the changes induced by CRL1505 in TNF-α and TM during pneumococcal infection. Identification of these unknown receptor(s) is an interesting topic for future investigations.

ACKNOWLEDGMENTS

This work was supported by grants from CIUNT 26 D/403 and PICT 2010 No. 1381. We also wish to thank Wiener Lab, Rosario, Argentina for their kind donation of assay kits.

DISCLOSURE

The authors have no conflicts of interest to disclose.

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