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Immunobiotic *Lactobacillus rhamnosus* improves resistance of infant mice against respiratory syncytial virus infection

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ABSTRACT

Previously we showed that orally administered *Lactobacillus rhamnosus* CRL1505 beneficially regulated the balance between pro- and anti-inflammatory mediators in the lungs of poly(I:C)-challenged mice, allowing an effective inflammatory response against the TLR3/RIG-I agonist but at the same time reducing tissue damage. The aim of the present study was to investigate whether oral administration of the CRL1505 strain was able to improve resistance against respiratory syncytial virus (RSV) infection in infant mice and to evaluate the immunological mechanisms involved in the immunobiotic effect. We demonstrated that treatment of 3-week old BALB/c mice with *L. rhamnosus* CRL1505 significantly reduce lung viral loads and tissue injuries after the challenge with RSV. Moreover, we showed that the protective effect achieved by the CRL1505 strain is related to its capacity to differentially modulate respiratory antiviral immune response. Our results shows that IFN-γ and IL-10 secreted in response to *L. rhamnosus* CRL1505 oral stimulation would modulate the pulmonary innate immune microenvironment conducting to the activation of $CD103^+$ and $CD11b^{high}$ dendritic cells and the generation of $CD3^+CD4^+IFN\text{-}\gamma^+$ Th1 cells with the consequent attenuation of the strong and damaging Th2 reactions associated with RSV challenge. Our results indicate that modulation of the common mucosal immune system by immunobiotics could favor protective immunity against respiratory viral pathogens with a high attack rate in early infancy, such as RSV.

1. Introduction

Respiratory syncytial virus (RSV), a pneumovirus in the family Paramyxoviridae, infects nearly all children within the first 3 years of life and is the chief cause of bronchiolitis and viral pneumonia in younger children [1]. It has been shown that viral and host factors contribute to severe disease in infants. RSV targets both type I alveolar and nonbasilar airway epithelial cells that results in impairment of the ciliary action and sloughing of infected epithelial cells. Despite its low cytopathicity, there is compelling evidence that the level of RSV replication correlates to the disease severity [1]. In addition, the host immune response contributes to the damage induced by RSV infection because a large number of inflammatory cells are accumulated and activated in the lungs after infection [2,3]. Therefore, as described for other respiratory viruses such as influenza virus, the immune response plays a critical role in the outcome of RSV-induced bronchiolitis and pneumonia. The exacerbated disease due to immune-mediated pulmonary injury during acute RSV infection results in severe morbidity and mortality [3]. Then, identifying novel approaches to modulate virus-induced immunopathology could be important alternatives for treating acute RSV infections.

Several studies have demonstrated that certain lactic acid bacteria (LAB) strains can exert their beneficial effect on the host through their immunomodulatory activities. Moreover, some studies have centered on whether immunoregulatory probiotic LAB (immunobiotics) might sufficiently stimulate the common mucosal immune system to provide protection in other mucosal sites distant from the gut [4–7]. In this regard, our laboratory has made important advances in the demonstration of the capacity of certain immunobioitc strains to beneficially modulate the respiratory immune system [4–7]. Among the strains studied in our laboratory, *Lactobacillus rhamnosus* CRL1505

Abbreviations: BAL, broncho-alveolar lavage; BCA, bicinchoninic; DCs, dendritic cells; dsRNA, double-stranded RNA; ELISA, enzyme-linked immunosorbent assay; IL, interleukin; IFN, interferon; LAB, lactic acid bacteria; LDH, lactate dehydrogenase; NAD, nicotinamide adenine dinucleotide; PBS, phosphate buffer saline; RIG-I, retinoic acid-inducible gene I; RSV, respiratory syncytial virus; TLR3, toll-like receptor 3; TNF, tumor necrosis factor.

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showed the capacity to improve respiratory defenses against bacterial infection when orally administered to immunocompetent and immunocompromised mice [4–6]. Moreover, we performed a randomized controlled clinical trial in order to evaluate the effect of a probiotic yogurt containing *L. rhamnosus* CRL1505 on both gut and non-gut related illnesses among children [8]. We showed that administration of *L. rhamnosus* CRL1505 improved mucosal immunity and reduced the incidence and severity of intestinal and respiratory infection in children. When we studied the occurrence of infections and classified them according to their location and symptoms, their frequency was consistent with the prevalence reported in Argentina. The most common infectious diseases were upper respiratory infections and angina, followed by lower respiratory infections (acute bronchitis) and diarrhea [9,10]. We registered that 34% of the children who consumed the probiotic yogurt showed some type of infectious event, while in the placebo group this value was higher reaching a 66% of the children. These results demonstrate a significant reduction in occurrence of infectious events associated with consumption of *L. rhamnosus* CRL1505 [8]. Although we did not evaluate the etiology of the respiratory infections during the clinical study, previous data showed that viral pathogens, such as RSV, human metapneumovirus, influenza A virus, parainfluenza viruses, and rhinoviruses are the major pathogens that can cause respiratory tract diseases in children in northern Argentina [9,11]. Therefore, the findings of our study suggested that administration of *L. rhamnosus* CRL1505 may provide one potential intervention to reduce the burden of common childhood morbidities, especially those associated with viral infections [8].

Considering the results of this clinical study, our laboratory evaluated later the capacity of the immunobiotic CRL1505 strain to beneficially modulate antiviral immune responses in the respiratory tract. To mimic the pro-inflammatory and physiopathological consequences of RNA viral infections in the lung such as those induced by RSV infection, we used an experimental model of lung inflammation based on the administration of the artificial toll-like receptor 3 (TLR3) and retinoic acid-inducible gene I (RIG-I) ligand and dsRNA analog poly(I:C) [12]. In our experiments, nasal administration of poly(I:C) to adult BALB/c mice induced a marked impairment of lung function accompanied by inflammatory cell recruitment into the airways and the production of TNF-α, IL-6, IL-8, MCP-1 and type I interferons [13]. When we evaluated the effect of the oral administration of *L. rhamnosus* CRL1505 in this model, we found that the CRL1505 strain beneficially regulated the balance between pro-inflammatory mediators and IL-10 in the lungs of poly(I:C)-challenged mice, allowing an effective inflammatory response against the TLR3/RIG-I agonist but at the same time reducing tissue damage [12]. 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 antigen presenting cells (APCs) [12].

To summarize, our previous results indicate that *L. rhamnosus* CRL1505 is a potent inducer of antiviral cytokines and may be useful as a prophylactic agent to control respiratory virus infections as observed in the clinical study [8,12]. However, further studies are needed in order to conclusively demonstrate the protective effect of *L. rhamnosus* CRL1505. Therefore, the aim of this study was to investigate whether oral administration of *L. rhamnosus* CRL1505 has the ability to improve resistance against RSV-challenged infant mice and to evaluate the immunological mechanisms involved in the probiotic effect.

2. Materials and methods

2.1. Microorganisms

L. rhamnosus CRL1505 and *L. rhamnosus* CRL1506 were obtained from the CERELA culture collection (Chacabuco 145, San Miguel de Tucumán, Argentina). Strains were kept freeze–dried and activated as explained before [5].

2.2. Animals and feeding procedures

Female 3-week-old BALB/c mice were obtained from the closed colony kept at Tohoku University. They were housed in plastic cages at room temperature. Mice were housed individually during the experiments and the assays for each parameter studied were performed in 5–6 mice per group for each time point. *L. rhamnosus* CRL1505 or *L. rhamnosus* CRL1506 was orally administered to different groups of mice for 5 consecutive days at a dose of 10^8 cells/mouse/day (Fig. 1) as previously described [12]. The treated groups and the untreated control group were fed a conventional balanced diet *ad libitum*. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the Guidelines for Animal Experimentation of Tohoku University, Sendai, Japan and all efforts were made to minimize suffering.

2.3. Virus and infection

Human RSV strain A2 was grown in Vero cells as described by Murawski et al. [14]. 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 (Sigma, Tokyo, Japan), 0.1% penicillin–streptomycin (Pen/Strep) (Sigma, Tokyo, Japan), and 0.001% ciprofloxacin (Bayer) were added to the flask and further incubated. When extensive syncytium formation was observed, cells were scraped from the flask and sonicated three times, 5 s per pulse, at 25 W on ice. Cell debris was removed by centrifugation at 700 *g* for 10 min at 4 °C. Virus supernatant was stored in 30% sucrose at −80 °C. Uninfected cells were treated identically to generate a Vero cell lysate control. For *in vivo* infection, mice were lightly anesthetized with isoflurane and intranasally challenged with 2.4×10^6 PFU of RSV strain A2 or an equal volume of Vero cell lysate control on day 6 after lactobacilli treatment (Fig. 1).

2.4. RSV immunoplaque assay

Intact lung tissue was removed and stored in 30% sucrose for plaque assays. For this, the lungs were homogenized using a pellet pestle and centrifuged at 2,600 *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 medium 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 Tehcnology) for 1 h. Plates 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 manufacture's specifications. Results for immunoplaque assay were expressed as log10 PFU/g of lung.

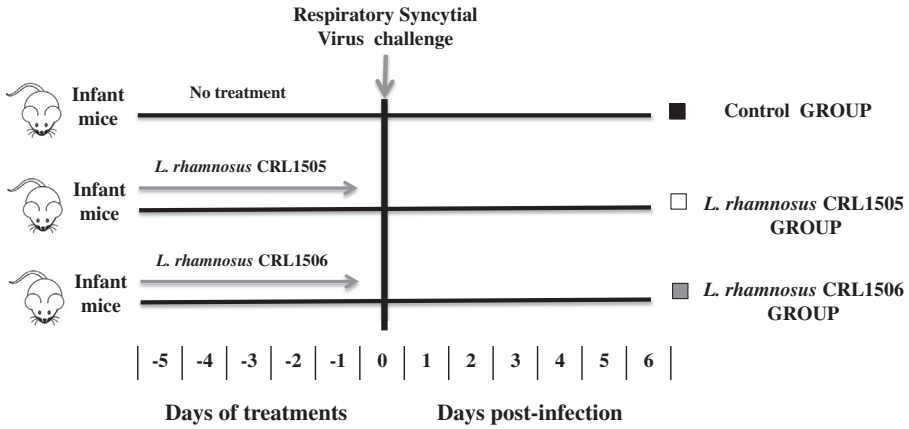


Fig. 1. Feeding protocols used in this work. Female 3-week-old BALB/c were fed with *Lactobacillus rhamnosus* CRL1505 or *L. rhamnosus* CRL1506 for 5 consecutive days at a dose of 10^8 cells/mouse/day. The treated groups and the untreated control group were fed a conventional balanced diet *ad libitum*. After the treatment treated and control mice were lightly anesthetized with isoflurane and intranasally challenged with 2.4×10^6 PFU of respiratory syncytial virus.

2.5. Cytokine concentrations in broncho-alveolar lavages (BAL)

BAL samples were obtained as described previously [7]. Briefly, the trachea was exposed and intubated with a catheter, and 2 sequential bronchoalveolar lavages were performed in each mouse by injecting sterile PBS; the recovered fluid was centrifuged for 10 min at 900 g; the pellet was used to make smears that were stained for cell counts; and the fluid was frozen at −70 °C for subsequent cytokines analyses. Tumor necrosis factor (TNF)-α, IFN-α, IFN-β, IFN-γ, IL-6 and IL-10 concentrations in serum and BAL were measured with commercially available enzyme-linked immunosorbent assay (ELISA) technique kits following the manufacturer's recommendations (R&D Systems, MN, USA) [5].

2.6. Lung cell suspensions

Single lung cells were prepared using the previously described method [12]. Briefly, mice were anesthetized with diethyl ether. Lungs were removed, finely minced and incubated for 90 min with 300 U of collagenase (Yakult Honsha Co., Tokyo, Japan) in 15 ml of RPMI 1640 medium (Sigma, Tokyo, Japan). To dissociate the tissue into single cells, collagenase-treated minced lungs were gently tapped into a plastic dish. After removal of debris, erythrocytes were depleted by hypotonic lysis. The cells were washed with RPMI medium supplemented with 100 U/ml of penicillin and 100 mg/ml of streptomycin and then resuspended in a medium supplemented with 10% heat-inactivated fetal calf serum (FCS). Cells were counted using Trypan Blue exclusion and then resuspended at an appropriate concentration of 5×10^6 cells/ml.

2.7. Flow cytometry studies

Lung cell suspensions were pre-incubated with anti-mouse CD32/CD16 monoclonal antibody (Fc block) for 15 min at 4 °C. Cells were incubated in the antibody mixes for 30 min at 4 °C and washed with FACS buffer. The following antibodies from BD PharMingen were used: anti-mouse CD3-FITC, anti-mouse CD4-PE, anti-mouse CD8-PE, anti-mouse CD11b-FITC, anti-mouse CD11c-PE, anti-mouse MHC-II-PE, and anti-mouse CD103-biotin. Following incubation with biotinylated primary antibodies, the labeling was revealed using streptavidin-PercP. For the determination of cytokine production by $CD4^+$ and $CD8^+$ T lymphocytes, lung cells were washed with FACS buffer and stained for surface markers. For intracellular staining, cells were fixed and permeabilized with CytoFix/CytoPerm (BD PharMingen) solution and Perm/Wash buffer (BD PharMingen) and stained with anti-mouse IFN-γ-APC, anti-mouse IFN-γ-PE or anti-mouse IL-10-PE (BD PharMingen). In all cases, cells were then acquired on a BD FACSCalibur™ flow cytometer (BD Biosciences) and data were analyzed with FlowJo software (TreeStar). The total number of cells in each population was determined by multiplying the percentages of subsets within a series of marker negative or positive gates by the total cell number determined for each tissue [12,15].

2.8. Lung tissue injury markers

Protein and albumin content, a measure to quantitate increased permeability of the bronchoalveolar–capillarity barrier, and lactate dehydrogenase (LDH) activity, an indicator of general cytotoxicity, were determined in the cell-free BAL fluid. Protein and albumin content were determined as explained before [7,12]. Lung wet:dry weight ratio was measured as previously described [12]. Briefly, mice were euthanized and exsanguinated, and their lungs removed, weighed, and dried in an oven at 55 °C for 7 days. After drying, the lungs were weighed again. Wet:dry weight ratio was then calculated as an index of intrapulmonary fluid accumulation, without correction for blood content.

2.9. Blocking experiments

In order to evaluate the role of IFN-γ and IL-10 in the immunoprotective effect of *L. rhamnosus* CRL1505, anti-IFN-γ and 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 (Fig. 1). On day 6 the mice were injected intraperitoneally with 50 μg of purified anti-IL10R (LEAF™ Purified anti-mouse IL-10R antibody, #112708 BioLegend, Tokyo, Japan) or anti-IFN-γ (LEAF™ Purified anti-mouse IFN-γ antibody, #505706 BioLegend) antibodies or 250 μg isotype control antibodies (LEAF™ Purified Rat IgG1, κ Isotype Ctrl, LEAF™ Purified Rat IgG1, κ Isotype Ctrl, BioLegend) and two hours later they were challenged with RSV. Virus titer, wet:dry lung weight ratio and BAL LDH and albumin concentrations were determined as described previously.

2.10. 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 but not L. rhamnosus CRL1506 improve resistance against respiratory syncytial virus infection

We first addressed the question of whether changes observed previously in respiratory immune system caused by the intervention with *L. rhamnosus* CRL1505 [12] affected the outcome of RSV infection in mice. Treated and control mice were nasally challenged with 10^6 PFU of RSV strain A2 and lungs were harvested during 5 days after infection. This time period was selected considering previous studies that demonstrated that peak viral titers in mouse models occurs approximately on day 4 post-infection [14,16]. Oral administration of *L. rhamnosus* CRL1505 markedly increased resistance to infection as observed in the reduced viral load titrated over time, which becomes even more evident at day 4 with a 1.5-log reduction respective to untreated mice or mice fed with *L. rhamnosus* CRL1506 (Fig. 2). Mice fed with *L. rhamnosus* CRL1505 reached the highest mean body weight, suggesting a correlation between viral load and general health state. In contrast, mice administered the CRL1506 strain or without lactobacilli did not show differences in body weight (Fig. 2) and weighted at day 5 a 9% less than CRL1505-treated mice.

3.2. L. rhamnosus CRL1505 reduce lung tissue injuries induced by respiratory syncytial virus infection

Lung wet:dry weight ratio and LDH activity in BAL as well as total protein and albumin concentrations were used to evaluate lung injury as previously described [12]. RSV infection resulted in lung water content retention as expected. The level of lung water retention was exactly the same in RSV-infected mice fed with *L. rhamnosus* CRL1506 and control mice. However, lung wet:dry weight ratio reached significantly lower levels in RSV-infected mice fed with *L. rhamnosus* CRL1505 reaching levels close to unchallenged mice (Fig. 3). RSV infection also increased LDH activity and albumin content in BAL earlier at hour 3 post-infection. The levels of both markers of tissue damage gradually increased until reaching the highest levels between days 1 and 2 post-infection (Fig. 3). Already at day 3 post-infection, there was a moderate reduction in cell cytotoxicity, as indicated by the reduction of BAL LDH activity in *L. rhamnosus* CRL1505-treated mice. Moreover, levels of LDH and albumin were significantly lower than controls from hour 6 to 48 (Fig. 3). *L. rhamnosus* CRL1506-treated mice showed no differences when compared to controls (Fig. 3). These findings indicated that in addition to the reduction in viral replication, *L. rhamnosus* CRL1505 was able to protect against lung tissue damage in agreement with results previously obtained in a poly(I:C)-challenge model in BALB/c mice orally treated with the same immunobiotic lactobacilli [12].

3.3. L. rhamnosus CRL1505 beneficially modulate immune response during respiratory syncytial virus infection

In order to evaluate the effect of CRL1505 and CRL1506 strains in the pulmonary immune response to RSV we analyzed the cytokine profiles in BAL and different immune cell populations in lungs. Challenge of infant mice with RSV significantly increased the levels of IFN-β, TNF-α, IL-6, IFN-γ and IL-10 in BAL samples (Fig. 4). Whereas the oral administration of *L. rhamnosus* CRL1506 did not significantly changed in BAL cytokines levels when compared to control infected mice, the strain CRL1505 significantly modified the cytokine expression patterns of all cytokines tested (Fig. 4). Thus, IFN-γ, IFN-β and IL-10 were induced at significantly ($P < 0.05$) higher levels by *L. rhamnosus* CRL1505 at all times tested. In the case of IL-6 and TNF-α, the absolute levels induced by the CRL1505 strain were the same as in BAL of CRL1506 and control mice, but the kinetics were noticeable different: CRL1505-treated mice presented the highest peak for both cytokines at day 2, whereas control and CR1506-treated mice showed the peak at day 3 (Fig. 4).

The influence of lactobacilli administration on lung DCs was studied by flow cytometry. As previously reported, lung DCs can be separated into two distinct populations according to the expression of CD11c, CD11b and CD103: $CD11c^+CD103^+CD11b^{low}$ cells and $CD11c^+CD103^-CD11b^{high}$ cells [12]. We observed that *L. rhamnosus* CRL1505 treatment was able to significantly increase ($P < 0.05$) cell counts of both populations of lung DCs (Fig. 5). Furthermore, oral treatment with the immunobiotic strain upregulated the expression of MHC-II in both $CD11c^+CD103^+CD11b^{low}$ and $CD11c^+CD103^-CD11b^{high}$ cells. In contrast, administration of *L. rhamnosus* CRL1506 did not modify the DCs numbers or the MHC-II expression levels with respect to the RSV infected mouse without lactobacilli treatment (Fig. 5).

We also analyzed the effect of both CRL1505 and CRL1506 strains on lung T cells after the challenge with RSV. As described by others [17] challenge with RSV significantly increased the numbers of $CD3^+CD4^+IFN\text{-}\gamma^+$, $CD3^+CD8^+IFN\text{-}\gamma^+$ and $CD3^+CD4^+IL\text{-}10^+$ in lungs (Fig. 6). *L. rhamnosus* CLR1505 administration resulted in higher $CD3^+CD4^+IFN\text{-}\gamma^+$ and

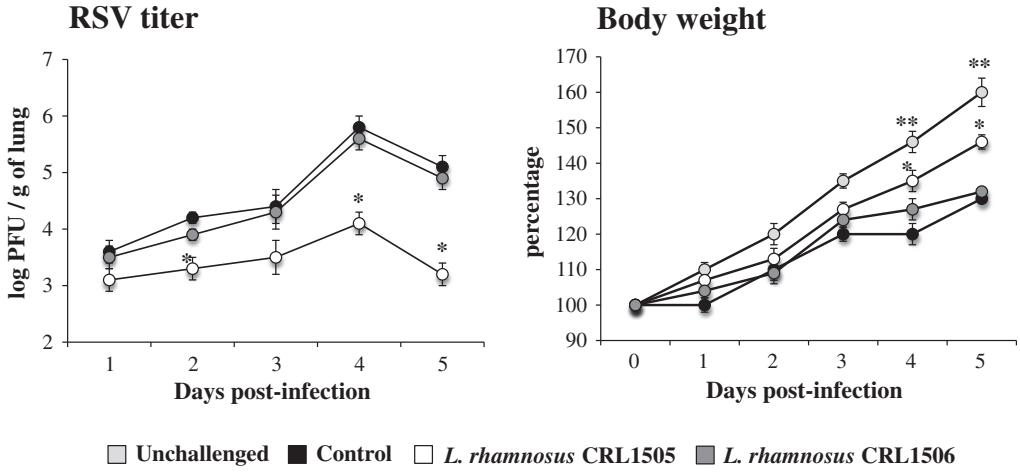


Fig. 2. Effect of lactobacilli on the resistance against respiratory syncytial virus (RSV) infection. Effect of *Lactobacillus rhamnosus* CRL1505 and *L. rhamnosus* CRL1506 oral administration on lung RSV titers and changes in body weight on different time points after the challenge. The results represent data from three independent experiments. (*) Significantly different from control mice ($P < 0.05$).

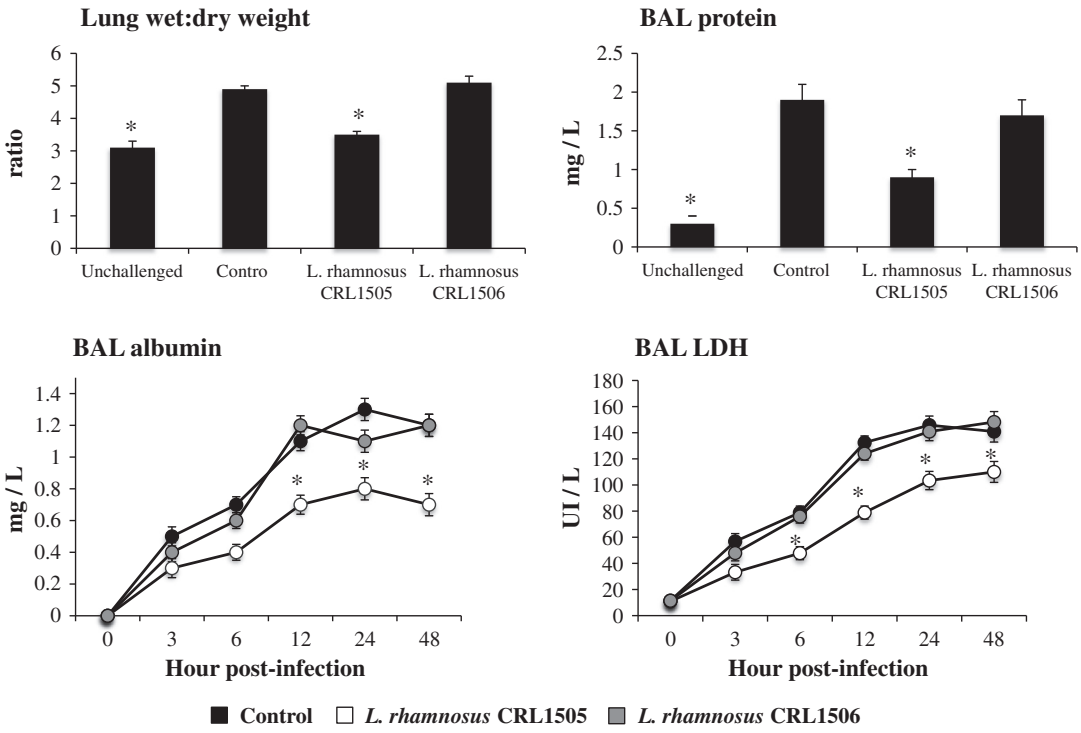


Fig. 3. Effect of lactobacilli on lung injuries induced by respiratory syncytial virus (RSV) infection. Effect of *Lactobacillus rhamnosus* CRL1505 and *L. rhamnosus* CRL1506 oral administration on lung wet:dry weight ratio, lactate dehydrogenase (LDH) activity and, albumin and protein concentrations in broncho-alveolar lavages (BAL) after the challenge with RSV. The results represent data from three independent experiments. (*) Significantly different from control infected mice ($P < 0.05$).

$CD3^+CD4^+IL\text{-}10^+$ cell numbers in lungs after RSV challenge, whereas $CD3^+CD8^+IFN\text{-}\gamma^+$ cell counts remained unchanged when compared to control RSV-infected mice. In contrast, numbers of lung $CD3^+CD4^+IFN\text{-}\gamma^+$, $CD3^+CD8^+IFN\text{-}\gamma^+$ and $CD3^+CD4^+IL\text{-}10^+$ cells in CRL1506-treated mice were not different from those in controls (Fig. 6).

3.4. IFN-γ and IL-10 are differently involved in the immunoprotective effect of L. rhamnosus CRL1505 during respiratory syncytial virus infection

In order to evaluate the role of IFN-γ and IL-10 in the immunoregulatory effect of *L. rhamnosus* CRL1505 during RSV infection we used

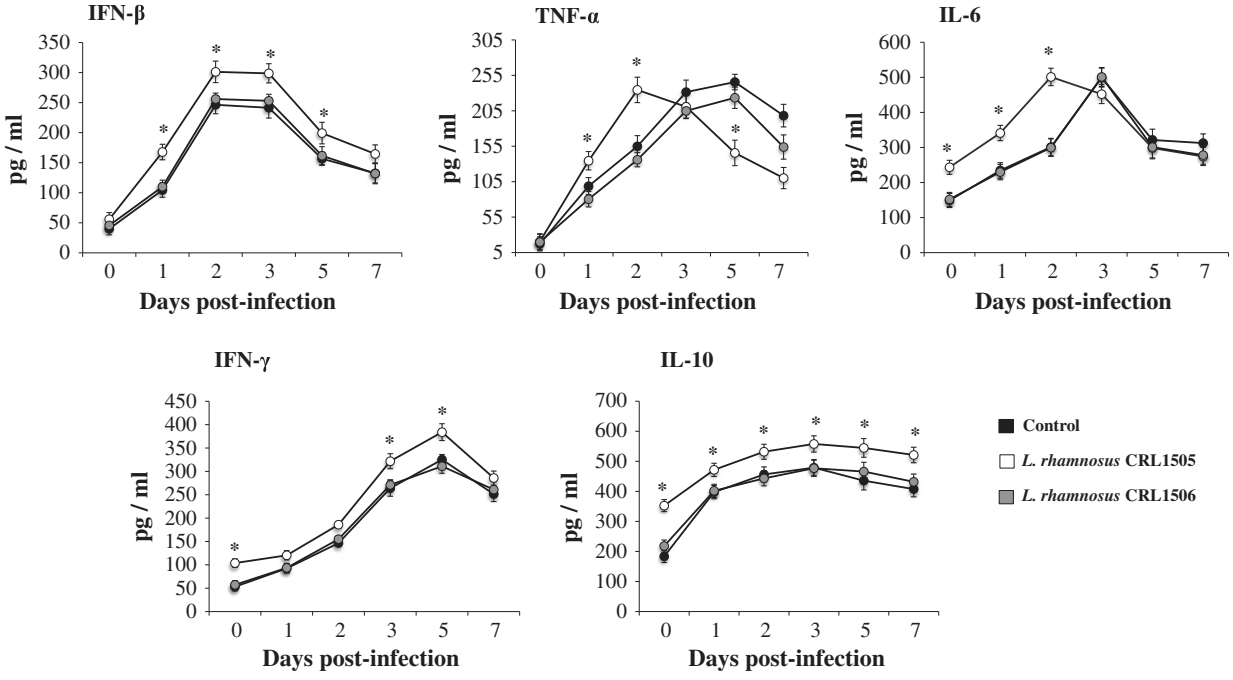


Fig. 4. Effect of lactobacilli on the production of cytokines induced by respiratory syncytial virus (RSV) infection. Effect of *Lactobacillus rhamnosus* CRL1505 and *L. rhamnosus* CRL1506 oral administration on the tumor necrosis factor (TNF)-α, interferon (IFN)-α, IFN-γ, interleukin (IL)-6 and IL-10 concentrations in broncho-alveolar lavages on different time points after the challenge. The results represent data from three independent experiments. (*) Significantly different from control infected mice ($P < 0.05$).

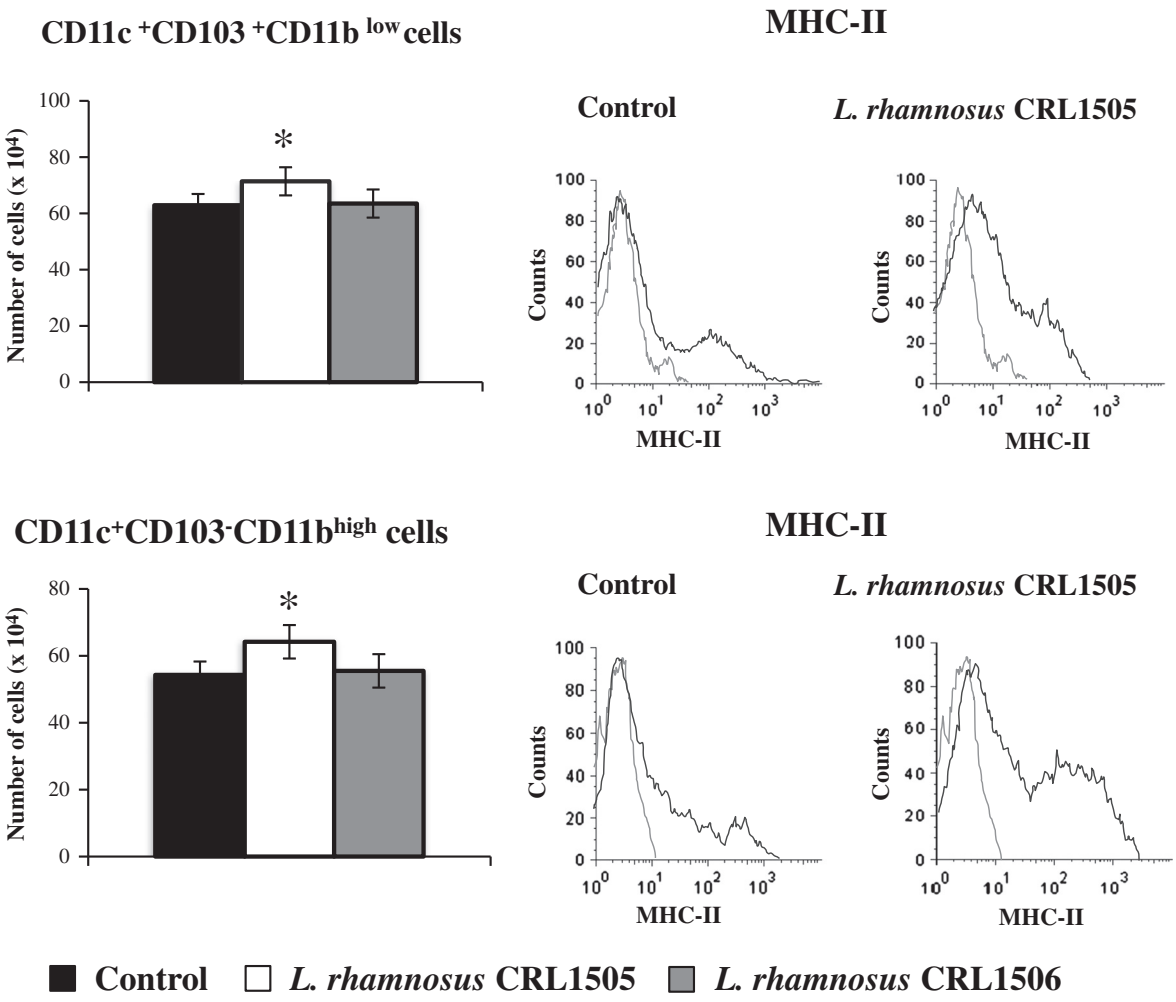


Fig. 5. Effect of lactobacilli on respiratory dendritic cells populations after the challenge with respiratory syncytial virus (RSV). Effect of *Lactobacillus rhamnosus* CRL1505 and *L. rhamnosus* CRL1506 oral administration on $CD11c^{+}CD103^{+}CD11b^{low}$ and $CD11c^{+}CD11b^{high}CD103^{-}$ dendritic cells from lung and their expression of MHC-II. The results represent data from three independent experiments. (*) Significantly different from control infected mice ($P < 0.05$).

blocking anti-IFN-γ and anti-IL-10R antibodies. As shown in Fig. 7A, anti-IFN-γ antibodies significantly abolish the reduction of RSV titers induced by the CRL1505 strain. In addition, the reductions of lung wet:dry weight and BAL albumin concentrations induced by the immunobiotic strain were partially abolished with anti-IFN-γ antibodies while no effect was observed in BAL LDH (Fig. 7A). Treatment of mice with anti-IL-10R antibodies did not induce modification in the reduction of RSV titer induced by *L. rhamnosus* CRL1505 (Fig. 7B). However, blocking IL-10/IL-10R interaction significantly abolished the capacity of the immunobiotic strain to protect against lung tissue damage as observed in the values of lung wet:dry weight, BAL albumin and BAL LDH in anti-IL-10R-treated mice (Fig. 7B).

4. Discussion

Viral pathogens are the most common etiological agents of acute respiratory disease and despite years of research, and current prophylactic and therapeutic strategies to combat infections remain largely inadequate. Influenza viruses and RSV are among the most common causes of human respiratory infections, and among the most significant because they cause high morbidity and mortality. In the past decade, several lines of evidence showed that oral administration of immunobiotics is able to increase resistance against respiratory virus infections [18]. It was reported that the oral administration of the *Bifidobacterium breve* YIT4064 protected mice against influenza virus challenge through an enhancement of the humoral immune response [19]. In addition, *Lactobacillus casei* Shirota activates the systemic and respiratory immune systems and ameliorates influenza virus infection in the respiratory tract of aged [20] and infant mice [21]. Other studies have also emphasized the capacity of immunobiotics to improve protection against influenza infection (reviewed in [18]). However, to the best of our knowledge, there are no reports regarding the protective effects of immunobioitcs against RSV. Then, this work is the first demonstration of the beneficial immunoregulatory effect of an immunobiotic strain during RSV infection. We demonstrated that oral administration of *L. rhamnosus* CRL1505 to 3-week old BALB/c mice significantly reduces lung viral loads and tissue injuries after the challenge with RSV. Moreover, this study shows that the protective effect achieved by the CRL1505 strain is related to its capacity to differentially modulate the respiratory antiviral immune response.

Natural human RSV infection in children and experimental RSV inoculation in mice result in prominent local secretion of pro-inflammatory cytokines, such as TNF-α, IL-6, IL-8, MIP-1, RANTES, and MCP-1 [22]. The excessive TNF-α, IL-8 and MCP-1 response can lead to increased immunopathology. These pro-inflammatory cytokines contribute to clearance of the virus during the early stages of RSV infection, however continued production of these factors exacerbate illness and tissue injuries during the late stages of infection [23]. Interestingly, recent studies demonstrate a role for IL-10 in

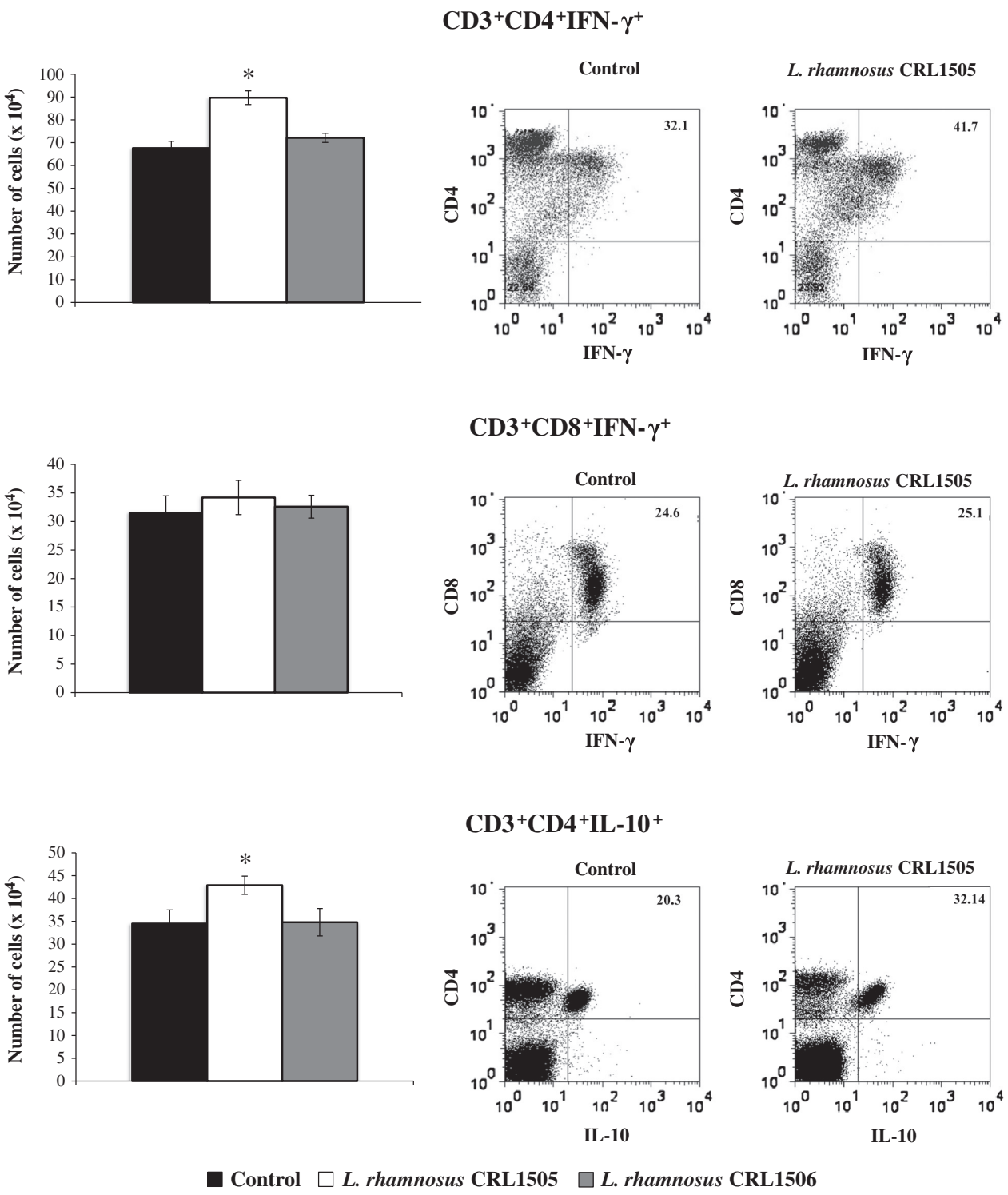
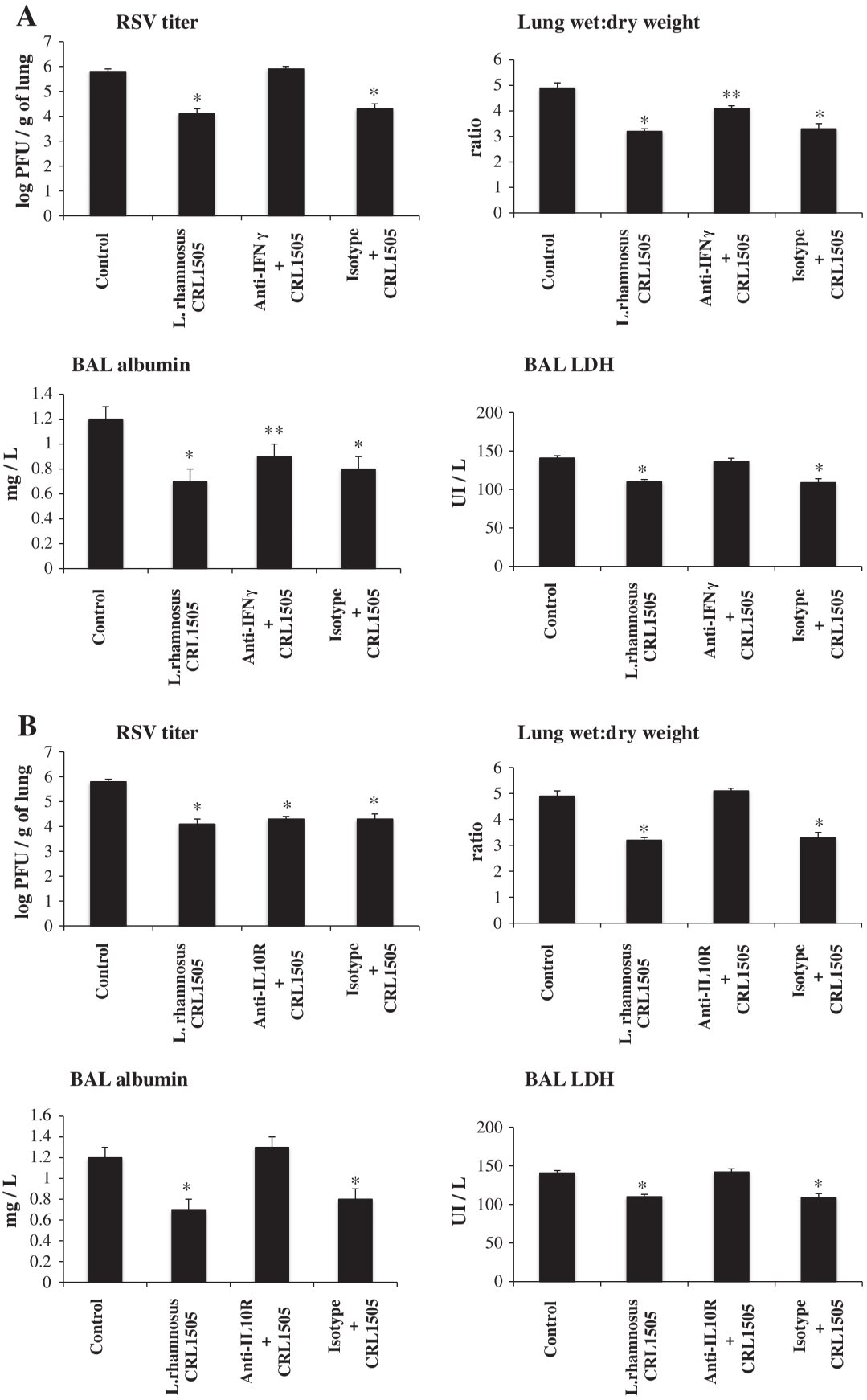


Fig. 6. Effect of lactobacilli on respiratory T cells populations after the challenge with respiratory syncytial virus (RSV). Effect of *Lactobacillus rhamnosus* CRL1505 and *L. rhamnosus* CRL1506 oral administration on $CD3^+CD8^+IFN\text{-}\gamma^+$, $CD3^+CD4^+IFN\text{-}\gamma^+$ and $CD3^+CD4^+IL\text{-}10^+$ T cells from the lung. The results represent data from three independent experiments. (*) Significantly different from control infected mice ($P < 0.05$).

controlling immunopathology during RSV infections [24,25]. It was found that IL-10 deficiency during RSV challenge did not affect viral load, but led to markedly increased disease severity with enhanced influx of inflammatory cells into the lung and airways and enhanced release of inflammatory mediators [17]. Therefore, an adequate balance of pro-inflammatory and anti-inflammatory factors is essential for a safe and effective antiviral immune response enabling virus elimination but limiting the detrimental effects of inflammation on the lung tissue [26,27].

We have demonstrated previously that orally administered *L. rhamnosus* CRL1505 modulates the balance between pro-inflammatory cytokines and IL-10 in the respiratory tract after the nasal challenge with poly(I:C) [12]. In this work we extend this finding by demonstrating that the CRL1505 strain beneficially modulates


A
RSV titer
log PFU / g of lung
Control
L. rhamnosus CRL1505
Anti-IFNγ + CRL1505
Isotype + CRL1505
Lung wet:dry weight
ratio
BAL albumin
mg / L
BAL LDH
UI / L
B
RSV titer
log PFU / g of lung
Control
L. rhamnosus CRL1505
Anti-IL10R + CRL1505
Isotype + CRL1505
Lung wet:dry weight
ratio
BAL albumin
mg / L
BAL LDH
UI / L

the balance between pro- and anti-inflammatory cytokines in response to RSV infection. We observed that CRL1505-treated mice were able to early increase the levels of TNF-α and IL-6 in the respiratory tract when compared to controls while the levels of TNF-α were significantly lower later in the infection. These findings indicate that the immunobiotic treatment induces changes in the respiratory tract that allow an early and improved inflammatory response which is later tightly regulated. These observations are in line with our previous results evaluating the effect of immunobiotic strains in the response to pneumococcal infection in which we showed an earlier and improved production of pro-inflamatory cytokines, related to pulmonary macrophages activation, with a significant reduction later in the infection when compared to untreated controls [4,7]. Orally administered *L. rhamnosus* CRL1505 significantly increased IL-10 levels that contribute to protection against inflammatory damage. In fact, blocking IL-10R significantly reduced the capacity of the CRL1505 strain to protect against lung tissue damage although it did not affected viral load. In addition, we demonstrated in this study that blocking IFN-γ significantly reduced the ability of *L. rhamnosus* CRL1505 treatment to reduce RSV replication. Then, the early increase of pro-inflamatory cytokines together with the improved levels of IFN-γ explains the higher capacity of CRL1505-treated mice to reduce viral loads while the improved levels of IL-10 during RSV infection led to markedly reduced severity in lung damage.

During RSV infection pattern recognition receptors such as TLR3 and RIG-I expressed in airway epithelial cells and APCs initiate complex signaling events that promote the production of cytokines, chemokines, IFN-α and IFN-β in the lung that link innate with adaptive immune response. In this regard, DCs have a central role in this shape of innate and acquired immune responses, and then we also examined the effect of immunobiotics on DCs activation. In the mouse lung two major subsets of myeloid DCs are derived from blood monocytes MHC-II$^+$CD11c$^+$CD11b^{low}CD103$^+$ (CD103$^+$ DCs) and MHC-II$^+$CD11c$^+$CD11b^{high}CD103$^-$(CD11b^{high} DCs) cells [28]. CD103$^+$ DCs are found mainly at the basal lamina of the bronchial epithelia and arterioles and are able to sample the airways with their extensions [28]. CD11b^{high} DCs are localized in the parenchyma and are the main producers of chemokines for the recruitment of leukocytes [29]. We have previously demonstrated that orally administered *L. rhamnosus* CRL1505 is able to modulate the expression of MHC-II in both lung DCs subsets, the subepithelial CD103$^+$ and the parenchymal CD11b^{high} DCs [12]. In the present work we also observed that the CRL1505 strain improved the numbers of both DCs populations as well as the expression of MHC-II in response to RSV challenge. This effect would have a significant impact in the immune response against RSV since it was reported that both DCs populations are important in the generation of RSV-specific CD4$^+$ and CD8$^+$ T cells. In this sense, Lukens et al. [30], using effectors T cells as a readout system to measure antigen display by MHC-I and MHC-II molecules, found that both migrating CD103$^+$ and CD11b^{high} lung DCs presented RSV-derived antigens to CD4$^+$ and CD8$^+$ T cells. Then, considering that RSV interaction with lung DCs results in activation and maturation events that play important roles in establishing virus-specific immunity and, that these early events during the initial immune response may determine the quality and durability of host immunity and influence susceptibility to reinfection, we can speculate that *L. rhamnosus* CRL1505 preventive treatment would significantly improve resistance against the infection by beneficially modulating DCs activity for the generation of a protective adaptive immune response. To evaluate in detail the impact of the immunobiotic treatment in the adaptive immune response against RSV infection is an interesting topic for future research.

It was reported that RSV susceptible infants exhibit characteristics of Th2 responses [31]. In general, a Th2 immune response is favored during RSV infection, especially in younger hosts. RSV-induced pulmonary inflammation in mice was previously found to cause a shift from Th1 to Th2 cell inflammation. RSV uses multiple mechanisms to induce a Th2 cell response in the host, including RSV G protein-mediated effects [32], increasing IL-4 production from basophils [33] and induction of alternatively activated macrophages [34]. Then, it is considered that strategies able to improve Th1 responses against RSV would beneficially modulate the outcome of the infection especially in young individuals. It was demonstrated that IFN-γ is able to upregulate the expression of MHC-II and MCH-I molecules in APCs and thereby enhances the cellular immune response to viral infection and suppresses the proliferation of Th2-type T cells [35]. Consistent with this notion, we showed that treatment of infant mice with *L. rhamnosus* CRL1505 significantly improved the production of IFN-γ in response to RSV infection and increased the capacity of mice to clear the virus. This observation is in line with our previous studies showing improved levels of serum and BAL IFN-γ in mice receiving *L. rhamnosus* CRL1505 prior to nasal challenge with poly(I:C) [12]. Then, modulation of respiratory and systemic immunity potentiated by the CRL1505 strain might contribute to an improved Th1 to Th2 shift and thereby favors protective immunity against viral infections such as RSV. We have recently demonstrated that orally administered *L. rhamnosus* CRL1505 induces a mobilization of CD3$^+$CD4$^+$IFN-γ$^+$ T cells from the gut into the respiratory mucosa and improve local production of IFN-γ [12]. Probably, IFN-γ secreted in response to *L. rhamnosus* CRL1505 stimulation would modulate the pulmonary innate immune microenvironment conducting to the activation of pulmonary macrophages [36] and DCs [12] and the generation of a Th1 response with the consequent attenuation of the strong and damaging Th2 reactions associated with the subsequent intranasal RSV challenge.

5. Conclusions

It has been estimated that 5.2 million children under five years of age die every year due to preventable infectious diseases like pneumonia and diarrhea. Moreover, recent findings suggest that 21% global mortality of children younger than 5 years of age are attributable to malnutrition and its synergistic relationship with preventable infectious diseases [37,38]. Fortification with probiotics may provide one feasible intervention to reduce the burden of common childhood morbidities such as viral respiratory infections through the modulation of intestinal microbiota and common mucosal immune system. It was showed that immune responses against respiratory tract influenza A virus infections could be influenced by gut commensal bacteria. Administration of broad-spectrum antibiotics in mice resulted in incompetent virus-specific CD4$^+$ and CD8$^+$ T cell responses, a defect that could be completely restored by intrarectal injection of the TLR ligands [39]. Another recent study of mice showed that specific dietary oligosaccharides can influence trafficking and effector functions of innate immune, CD4$^+$, and CD8$^+$ T cell subsets in the lungs of RSV-infected mice. Similarly to our present work, authors showed that changes in the microbiota induced by dietary oligosaccharides increased viral clearance and systemic Th1 responses [40]. The present work demonstrates that *L. rhamnosus* CRL1505 significantly reduces lung viral loads and tissue injuries after the challenge with

Fig. 7. Effect of IFN-γ (A) and IL-10 (B) in the immunoregulatory effect of lactobacilli during respiratory syncytial virus (RSV) infection. Mice were orally treated with *Lactobacillus rhamnosus* CRL1505 or *L. rhamnosus* CRL1506, intraperitoneally injected with anti-IFN-γ or anti-IL-10R antibodies and then challenged with RSV. Untreated and isotype-treated mice were used as controls. Lung RSV titers, lung wet:dry weight ratio, lactate dehydrogenase (LDH) activity and albumin concentrations in broncho-alveolar lavages (BAL) were determined on day 2 after the challenge with RSV. The results represent data from three independent experiments. (*) Significantly different from control mice ($P < 0.05$); (**) Significantly different from *L. rhamnosus* CRL1505-treated mice ($P < 0.05$).

RSV through its capacity to beneficially modulate pro-inflammatory/IL-10 and Th1/Th2 balances in the respiratory tract. Then, the previous studies and the present work indicate that modulation of the common mucosal immune system by immunobiotics could favor protective immunity against respiratory viral pathogens with a high attack rate in early infancy, such as RSV.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

EC, YT, MGV, and JV carried out experiments, analyzed data and performed the statistical analysis. HK, SA and JV conceived the study, and participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

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