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Non-viable immunobiotic *Lactobacillus rhamnosus* CRL1505 and its peptidoglycan improve systemic and respiratory innate immune response during recovery of immunocompromised-malnourished mice

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ABSTRACT

The effect of non-viable *Lactobacillus rhamnosus* CRL1505 and its cell wall and peptidoglycan on respiratory immunity in malnourished mice was studied. Weaned mice were malnourished with a protein-free diet for 21d and received BCD during 7d (BCD) or BCD with nasal non-viable *L. rhamnosus* CRL1505 (BCD + UV) or its cell wall (BCD + CW) or peptidoglycan (BCD + PG) supplementation during last 2d of the treatment. Malnourished mice without treatment (MNC) and well-nourished mice (WNC) were used as controls. Mice were infected nasally with *Streptococcus pneumoniae* after treatments. Resistance against pneumococci was reduced in MNC mice. Repletion with BCD reduced lung and blood bacterial cell counts when compared to MNC mice but the counts did not reach the levels of the WNC group. However, when malnourished mice received BCD + UV, BCD + CW or BCD + PG, pneumococci was not detected in lung or blood samples. Pneumococcal infection increased the levels of TNF-α, IL-1β, IL-6, and IL-10 in the respiratory tract, however the values were lower in MNC than in WNC mice. BCD + UV and BCD + PG groups showed values of phagocytes, IL-1β and IL-6 that were similar to WNC mice, while TNF-α was significantly higher in those groups when compared to WNC mice. Moreover, BCD + UV and BCD + PG treatments improved levels of respiratory IL-10, reaching values that were superior to those observed in WNC mice. The work demonstrates for the first time that non-viable probiotic bacteria or their cellular fractions could be an interesting alternative as mucosal immunomodulators, especially in immunocompromised hosts in which the use of live bacteria might be dangerous.

1. Introduction

Acute lower respiratory tract infections are a persistent public health problem. Despite the remarkable advances in antibiotic therapies, diagnostic tools, prevention campaigns and intensive care, respiratory infections are still among the primary causes of death worldwide, and there have been no significant changes in mortality in the last decades [1]. Childhood acute community-acquired pneumonia is one of the leading causes of morbidity and mortality in developing countries. In children who have not received prior antibiotic therapy, one of the main bacterial causes of clinical pneumonia is *Streptococcus pneumoniae* [2]. Moreover, despite appropriate therapies, mortality due to the different pneumococcal pathologies remains high in immunocompromised malnourished children; one million children die every year from pneumococcal diseases, mainly in developing countries [3,4]. Conversely, malnutrition is an important factor in the occurrence of respiratory infections. In this regard, we have demonstrated previously in a mouse model of protein-malnutrition that the increased susceptibility of malnourished hosts to respiratory pneumococcal infection is associated with impaired emergency granulopoiesis, cell-mediated immunity, phagocyte function, secretory immunoglobulin A (IgA) antibody concentrations, cytokine production, B cell development and activity, and antigen presenting cells function [5–8].

Our previous studies also suggest that lactic acid bacteria (LAB) with immunomodulatory capacities (immunobiotics) are able to accelerate the recovery of the respiratory immune system and improve resistance against bacterial respiratory infection in malnourished hosts [5–8]. We have demonstrated that the supplementation of balance conventional diet (BCD) with nasally or orally administered viable immunobiotics, during the recovery of malnourished mice, is able to improve respiratory innate immune response [5], emergency granulopoiesis [8], and humoral anti-pneumococcal response [7]. Our studies also suggest that non-viable immunobiotics are effective in the immunomodulation of the systemic and respiratory immune system in malnourished hosts under repletion treatments [9]. Therefore, immunobiotic bacteria in the form of live cells may not be required for this purpose. However, the capacity of non-viable immunobiotic strains or their cellular fractions such as the cell wall or peptidoglycan to influence innate immunity during recovery of malnourished hosts and the impact of such effect

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on the resistance against respiratory pathogens have not been evaluated in depth.

Non-viable bacteria or their cellular fractions could be an interesting alternative as mucosal immunomodulators, especially in immunocompromised hosts in which the use of live bacteria might be dangerous. Therefore, in the current study we aimed to study the effect of non-viable *Lactobacillus rhamnosus* CRL1505 and its cell wall and peptidoglycan on the innate immune response against the respiratory pathogen *S. pneumoniae* in protein-malnourished mice under repletion treatments.

2. Materials and methods

2.1. Microorganisms

L. rhamnosus CRL1505 was obtained from the CERELA culture collection (Chacabuco 145, San Miguel de Tucumán, Argentina). This strain was isolated from goat milk from northwestern Argentina and was selected because of its immunomodulatory capacity [10,11]. 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, 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). The bacteria were harvested by centrifugation at 3000 *g* for 10 min, washed three times with sterile 0.01 mol/l phosphate buffer saline (PBS), pH 7.2, and resuspended in sterile on PBS. Non-viable *L. rhamnosus* CRL1505, designated as UV was obtained by exposition to ultraviolet radiation for 2 h. The absence of bacterial growth was confirmed using MRS agar plates. For obtaining cell-wall and peptidoglycan from *L. rhamnosus* CRL1505, the bacterium was grown in MRS broth for three days, performing periodic refills means to concentrate the cells. Cell wall and peptidoglycan from *L. rhamnosus* CRL1505 were obtained using the method of Shida K. et al. [12] with minor modifications. Briefly, the bacterium was grown in MRS broth for 18 h at 37 °C, washed 3 times with sterile PBS and lyophilized. Then the cells were resuspended in sterile water (0.1 g/mL) and were lysed by sonication in an Ultrasonic Homogenizer (Cole Parmer) with cycles of 2.5 min and an amplitude of 70%. The wall obtained in this way was delipidated by successive refluxing with methanol, methanol–chloroform (1:1), and chloroform. The delipidated preparation was resuspended in Tris–HCl buffer pH 50 μM 7.2 to 7.5 and treated with bovine pancreatic DNAse I (Sigma) (50 μg/mL) and ribonuclease A (Sigma) (100 μg/mL) at 37 °C with stirring for 4 h. The insoluble material was washed with distilled water and lyophilized; the resultant product was used as the cell wall (CW) preparation. To obtain intact peptidoglycan, CW was treated with 50% hydrogen chloride at 4 °C for 20 h. Finally, the product obtained was washed with sterile water, adjusted to pH 7.2, lyophilized and was used as intact peptidoglycan (PG).

2.2. Animals and feeding procedures

Male or female, 3-wk-old, Swiss-albino mice were obtained from CERELA. Weaned mice were fed a protein-free diet (PFD) for 21 d and the mice that weighed 45–50% less than the well-nourished mice were selected for the experiments [5] (Fig. 1). Malnourished mice were divided into 3 groups for treatments: mice were fed for 7 days with a balanced conventional diet (BCD; BCD group), BCD for 7 days and nasally treated during the last 2 days with UV (10^8 cells/mouse/day, UV group), CW (8 μg/ml, CW group); or PG (8 μg/ml, PG group) (Fig. 1). The doses of CW and PG were chosen on the basis of preliminary experiments [Salva and Alvarez, unpublished results]. A fourth group of malnourished mice was used as the malnourished control group (MNC). The MNC mice received only a PFD during experiments. Normal mice were used as the well-nourished control (WNC) group. The WNC mice consumed only BCD ad libitum during experiments. The compositions of the BCD and PFD diets were previously described [6]. Experiments with animals were approved by the CERELA Ethical Committee of Animal Care (protocol BIOT-CRL-10).

2.3. Experimental infection

S. pneumoniae serotype 6B was obtained from the respiratory tract of a patient from the Department of Bacteriology of Malbran Institute in Buenos Aires, Argentina [5]. The experimental animal model of respiratory infection was used as previously described [5]. Briefly, the different experimental groups of mice were nasally challenged with *S. pneumoniae* (10^9 cfu/ml in PBS) at the end of each treatment (day 8) (Fig. 1). WNC and MNC (without repletion treatment) groups were infected equally. Animals were euthanized on day 0 (before challenge) and at 48 h after infection.

2.4. Bacterial cell counts in lung homogenates and blood

Mice were euthanized on day 2 after the challenge and their lungs were excised, weighed and homogenized in 5 ml of sterile 0.1% peptone water. Homogenates were diluted appropriately, plated in duplicate on blood agar and incubated for 18 h at 37 ° C. Results were expressed as log of cfu/g of lung. Progression of bacterial growth to the bloodstream was monitored by sampling blood obtained through cardiac puncture and plating on blood agar. Results were reported as log of cfu/ml of blood [9].

2.5. Lung tissue injury

Forty eight hours after the 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 determined in the acellular broncho-alveolar lavage (BAL) fluid. BAL samples were obtained as described previously [13]. 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. Albumin content was determined colorimetrically based on albumin binding to bromocresol 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].

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

Blood samples were obtained through cardiac puncture at the end of each treatment and collected in heparinized tubes. BAL samples were obtained as described above. Total number of blood and BAL 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 [5,9].

2.7. Activation of blood neutrophils

Measurement of myeloperoxidase (MPO) activity of blood neutrophils was carried out by use of the Washburn test, which is a

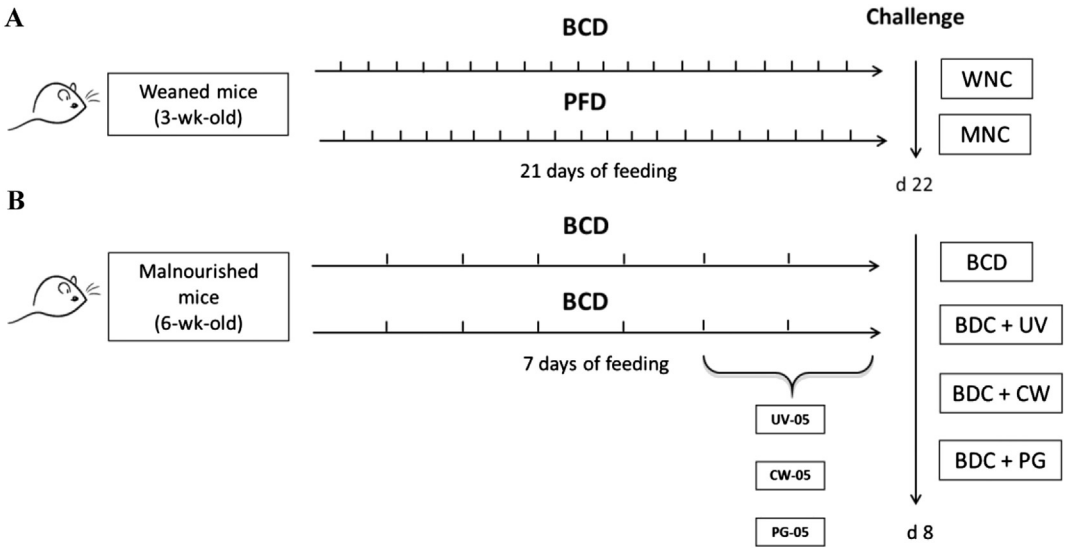

Fig. 1. Feeding protocols used in this study. (A) Obtainment of malnourished mice (MNC) and well-nourished (WNC) infected controls. (B) Repletion of malnourished mice by feeding balanced conventional diet (BCD) or BCD supplemented with non-viable *Lactobacillus rhamnosus* CRL1505 (BCD + UV), intact cell wall (BCD + CW) or peptidoglycan (BCD + PG), for the nasal route for 2 d. PFD, protein-free diet.

cytochemical method that uses benzidine as an MPO chromogen [6]. 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.

2.8. Cytokine concentrations in serum and bronchoalveolar lavages

Tumor necrosis factor (TNF)-α, interleukin (IL)-6, IL-1β, and IL-10 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).

2.9. Culture of peritoneal and alveolar macrophages

Alveolar and peritoneal macrophages were collected aseptically from uninfected mice as described previously [14]. The macrophages were washed twice with PBS containing BSA and adjusted to a concentration of 10^6 cells/ml. In order to determinate the influence of dietary treatments on the capacity of macrophages to produce cytokines; alveolar and peritoneal macrophages were challenged in vitro with heat-killed *S. pneumoniae* serotype 6B at a concentration of 10^7 cells/ml. Twelve hours after the challenge total RNA was isolated for quantitative expression analysis by real-time PCR.

2.10. Quantitative expression analysis by real-time PCR

We performed two-step real-time quantitative PCR to characterize the expression of TNF-α, IL-1β, IL-6, IL-10, TLR2 and TLR9 mRNAs in peritoneal and alveolar macrophages. The primers used were previously described [8,15]. 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 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.

2.11. 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. Non-viable L. rhamnosus CRL1505 and its cellular fractions significantly increase the resistance of immunocompromised-malnourished mice against pneumococcal infection

Malnourished mice were obtained after 21 days of feeding a protein free-diet. Then, mice were replete for 7 days with a balanced conventional diet (BCD) or BCD supplemented with nasally administered non-viable *L. rhamnosus* CRL1505 (BCD + UV) or its cell-wall or peptidoglycan (BCD + CW and BCD + PG respectively) (Fig. 1). Malnourished (MNC) and well-nourished (WNC) mice were used as controls. As shown in Table 1, resistance against *S. pneumoniae* infection was significantly reduced in MNC mice since the levels of lung and blood bacterial cell counts were higher than those detected in WNC mice. Repletion of malnourished mice with BCD significantly reduced lung and blood bacterial cell counts when compared to MNC mice but the counts did not reach the levels of the WNC group (Table 1). However, when malnourished mice were repleted with BCD + UV, BCD + CW or BCD + PG, *S. pneumoniae* was not detected in lung or blood samples (Table 1).

We also evaluated lung tissue injury by using histological studies and BAL biochemical markers such as albumin content, to measure the increase of bronchoalveolar–capillarity barrier permeability, and LDH activity, as indicator of general cytotoxicity (Fig. 2). Challenge with pneumoccocci induced an intense inflammatory response in lung, hemorrhage and a reduction of alveolar spaces in the MNC group when compared to WNC mice (Fig. 2A). Lung tissue changes in BCD mice were always intermediate between MNC and WNC mice. Mice receiving BCD + UV, BCD + CW or BCD + PG showed a moderate inflammatory infiltrate and no hemorrhage or reduction of alveolar spaces (Fig. 2A).

Table 1
Effect of *Lactobacillus rhamnosus* CRL1505 on the resistance to pneumococcal infection. Malnourished mice were replete for 7 days with a balanced conventional diet (BCD) or BCD supplemented with nasally administered non-viable *L. rhamnosus* CRL1505 (BCD + UV), intact cell wall (BCD + CW) or peptidoglycan (BCD + PG), and then challenged with *Streptococcus pneumoniae*. Malnourished (MNC) and well-nourished (WNC) mice were used as controls. Bacterial cell counts in lung (log CFU/g of lung) and blood (CFU/ml) after challenge. The results represent data from three independent experiments. Results are expressed as mean ± SD. Six mice per group per each time point were used. [a,b,c,d] Means in a column with different letters (a < b < c < d) were significantly different ($P < 0.05$).

	Lung	Blood
WNC	2.90 ± 1.10^{b}	3.62 ± 0.36^{c}
MNC	5.57 ± 1.02^{d}	4.06 ± 0.19^{d}
BCD	3.85 ± 0.46^{c}	2.28 ± 0.09^{b}
BCD + UV	$<1.5^{a}$	$<1.5^{a}$
BCD + CW	$<1.5^{a}$	$<1.5^{a}$
BCD + PG	$<1.5^{a}$	$<1.5^{a}$

However, BCD + UV and BCD + PG treatments were more effective than the BCD + CW treatment to reduce lung histological alterations (Fig. 2A).

S. pneumoniae infection significantly increased LDH activity and albumin concentration in BAL of all experimental groups (Fig. 2B). Values of both biochemical markers of lung injury were significantly higher in MNC mice than in the WNC group. BCD, BCD + UV, BCD + CW or BCD + PG groups showed reduced levels of BAL LDH and albumin when compared to MNC mice; however the nasal treatment with non-viable *L. rhamnosus* CRL1505, CW or PG was more effective than BCD treatment to reduce albumin concentration and LDH activity in BAL (Fig. 2B).

3.2. Non-viable *L. rhamnosus* CRL1505 and its cellular fractions enhance respiratory innate immune response against pneumococcal infection in immunocompromised-malnourished mice

We next studied the respiratory innate immune response by evaluating number of BAL leukocytes and levels of respiratory cytokines. Before the challenge with *S. pneumoniae* reduced numbers of BAL leukocytes, macrophages and neutrophils was observed in MNC mice when compared to the WNC group (Fig. 3A). Treatment with BCD or BCD + UV normalized the numbers of BAL leukocytes and macrophages. In addition, BCD + CW or BCD + PG mice showed numbers of BAL

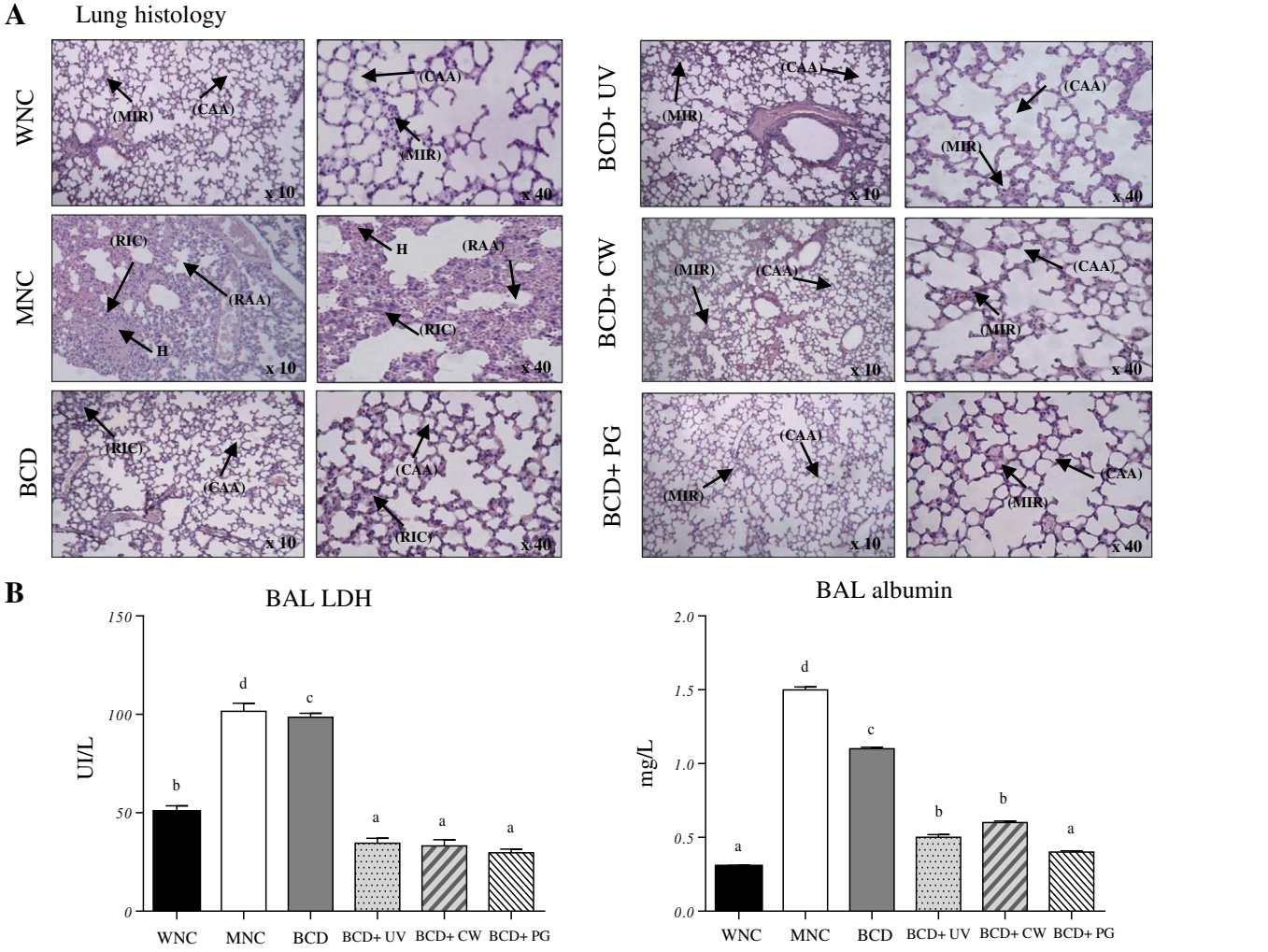

Fig. 2. Effect of *Lactobacillus rhamnosus* CRL1505 on lung injuries induced by pneumococcal infection. Malnourished mice were replete for 7 days with a balanced conventional diet (BCD) or BCD supplemented with nasally administered non-viable *L. rhamnosus* CRL1505 (BCD + UV), intact cell wall (BCD + CW) or peptidoglycan (BCD + PG), and then challenged with *Streptococcus pneumoniae*. Malnourished (MNC) and well-nourished (WNC) mice were used as controls. Lung tissue injury markers were studied 48 hours after the challenge with *S. pneumoniae*. The lungs were removed, fixed, and stained with hematoxylin and eosin for histological analysis (A). Light micrographs, original magnification × 10 and × 40. Recruited inflammatory cells (RIC), reduction of alveolar airspaces (RAA), hemorrhage (H), moderate inflammatory response (MIR), conserved alveolar air spaces (CAA). (B) Lactate dehydrogenase (LDH) activity and, albumin concentration in broncho-alveolar lavages (BAL) after the challenge with *S. pneumoniae*. The results represent data from three independent experiments. Results are expressed as mean ± SD. Six mice per group per each time point were used. [a,b,c,d] Means in a bar with different letters (a < b < c < d) were significantly different ($P < 0.05$).

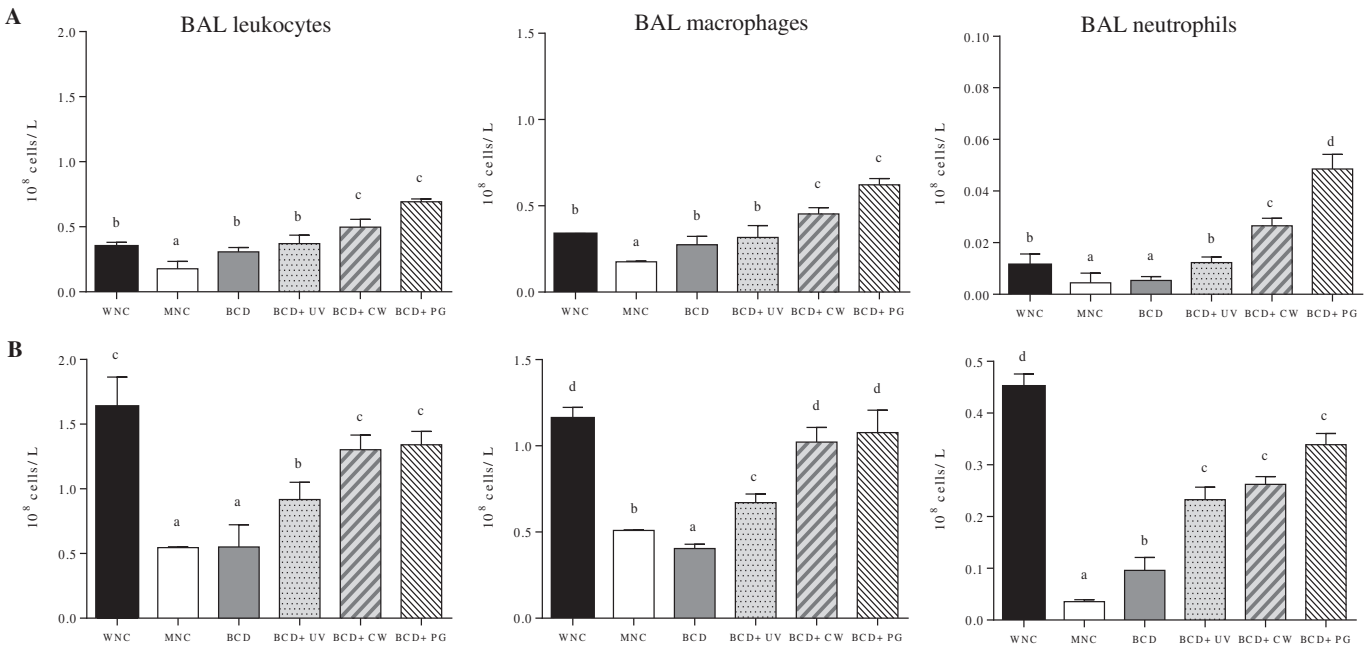

Fig. 3. Effect of *Lactobacillus rhamnosus* CRL1505 on respiratory immune response. Malnourished mice were replete for 7 days with a balanced conventional diet (BCD) or BCD supplemented with nasally administered non-viable *L. rhamnosus* CRL1505 (BCD + UV), intact cell wall (BCD + CW) or peptidoglycan (BCD + PG), and then challenged with *Streptococcus pneumoniae*. Malnourished (MNC) and well-nourished (WNC) mice were used as controls. Number of leukocytes, macrophages and neutrophils in broncho-alveolar lavages (BAL) before challenge (A) and on day 2 after the challenge with *S. pneumoniae* (B). The results represent data from three independent experiments. Results are expressed as mean ± SD. Six mice per group per each time point were used. [a,b,c,d] Means in a bar with different letters (a < b < c < d) were significantly different ($P < 0.05$).

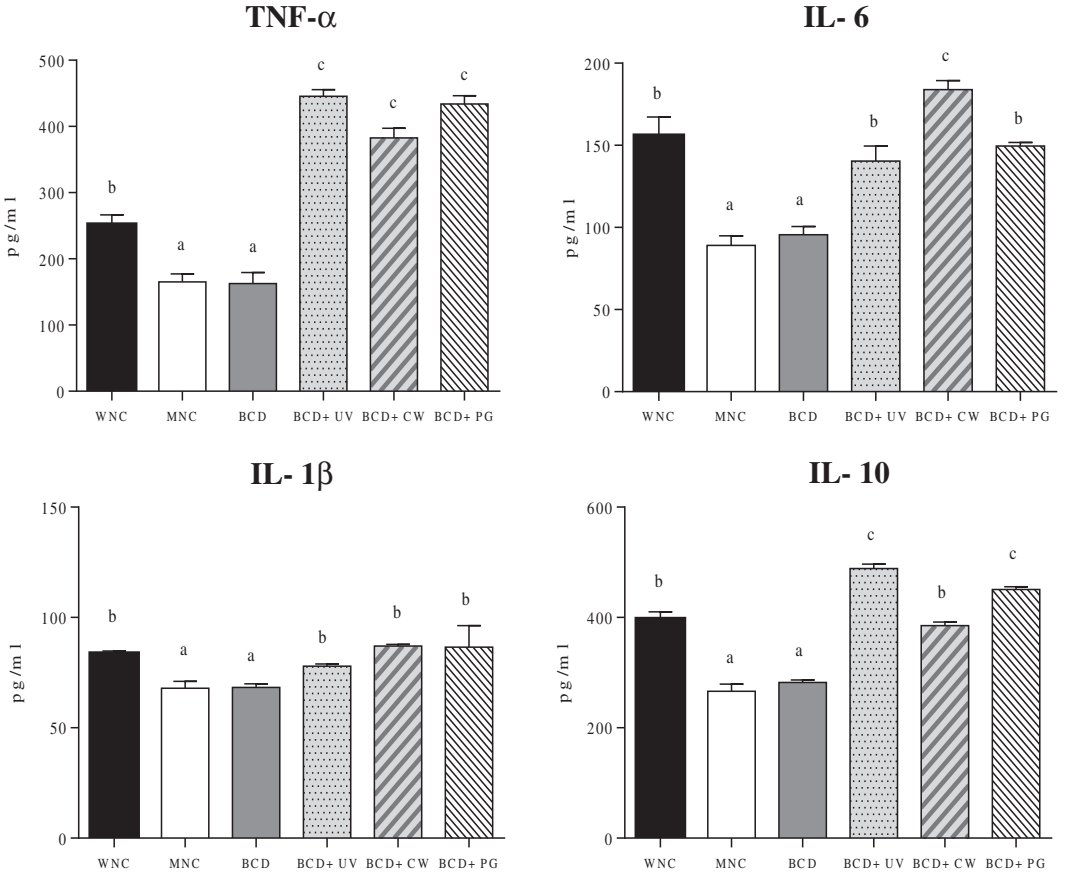

Fig. 4. Effect of *Lactobacillus rhamnosus* CRL1505 on respiratory immune response. Malnourished mice were replete for 7 days with a balanced conventional diet (BCD) or BCD supplemented with nasally administered non-viable *L. rhamnosus* CRL1505 (BCD + UV), intact cell wall (BCD + CW) or peptidoglycan (BCD + PG), and then challenged with *Streptococcus pneumoniae*. Malnourished (MNC) and well-nourished (WNC) mice were used as controls. Tumor necrosis factor (TNF)-α, interleukin (IL)-6, IL-1β and IL-10 concentrations in broncho-alveolar lavages (BAL) on day 2 after the challenge with *S. pneumoniae*. The results represent data from three independent experiments. Results are expressed as mean ± SD. Six mice per group per each time point were used. [a,b,c] Means in a bar with different letters (a < b < c) were significantly different ($P < 0.05$).

leukocytes, macrophages and neutrophils that were superior to those observed in the WNC group (Fig. 3A). *S. pneumoniae* infection significantly increased BAL leukocytes in all experimental groups ($p < 0.05$) (Fig. 3B). BAL leukocytes, macrophages and neutrophils were significantly lower in MNC mice than in the WNC group. BAL leukocyte numbers in BCD mice were not different from the MNC group (Fig. 3B). On the contrary, BCD + UV, BCD + CW or BCD + PG treatments were able to increase significantly leukocyte numbers in BAL when compared to malnourished mice (Fig. 3B). However, only BCD + CW or BCD + PG reached values of BAL leukocytes that were similar to WNC mice.

The levels of TNF-α, IL-1β, IL-6, and IL-10 were also studied in BAL samples after the challenge with *S. pneumoniae* (Fig. 4). Challenge with the respiratory pathogen significantly increased the levels of the four cytokines studied in all the experimental groups; however values of TNF-α, IL-1β, IL-6, and IL-10 were lower in MNC than in WNC mice (Fig. 4). BCD treatment was not able to improve the levels of respiratory cytokines in response to the infection. Values of BAL TNF-α in BCD + UV, BCD + CW or BCD + PG groups were higher than in WNC mice. The three treatments normalized the levels of BAL IL-1β. In addition, BCD + UV and BCD + PG groups showed levels of IL-6 that were similar to WNC mice, while this cytokine was significantly higher in BCD + CW mice when compared to WNC group (Fig. 4). BCD + UV, BCD + CW and BCD + PG treatments also improved levels of BAL IL-10; however values of this cytokine in BCD + UV and BCD + PG mice were superior to those observed in the WNC group.

3.3. Non-viable L. rhamnosus CRL1505 and its peptidoglycan enhance systemic innate immune response against pneumococcal infection in immunocompromised-malnourished mice

Reduced numbers of blood leukocytes and neutrophils as well as blood peroxidase scores were observed in MNC mice when compared to the WNC group before the challenge with *S. pneumoniae* (Fig. 5A). Treatment with BCD normalized blood peroxidase score but was not able to normalize blood leukocytes and neutrophils levels. In addition, BCD + UV, BCD + CW and BCD + PG treatments improved blood leukocytes and neutrophils; however only BCD + UV mice reached values that were similar to the WNC group (Fig. 5A). Blood peroxidase score in BCD + UV, BCD + CW and BCD + PG mice was superior to WNC group. *S. pneumoniae* infection significantly increased blood leukocytes and neutrophils in all experimental groups ($p < 0.05$) (Fig. 5B). As observed before infection, MNC mice showed values of blood neutrophils and blood peroxidase scores that were significantly lower than those in the WNC group. BCD, BCD + CW and BCD + PG treatments improved blood neutrophil numbers after the challenge, but these groups did not reach the levels of WNC mice. In addition, BCD + UV mice showed normal levels of blood neutrophils in response to the infection (Fig. 5B). BCD + UV, BCD + CW and BCD + PG treatments improved levels of blood peroxidase, reaching values that were similar to those observed in the WNC group.

The levels of blood TNF-α, IL-1β, IL-6, and IL-10 were also increased after the challenge with *S. pneumoniae* (Fig. 6). MNC mice showed significantly lower levels of the four cytokines when compared to the WNC group. BCD mice showed values of blood TNF-α, IL-1β, IL-6, and IL-10 that were similar to MNC mice. BCD + UV, BCD + CW and BCD + PG improved the levels of the pro-inflammatory cytokines TNF-α, IL-1β and IL-6 (Fig. 6). The levels of these three cytokines in BCD + UV and BCD + PG were similar to WNC mice, while BCD + CW showed values of pro-inflammatory cytokines that were superior to the WNC group. In addition, BCD + UV, BCD + CW and BCD + PG increased the levels of blood IL-10. BCD + CW showed normal values of this cytokine while BCD + UV and BCD + PG showed levels of blood IL-10 that were superior to those observed in the WNC group (Fig. 6).

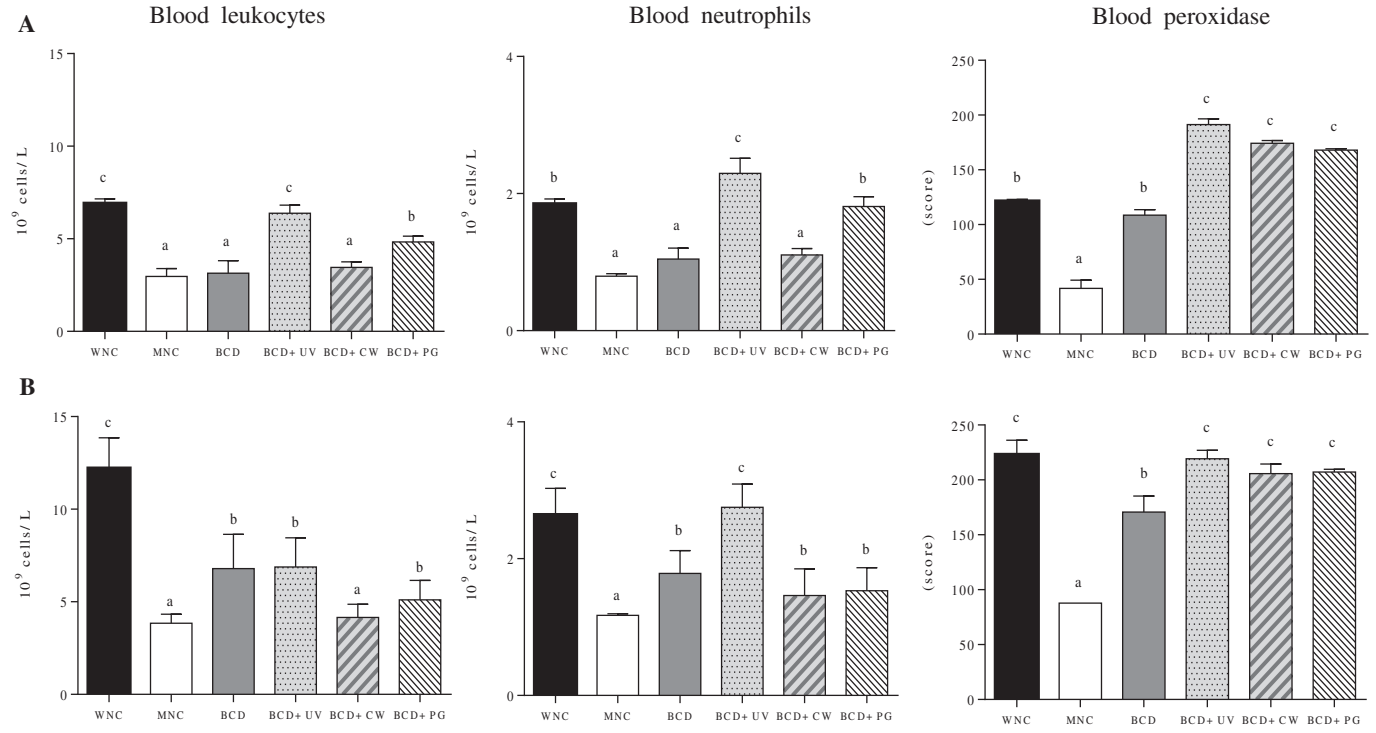

Fig. 5. Effect of *Lactobacillus rhamnosus* CRL1505 on sytemic immune response. Malnourished mice were replete for 7 days with a balanced conventional diet (BCD) or BCD supplemented with nasally administered non-viable *L. rhamnosus* CRL1505 (BCD + UV), intact cell wall (BCD + CW) or peptidoglycan (BCD + PG), and then challenged with *Streptococcus pneumoniae*. Malnourished (MNC) and well-nourished (WNC) mice were used as controls. Number of leukocytes, and neutrophils, and peroxidase activity in blood before challenge (A) and on day 2 after the challenge with *S. pneumoniae* (B). The results represent data from three independent experiments. Results are expressed as mean ± SD. Six mice per group per each time point were used. [a,b,c] Means in a bar with different letters (a < b < c) were significantly different ($P < 0.05$).

3.4. Non-viable L. rhamnosus CRL1505 and its peptidoglycan augment peritoneal and alveolar macrophages activity in immunocompromised-malnourished mice

Finally we aimed to evaluate the effect of repletion treatments in macrophages activity by using *ex vivo* experiments. For this purpose, alveolar and peritoneal macrophages were collected aseptically from uninfected mice, cells cultured and challenged in vitro with heat-killed *S. pneumoniae* serotype 6B. Twelve hours after the challenge total RNA was isolated for quantitative expression analysis by real-time PCR. As shown in Fig. 7, stimulation of alveolar macrophages with heat-killed pneumococci significantly increased the expression of TNF-α, IL-1β, IL-6, IL-10, TLR2 and TLR9 in all the experimental groups. As we previously observed in the in vivo experiments, values of TNF-α, IL-1β, IL-6, and IL-10 were lower in MNC mice than in the WNC group. In addition, both TLR2 and TLR9 mRNA were lower in MNC mice when compared with WNC group (Fig. 7). Values of all cytokines and TLRs studied in BCD mice were not different from those in MNC mice. In addition, alveolar macrophages coming from BCD + UV or BCD + PG showed increased levels of cytokines and TLR expressions. Values of TNF-α, IL-1β, IL-6, and IL-10 were superior than those observed in the WNC group, while TLR2 and TLR9 were equal to the well-nourished controls (Fig. 7). A similar trend was observed when analyzing TNF-α, IL-1β, IL-6, IL-10, TLR2 and TLR9 expression in peritoneal macrophages after the challenge with heat-killed pneumococci (Fig. 8). Malnutrition significantly reduced the expression of cytokines and TLRs in peritoneal macrophages. BCD treatment was not able to improve the expression of TNF-α, IL-1β, IL-6, IL-10, TLR2 or TLR9 while BCD + UV or BCD + PG significantly increased the levels cytokines and TLR expressions to levels that were superior to the WNC group (Fig. 8).

4. Discussion

In the last years, several investigations aimed at finding alternative treatments to promote hematopoiesis, enhance immunity, and potentiate resistance against infection in malnourished hosts. Considering that several works in animal models and clinical trials have shown the potential of probiotics to beneficially modulate the outcome of respiratory infections, our laboratory hypothesized that the use of probiotic during the recovery of malnourished host would be of value to accelerate immune defense mechanisms [16].

Our results demonstrated that LAB strains that are able to beneficially modulate the immune system (immunobiotics) represent an attractive safe way to regulate and enhance immune function in immunocompromised malnourished hosts [16,17]. In this regard, we recently demonstrated that repletion of malnourished mice with treatments including nasally administered viable *L. rhamnosus* CRL1505 is able to significantly accelerate the recovery of granulopoiesis and improve innate immunity [18]. We previously observed that malnutrition significantly impaired the production of TNF-α, IL-1β and IL-6 and the recruitment of

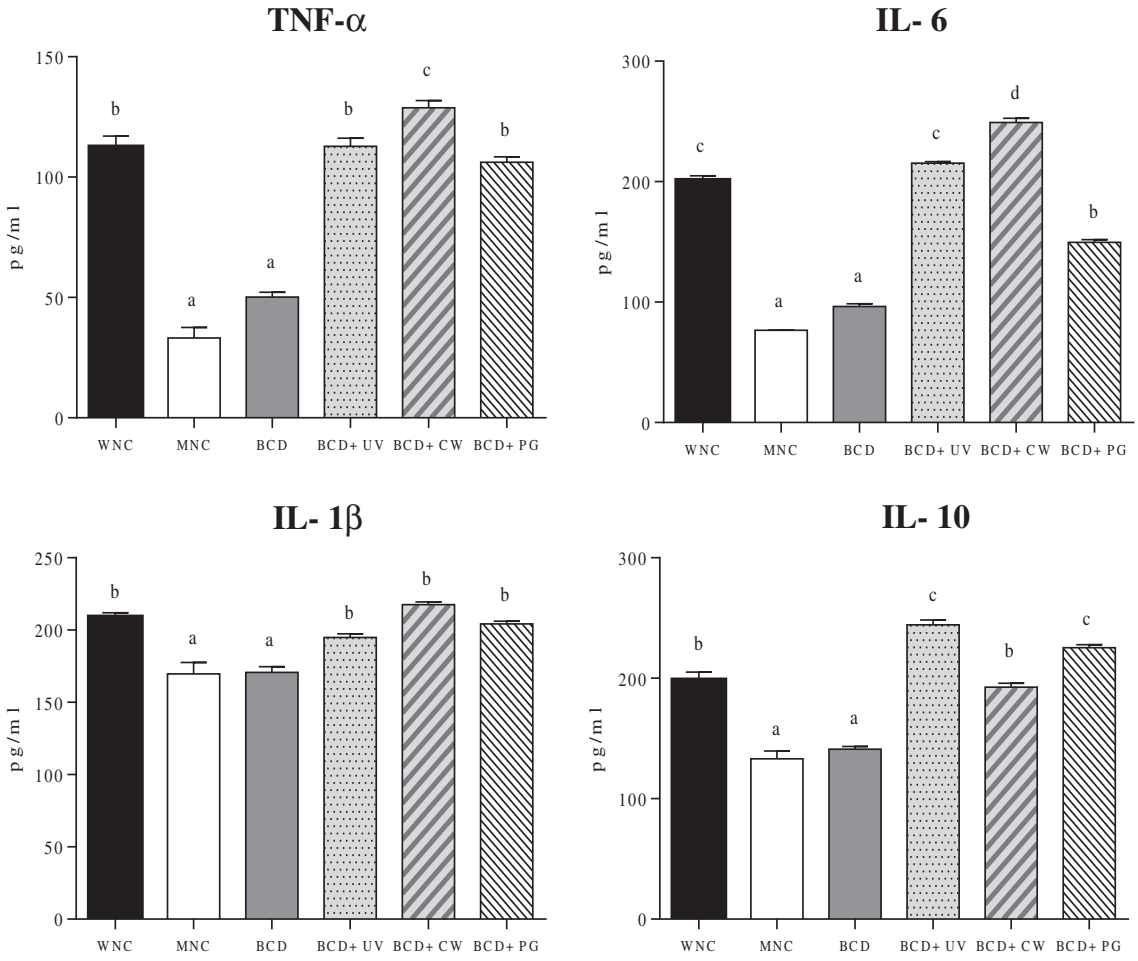

Fig. 6. Effect of *Lactobacillus rhamnosus* CRL1505 on systemic immune response. Malnourished mice were replete for 7 days with a balanced conventional diet (BCD) or BCD supplemented with nasally administered non-viable *L. rhamnosus* CRL1505 (BCD + UV), intact cell wall (BCD + CW) or peptidoglycan (BCD + PG), and then challenged with *Streptococcus pneumoniae*. Malnourished (MNC) and well-nourished (WNC) mice were used as controls. Tumor necrosis factor (TNF)-α, interleukin (IL)-6, IL-1β and IL-10 concentrations in blood on day 2 after the challenge with *S. pneumoniae*. The results represent data from three independent experiments. Results are expressed as mean ± SD. Six mice per group per each time point were used. [a,b,c,d] Means in a bar with different letters (a < b < c < d) were significantly different ($P < 0.05$).

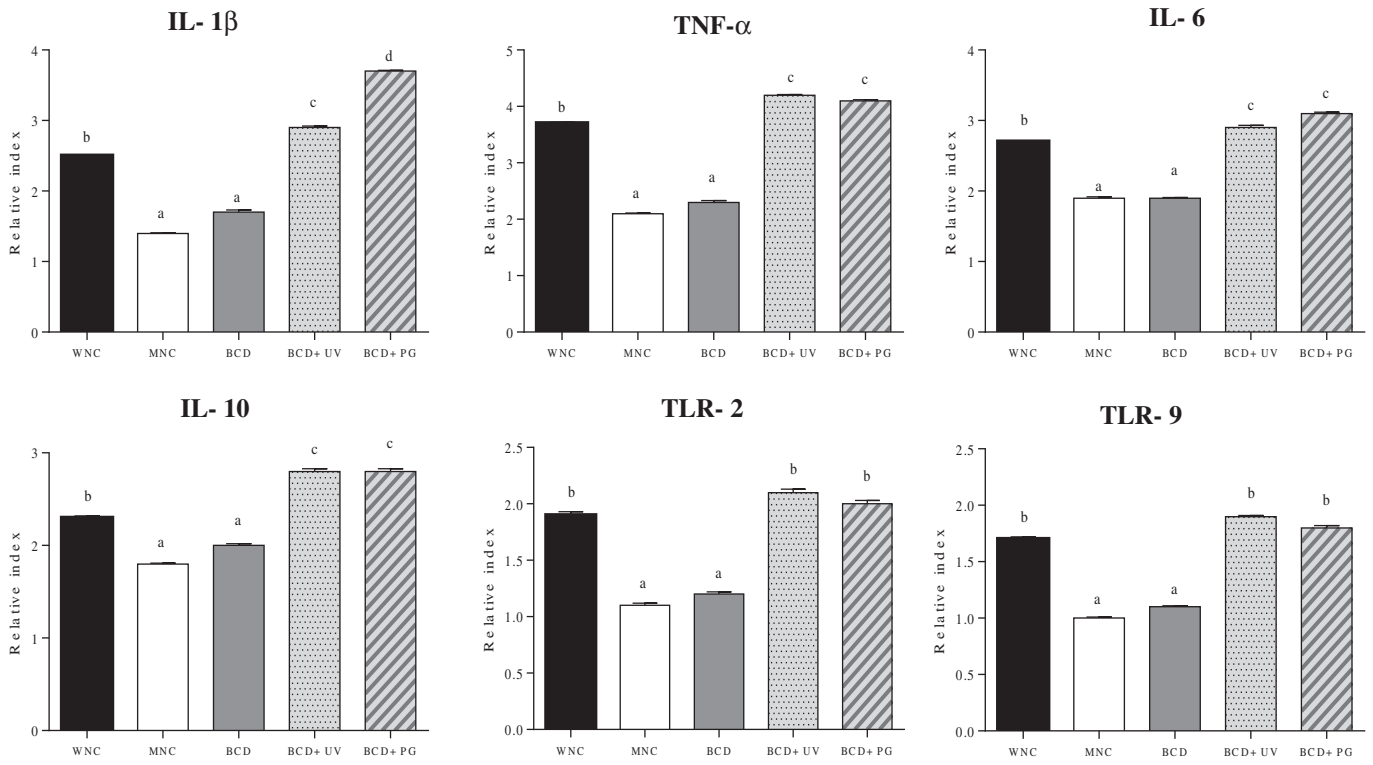

Fig. 7. Effect of *Lactobacillus rhamnosus* CRL1505 on alveolar macrophages. Malnourished mice were replete for 7 days with a balanced conventional diet (BCD) or BCD supplemented with nasally administered non-viable *L. rhamnosus* CRL1505 (BCD + UV), intact cell wall (BCD + CW) or peptidoglycan (BCD + PG). Malnourished (MNC) and well-nourished (WNC) mice were used as controls. Alveolar macrophages were removed from the different groups and challenged in vitro with *Streptococcus pneumoniae*. Tumor necrosis factor (TNF)-α, interleukin (IL)-6, IL-1β and IL-10, Toll-like receptor (TLR)-2 and TLR9 mRNA expressions were determined after the challenge with *S. pneumoniae*. The results represent data from three independent experiments. Results are expressed as mean ± SD. Six mice per group per each time point were used. [a,b,c,d] Means in a bar with different letters (a < b < c < d) were significantly different ($P < 0.05$).

neutrophils into the respiratory tract after pneumococcal challenge. Moreover, protein-malnutrition significantly affected emergency granulopoiesis, which is the hematopoietic response to infection and is characterized by mobilization of neutrophils from the bone marrow, leading to leukocytosis and neutrophilia [18]. We also showed that the administration of the BCD during 7 days was not enough to induce the normalization of respiratory innate immunity and the emergency granulopoiesis in response to pneumococcal infection while the nasal supplementation of the BCD with viable *L. rhamnosus* CRL1505 provided significant advantages in the recovery of innate immunity and hematopoiesis [18]. In the present work, we demonstrated that non-viable *L. rhamnosus* CRL1505 was also effective for improving systemic and respiratory innate immune responses during recovery of immunocompromised-malnourished mice.

Few studies have demonstrated the ability of nasally administered non-viable immunobiotics to improve resistance against respiratory pathogens [9,19,20]. In this regard, earlier studies by Hori et al. [19] showed that the nasal administration of heat-killed *L. casei* Shirota stimulated cellular immunity in the respiratory tract and significantly increased the resistance of adult BALB/c mice to influenza virus infection. Later it was reported that intranasal administration of heat-killed *L. pentosus* S-PT84 strongly enhanced Th1 immunity, IFN-α production and NK activity in the respiratory immune system and protected against influenza virus infection [20,21]. In addition, priming of the respiratory mucosa with lactobacilli resulted in full protection from the otherwise lethal severe pneumovirus infection and that protection was observed in response to both live and heat-killed *L. plantarum* and *L. reuteri* [20]. Our laboratory also demonstrated that the administration of both viable and heat-killed *L. rhamnosus* CRL1505 was effective to improve resistance of infant mice to respiratory syncytial virus infection and reduce lung injuries [22]. Those studies were performed with viral infection challenges in immunocompetent mice. To our knowledge, the only study evaluating the effect of non-viable immunobiotics in immunocompromised malnourished hosts was performed by our laboratory. We demonstrated that the nasal treatment of malnourished mice with heat-killed *L. casei* CRL431 was able to increase their resistance against *S. pneumoniae*, although the effect was inferior to the one obtained with viable strain [9]. We speculated that the reduced immunostimulatory effect of non-viable *L. casei* CRL431 was related to the heat-treatment. It is possible that heat-treatment alters molecular structures in the surface of immunobiotics that are recognized by the mucosal immune system, and therefore, non-viable bacteria are less efficiently recognized by pattern recognition receptors (PRRs). It was shown that in addition to protein inactivation, heat-treatment of bacteria disrupt the bacterial cell wall, releasing the cellular contents [23]. On the contrary, it was not possible to differentiate microscopically UV-killed cells from live cells demonstrating that UV-killed cells maintain sufficient cellular integrity before lysis. Moreover, cell membranes of UV-killed cells retained not only the capacity to transport specific molecules acting as a permeability barrier, but also the capacity to maintain the electron transport via protein and lipid carriers and a residual cytoplasmic esterase activity. As stated by the authors, these results were not unexpected because the principal target of UV radiation is DNA [23]. Therefore, in this work we used UV treatment to obtain non-viable immunobiotics. The results from the present study demonstrated that UV-killed *L. rhamnosus* CRL1505 is effective in the immunomodulation of the respiratory innate immune system in malnourished immunocompromised hosts under repletion treatment.

Stimulation of respiratory antigen presenting cells (APCs) such as alveolar macrophages could be involved in the immunoregulatory effect of non-viable *L. rhamnosus* CRL1505. Ex vivo experiments showed that alveolar macrophages coming from CRL1505-treated mice produced higher levels of pro-inflammatory cytokines as well as IL-10. In this

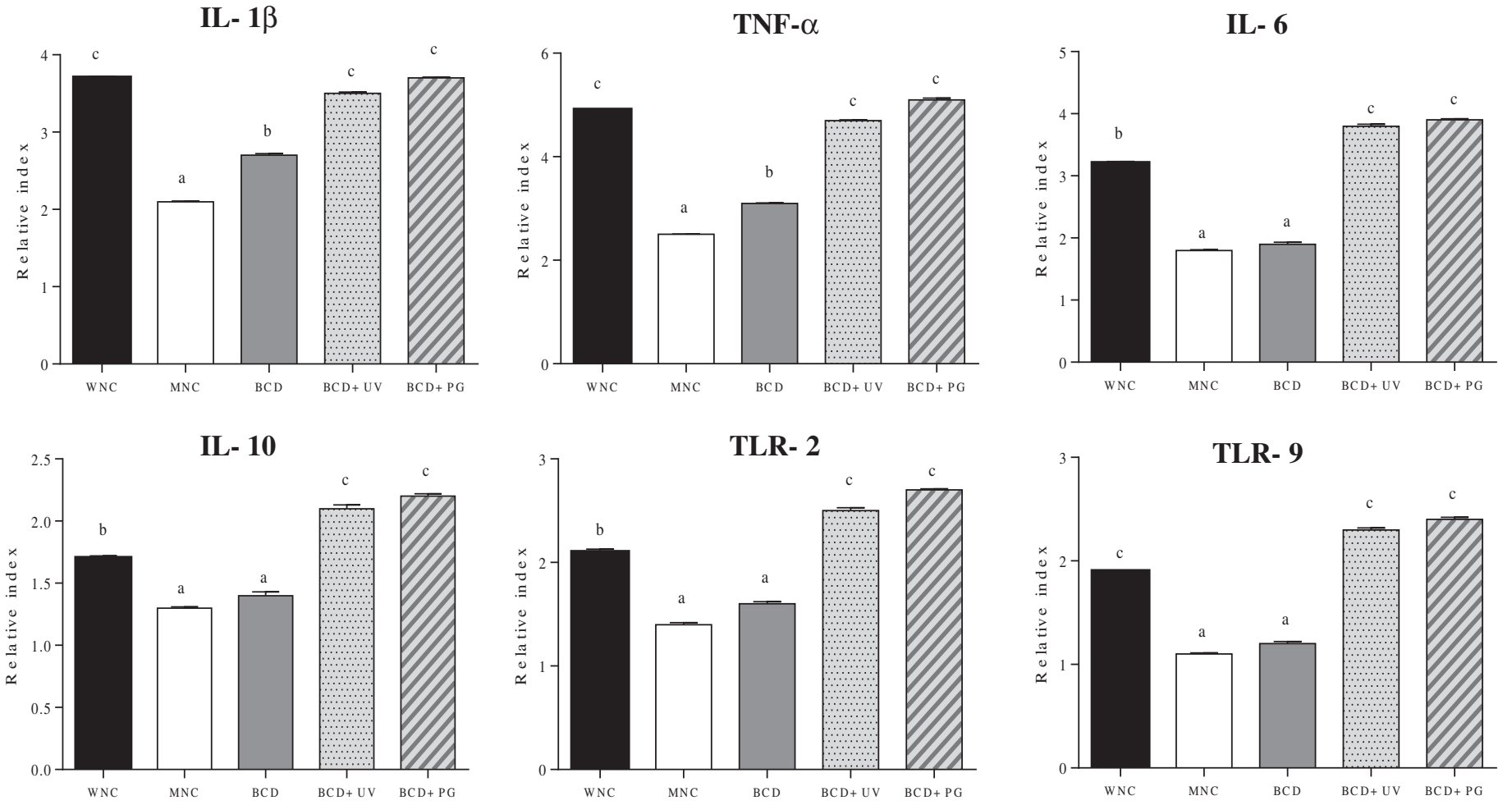

Fig. 8. Effect of *Lactobacillus rhamnosus* CRL1505 on peritoneal macrophages. Malnourished mice were replete for 7 days with a balanced conventional diet (BCD) or BCD supplemented with nasally administered non-viable *L. rhamnosus* CRL1505 (BCD + UV), intact cell wall (BCD + CW) or peptidoglycan (BCD + PG). Malnourished (MNC) and well-nourished (WNC) mice were used as controls. Peritoneal macrophages were removed from the different groups and challenged in vitro with *Streptococcus pneumoniae*. Tumor necrosis factor (TNF)-α, interleukin (IL)-6, IL-1β and IL-10, Toll-like receptor (TLR)-2 and TLR9 mRNA expressions were determined after the challenge with *S. pneumoniae*. The results represent data from three independent experiments. Results are expressed as mean ± SD. Six mice per group per each time point were used. [a,b,c,d] Means in a bar with different letters (a < b < c < d) were significantly different ($P < 0.05$).

regard, our previous studies with adult and infant immunocompetent mice demonstrated that both inflammatory cytokines and regulatory IL-10 are necessary to achieve full protection against respiratory pathogens such as *S. pneumoniae*, influenza virus or respiratory syncytial virus [15,22].

It was reported that treatment of *Lactococcus lactis* with hot acid destroys all the cellular components including intracellular components such as DNA. Cell wall components other than the rigid peptidoglycan matrix are also degraded. The result is a non-living particle of the same shape and size as the bacterium that is called bacterium like-particles (BLPs), that consist predominantly of a peptidoglycan surface, that are able to improve respiratory immunity when nasally administered [25–28]. It was also demonstrated that BLPs activate TLR2 by using in vitro and in vivo studies in TLR2$^{-/-}$ mice [29]. These studies further provided evidence that the mucosal immunostimulating activity of BLPs depend critically on the presence of peptidoglycan and related molecules that are able to stimulate TLR2 [24]. Similarly, we demonstrated in this work that cell wall and peptidoglycan from *L. rhamnosus* CRL1505, administered during recovery of malnourished mice, efficiently modulate systemic and respiratory innate immune responses and improve resistance against pneumococcal infection.

Several immunoregulatory effects were attributed to probiotics' peptidoglycan. It was described that both intact cells and a cell wall-derived polysaccharide–peptidoglycan complex from *L. casei* Shirota inhibited IL-6 production in lipopolysaccharide (LPS)-stimulated lamina propria mononuclear cells isolated from murine inflammatory bowel disease [30]. Moreover, authors compared the inhibitory effect of several wall-derived polysaccharide–peptidoglycan complexes isolated from other strains of lactobacilli on the production of IL-6 after LPS challenge, and found that polysaccharide–peptidoglycan complex derived from *L. casei* Shirota, but not from the other strains of lactobacilli, inhibited the production of IL-6 [30]. Similarly, by studying the role of peptidoglycan in the strain-specific anti-inflammatory activity of probiotic lactobacilli, Fernandez et al. [31] highlighted that the beneficial effect of peptidoglycan in the TNBS colitis model was correlated with the potency of the strain they derived from. Indeed peptidoglycan derived from a strain that does not protect in the specific TNBS colitis model used (*L. acidophilus* NCFM) lacked the anti-inflammatory activity. Therefore, we speculate that the immunomodulatory activity of *L. rhamnosus* CRL1505 peptidoglycan in malnourished mice can not be extrapolated to peptidoglycans of other lactobacilli strains. However, this hypothesis should be demonstrated in appropriate comparative experiments. In fact, to compare the immunomodulatory activity of peptidoglycans from different lactobacilli (immunobiotics and negative controls) in malnourished mice is an interesting topic for future near research.

Gram positive pathogens contain a rigid peptidoglycan cell wall that is reported to engage TLR2 and TLR6, the transmembrane peptidoglycan-recognition proteins (PGlyRPs), and nucleotide-binding oligomerization domain (NOD) receptors. Peptidoglycan from pathogens that bind these receptors on innate immune cells stimulate the production of pro-inflammatory cytokines including IL-8 and TNF-α. In fact, the peptidoglycans of *Bacillus anthracis* [32] or *Staphylococcus aureus* [33] are virulence factors that have a significant role in inflammation leading to septic shock and organ damage. Peptidoglycan from these pathogens induces high production of TNF-α in monocytes and macrophages. Although activation of inflammation and Th1 immunity by peptidoglycan from pathogenic bacteria serve as protection, unregulated immune responses could be detrimental, especially in immunocompromised host as we have been previously described in our malnutrition mice model [5,9]. In this regard, peptidoglycan from commensal or immunobiotic bacteria could function as immunoregulators for improving resistance against pathogens without the risk of detrimental inflammation in immunocompromised host. Resembling non-viable *L. rhamnosus* CRL1505, the peptidoglycan of this immunobiotic bacterium was also able to improve the production of both pro-inflammatory cytokines and IL-10 in the respiratory tract of malnourished mice. Induction of both types of cytokines by lactobacilli peptidoglycan has been described before. Studies by Sun et al. [34] reported that one dose of *Lactobacillus* peptidoglycan induces a sustained elevation of pro-inflammatory cytokines in peritoneal macrophages. Compared to one dose of peptidoglycan treatment, a greater number of inflammatory genes were upregulated in macrophages after three doses of *Lactobacillus* peptidoglycan. However, anti-inflammatory cytokines, such as TGF-β, were also upregulated during continuous peptidoglycan administration [34]. Similarly to lactobacilli, the response of APCs to the commensal bacteria *Streptococcus gordonii* involves the production of inflammatory and regulatory cytokines [35]. It was described that *S. gordonii* whole cells induce a dose-dependent production of TNF-α, IL-6, IL-12 and IL-10 in APCs. Moreover, studies performed with purified bacterial cell components: peptidoglycan, lipoteichoic acid, lipoproteins and DNA, showed that peptidoglycan increased the production of TNF-α and IL-6, which decreased with higher concentrations of peptidoglycan. Interestingly, peptidoglycan but not lipoteichoic acid or lipoproteins, induced the production of IL-10 even in higher doses [35]. Then, our results indicate that the peptidoglycan of *L. rhamnosus* CRL1505 could be an interesting tool as mucosal immunomodulator for improving protection against respiratory infections. Moreover, well-understood modes of action of *L. rhamnosus* CRL1505 peptidoglycan could allow rational design of safe and protective treatments able to improve respiratory immunity in different types of immunocompromised hosts.

In summary, our findings reveal for the first time that a non-viable immunobiotic bacterium and its peptidoglycan are able to modulate innate lung immune response in immunocompromised malnourished mice under repletion treatment. We demonstrated that non-viable *L. rhamnosus* CRL1505 and its peptidoglycan could be of value to beneficially modulate the balance between inflammatory and regulatory cytokines, improving the clearance of pneumococci and controlling inflammatory tissue damage, allowing normal gas exchange to be maintained in the face of the bacterial attack. It has been shown that cell wall components from immunobiotic lactobacilli can induce the immunoactivation of the intestinal immune system. Bacterial peptidoglycan is detected by several classes of PRRs including PGlyRPs, TLR2, NOD receptors. It was demonstrated that TLR2, NOD1 and NOD2 are able to recognize cell wall components of dietary immunobiotics in the intestine, thereby contributing to immunoregulation in the gut-associated lymphoid tissue [36]. A similar effect could occur in the nasal-associated lymphoid tissue with nasally administered immunobiotics or their cell wall components. Nasal priming of malnourished mice under dietary recovery with non-viable *L. rhamnosus* CRL1505 or its peptidoglycan would activate TLR2, NOD1 or NOD2 expressed in macrophages of the respiratory tract, and this effect would mediate an up-regulation of innate immune responses in both local and systemic compartments, improving the resistance of mice against pneumococcal infection. The immunoregulatory capacities of non-viable *L. rhamnosus* CRL1505 or its peptidoglycan offer interesting opportunities for the future development of immunomodulatory treatments directed to immunocompromised hosts.

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