

Nasally Administered *Lactobacillus rhamnosus* Accelerate the Recovery of Humoral Immunity in B Lymphocyte-Deficient Malnourished Mice^{1–3}

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Abstract

The ability of nasally administered *Lactobacillus rhamnosus* CRL1505 to accelerate the recovery of respiratory B cell-mediated immunity against pneumococcal infection in replete malnourished mice was evaluated. Weaned mice were malnourished after consumption of a protein-free diet for 21 d. Malnourished mice were fed a balanced conventional diet (BCD) for 7 d (BCD group) or a BCD for 7 d with supplemental *L. rhamnosus* CRL1505 by the nasal route during the last 2 d (BCD+Lr group). Nonreplete malnourished and normal mice were used as the malnourished (MNC) and the well-nourished (WNC) control groups, respectively. Mice were challenged with *Streptococcus pneumoniae* at the end of each dietary treatment. The immune response was studied before the challenge and at different times postinfection. The MNC mice had less resistance to pneumococcal infection, fewer mature and immature B cells in lung and spleen, and a reduced production of specific antibodies compared with WNC mice. The BCD treatment did not induce a complete normalization of the number B cell populations and antibody amounts. However, the BCD+Lr group had normal numbers of spleen and lung B cells. Moreover, the BCD+Lr mice had a significantly lower susceptibility to *S. pneumoniae* infection and higher amounts of anti-pneumococcal antibodies. Although further studies are necessary to clarify the effect of malnutrition and nasally administered lactobacilli in other immune cell populations involved in the protection against respiratory pathogens, this work gives evidence of the importance of using nasal priming with probiotics to accelerate the recovery of respiratory immunity in immunocompromised malnourished hosts. *J. Nutr.* 143: 227–235, 2013.

Introduction

The respiratory pathogen *Streptococcus pneumoniae* is responsible for most cases of meningitis and pneumonia in young children and of otitis media in infants (1). An increased frequency and severity of infections by *S. pneumoniae* and other encapsulated bacteria (*Neisseria meningitidis*, *Haemophilus influenzae*) is the first and most important symptom of primary B cell immunodeficiency and a sign of AIDS progression in HIV-infected children (2). In addition, pneumococcal diseases are 20–100 times more frequent in individuals with asplenia, splenectomy, and sickle-cell disease (3). Moreover, despite appropriate therapies, mortality due to the different pneumococcal pathologies remains high in immunocompromised malnourished children; ~1 million children die every year from pneumococcal diseases, mainly in developing countries (4,5).

Malnutrition suppresses immune function and confers a higher susceptibility to infectious diseases. Indeed, nutritional deprivation induces atrophy of lymphoid tissues such as spleen and thymus and decreases the number of circulating T and B cells (6). In this sense, we recently reported that protein malnutrition induces a significant reduction in bone marrow (BM)⁶ cell compartments, which is reflected in a decrease of B cells (7). Moreover, when we investigated the effect of nutritional deprivation on B cell populations in BM, we observed that the number of B220⁺ cells (the whole B cell compartment) was reduced in the BM of malnourished mice (7). In parallel with the total B cell decrease, the proportion of the different B cell subsets was markedly altered in malnourished mice. We observed that pro-B/pre-B (B220^{interm}IgM⁻) and immature B cell (B220^{interm}IgM⁺) numbers were lower in feed-deprived mice. The reduction of immature B cells was accompanied by an increase in the percentage of mature B cells (B220^{high}IgM⁺) but not by

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³ Supplemental Figures 1–6 are available from the "Online Supporting Material" link in the online posting of the article and from the same link in the online table of contents at <http://jn.nutrition.org>.

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⁶ Abbreviations used: BAL, bronchoalveolar lavage; BCD, balanced conventional diet; BCD+Lr, balanced conventional diet plus nasally administered *Lactobacillus rhamnosus* CRL1505; BM, bone marrow; LAB, lactic acid bacteria; MNC, malnourished control; PFD, protein-free diet; p.i., postinfection; WNC, well-nourished control.

changes in the total number of mature B cells. These observations suggest that nutritional deprivation leads to the alteration of B cell development in the BM (7).

During the last few decades, a large body of literature established strong links among nutrition, immune function, and infectious diseases. It was demonstrated that one of most important strategies for the prevention of infectious diseases is to improve healthy nutrition. Lactic acid bacteria (LAB) can be used for this strategy. LAB strains able to modulate the immune system (immunobiotics) (8,9) have been used to improve intestinal and respiratory immunity. In our laboratory, several *Lactobacillus* strains isolated from goat's milk were evaluated according to their capacity to modulate respiratory immunity and we found that *Lactobacillus rhamnosus* CRL1505, administered by the oral route at the proper dose, was able to increase *S. pneumoniae* clearance rates in lung and blood, reduce lung injuries, and increase the survival of infected mice (10). We also demonstrated that the protective effect of the CRL1505 strain can be achieved in immunocompromised malnourished mice and that it was related to an upregulation of both innate and specific immune responses in the respiratory tract (11). To elucidate the immunological mechanisms involved in the increased resistance to pneumococcal infection induced by *L. rhamnosus* CRL1505, we performed studies of B cell populations in BM. We observed that the alteration of B lineage cells in the BM of malnourished mice was reverted by the treatment with *L. rhamnosus* CRL1505 (7). A remarkable finding of our work was that oral administration of *L. rhamnosus* CRL1505 was able to normalize the number of immature B cells (7).

Considering that nasally administered antigens can induce respiratory and systemic immune responses superior to those obtained using oral stimulation (12), researchers more recently focused on the ability of nasal stimulation with immunobiotics to improve respiratory immune responses (13). Some studies demonstrated that nasal administration of LAB is able to improve respiratory immunity and significantly increase the resistance of immunocompetent mice against influenza virus (14), *S. pneumoniae* (15), and lethal pneumovirus infections (16). Moreover, we have evaluated whether the nasal administration of heat-killed immunobiotics during recovery of malnourished mice could improve respiratory immunity. Our results showed for the first time, to our knowledge, that nasal administration of heat-killed *Lactobacillus casei* CRL431 significantly increases the resistance of malnourished mice against respiratory pathogens (17).

The ability of viable LAB strains when nasally administered to immunocompromised mice to stimulate respiratory immunity has not, to our knowledge, been studied before. Thus, the aims of the present work were to deepen the knowledge of the effect of malnutrition on systemic and respiratory B lymphocyte populations and to evaluate the effectiveness of nasal administration of *L. rhamnosus* CRL1505 to enhance B cell-mediated immunity and the humoral immune response to pneumococcal infection in replete, malnourished, immunocompromised mice.

Materials and Methods

Microorganism. *Lactobacillus rhamnosus* CRL1505 was obtained from the CERELA culture collection. Lactobacilli (stored at -70°C) was activated and cultured for 12 h at 37°C (final log phase) in Man-Rogosa-Sharpe broth. The bacteria were harvested by centrifugation and washed with sterile 0.01 mol/L PBS, pH 7.2 (7). Capsulated *Streptococcus pneumoniae* was isolated from the respiratory tract of a patient from the Children's Hospital (Tucuman-Argentina).

Mice and treatment procedures. Male, 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 (18). Malnourished mice were divided into 2 groups for treatments: mice were fed for 7 d with a balanced conventional diet (BCD; BCD group) or BCD for 7 d; during the last 2 d, the mice received *L. rhamnosus* CRL1505 (10^8 cells \cdot mouse $^{-1}$ \cdot d $^{-1}$) by the nasal route (BCD+Lr group) (Fig. 1). The dose of *L. rhamnosus* was chosen on the basis of preliminary experiments (J. Villena, S. Salva and S. Alvarez, unpublished results). A third 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 ad libitum only the BCD during experiments. The compositions of the BCD and PFD diets were previously described (18). Experiments with mice were approved by the CERELA Ethical Committee of Animal Care (protocol BIOT-CRL-10).

Cellular recovery. Following thoracotomy, a right heart catheterization was performed and the pulmonary circulation was perfused with saline-EDTA to remove intravascular cells. Lungs were removed, minced, and incubated in digestion medium for 1 h at 37°C . The digestion medium consisted of RPMI-1640 supplemented with 5% FBS and 140 kU/L collagenase type I (Sigma). Subsequently, the samples were homogenized through a tissue strainer with RPMI 1640 with 5% FBS. Finally, samples were subjected to RBC lysis (Tris-ammonium chloride, BD Pharmingen) washed in FACS buffer (PBS with 2% FBS, Gibco) and passed through a 50- μm cell-strainer.

Spleens were collected and tissue was homogenized through a tissue strainer with RPMI 1640 with 2% FBS, followed by incubation with lysis buffer to eliminate erythrocytes (7). Isolated cells were suspended in FACS buffer, counted on a hemocytometer, and kept on ice until immunofluorescent labeling. Viability of the cells was assessed through Trypan blue exclusion.

Flow cytometry. Spleen or lung cells were preincubated with anti-mouse CD32/CD16 monoclonal antibody (Fc block) and stained with the following antibodies from BD Pharmingen: fluorescein isothiocyanate-labeled anti-mouse IgM, fluorescein isothiocyanate-labeled anti-mouse CD19, PE-labeled anti-mouse CD24, biotinylated anti-mouse B220, and biotinylated anti-mouse IgD antibodies. Following incubation with biotinylated primary mAbs, the labeling was revealed using streptavidin-Peridinin Chlorophyll-a Protein (SAV-PerCp). In all cases, cells were then acquired on a BD FACSCaliburTM flow cytometer (BD Biosciences) and data were analyzed with FlowJo software (TreeStar). The 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.

Pneumococcal infection. Challenge with *S. pneumoniae* was carried out on the day after the end of each treatment (d 8) by dropping 25 μL of the inoculum containing 10^5 log-phase cells of *S. pneumoniae* in PBS into each nostril (17,18). Survival of the infected mice was monitored for 15 d. Bacterial cell counts in lung were performed on d 2, 5, 10, and 15 postinfection (p.i.) as previously described (17,18). Results were expressed as log of CFU/g of lung. In addition, whole-lung samples from control and infected mice were excised and immersed in 4% paraformaldehyde and processed by standard histological techniques (17,18). Samples were stained with hematoxylin-eosin for light microscopy examination.

Serum and broncho-alveolar lavages antibodies. Anti-pneumococcal antibodies (IgA, IgM, and IgG) were determined by ELISA on d 10 p.i. (18). In brief, plates were coated with a 1:100 dilution of heat-killed *S. pneumoniae* overnight at 4°C and blocked with PBS containing 5% nonfat dry milk. Appropriate dilutions of the samples [serum 1:20; bronchoalveolar lavage (BAL) 1:2] were incubated for 1 h at 37°C . Peroxidase conjugated anti-mouse IgG, IgA, or IgM (1:500) (Sigma-Aldrich) was added and incubated for 1 h at 37°C . The reaction was

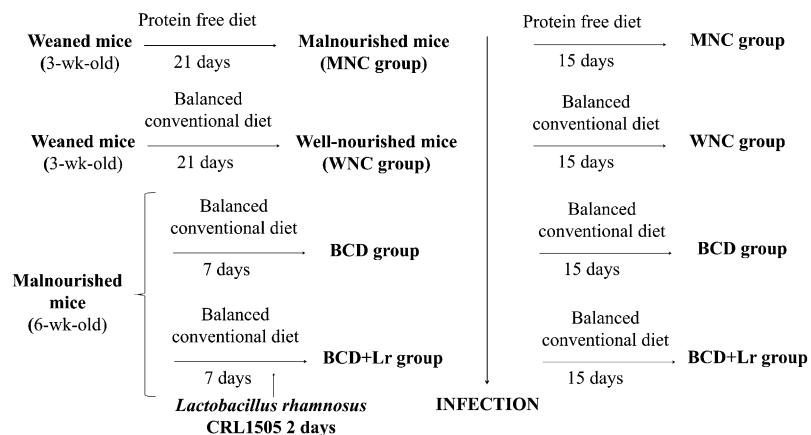


FIGURE 1 Different feeding protocols used in this work. BCD, balanced conventional diet; BCD+Lr, balanced conventional diet plus nasally administered *Lactobacillus rhamnosus*; MNC, malnourished control; WNC, well-nourished control.

developed with TMB Substrate Reagent (Sigma-Aldrich). The concentration was measured with reference to standard curves using known amounts of the respective murine Ig (Sigma-Aldrich).

Functional activity of antibodies. The opsonophagocytic activity of BAL and serum antibodies was determined by measuring the killing of live pneumococci by peritoneal macrophages in the presence of antibodies and complement as previously described (19). For the measurement of IgG, IgA, and IgM antibody avidity the ELISA-NaSCN technic was used (19).

Number and activity of phagocytes. The number of blood and BAL leukocytes was determined by using a hemocytometer as previously described (19). The myeloperoxidase activity of blood and BAL neutrophils was carried out using a cytochemical method with benzidine as a myeloperoxidase chromogen. Cells were graded as negative or positive weak, moderate, or strong and were used to calculate the score (15). The phagocytic bactericidal activity (oxidative burst) of macrophages and neutrophils was measured using the nitroblue tetrazolium reduction test (Sigma) (19).

Statistical analysis. Experiments were performed in duplicate and the results were expressed as mean \pm SD. Statistical analysis was conducted using MINITAB software (version 15 for Windows). Two-factor ANOVA was used to test the effects of experimental group, time (d 0 and d p.i), and their interaction. Tukey's post hoc test was used to test for differences between the mean values. Significance was set at $P < 0.05$. Survival statistics were performed using the log-rank test to compare different Kaplan-Meier curves.

Results

***L. rhamnosus* CRL1505 accelerates the recovery of B cell populations.** Malnutrition significantly reduced spleen weight and cellularity (Table 1). The number of spleen lymphocytes and CD19⁺B220⁺ cells (the whole B cell compartment) in MNC mice were lower than those observed in the WNC group. The BCD group had higher numbers of spleen lymphocytes and CD19⁺B220⁺ cells compared with the MNC mice; however, the amounts of cells did not reach the values of the WNC group. The number of CD19⁺B220⁺ cells was greater in the BCD+Lr group than in the BCD group (Table 1; Supplemental Fig. 1). In addition, B cell subpopulations were studied with CD19, B220, CD24, IgM, and IgD antibodies (Fig. 2; Supplemental Fig. 2). The MNC mice had lower numbers of both mature (CD19⁺B220^{high}CD24^{low}) and immature (CD19⁺B220^{low}CD24^{high}) spleen B cells than the WNC mice. Both BCD and BCD+Lr treatments augmented the number of spleen CD19⁺B220^{high}CD24^{low} B cells, but they were unable to normalize this cell population.

CD19⁺B220^{low}CD24^{high} B cell numbers were higher in both the BCD and BCD+Lr mice compared with the MNC group; however, only BCD+Lr mice had values similar to those observed in the WNC group (Fig. 2A). The spleen is a hematopoietic organ in adult mice (20,21). Immature B cells (IgM⁺B220^{low}) exit the BM and develop and mature into "transitional" T1 B cells (CD21⁻B220⁺CD24^{high}IgD⁻IgM^{high}), T2 B cells (CD21⁺B220⁺CD24^{high}IgD⁺IgM^{high}), and mature (CD21⁺B220⁺CD24^{low}IgD⁺IgM^{low}) B cells within spleen (3,22). A significant reduction of all the B cell populations studied (mature, immature, transitional, and pro/pre-B cells) was observed in MNC mice (Fig. 2B,C). The BCD and BCD+Lr groups had more mature (B220^{high}IgM⁺CD24^{low} and IgD⁺IgM^{low}CD24^{low}) B cells compared with MNC mice. In addition, both the BCD and BCD+Lr groups had more B220^{low}IgM⁺CD24^{high} immature B cells, but this population was normalized only in the BCD+Lr group (Fig. 2B). Moreover, only BCD+Lr mice had similar amounts of transitional T1 B cells (B220⁺IgD⁻IgM^{high}CD24^{high}) to the WNC group (Fig. 2C). The BCD+Lr treatment significantly increased pro/pre-B cells (B220^{low}IgM⁻CD24^{high}) to higher values than those in the WNC group (Fig. 2B). Therefore, the effect of *L. rhamnosus* nasal treatment was more remarkable in the earlier stages of B cell development.

MNC mice had significantly fewer lung lymphocytes and CD19⁺B220⁺ cells compared with the WNC group. Both the BCD and BCD+Lr groups had higher amounts of lung CD19⁺B220⁺ cells than the MNC mice; however, only the BCD+Lr group had values similar to those in the WNC group (Table 1; Supplemental Fig. 1). As expected, the majority of lung B cells displayed the mature phenotype CD19⁺B220^{high}CD24^{low}IgD⁺IgM^{-/+}, whereas few lung B cells were immature CD19⁺B220^{low}CD24^{high}IgM⁺ cells (Fig. 2; Supplemental Fig. 3). The MNC mice had fewer lung mature and immature B cells compared with the WNC mice. The BCD and BCD+Lr mice normalized the number of lung immature B cells (Fig. 2D,E), whereas the number of lung mature B cells was normalized only in the BCD+Lr group (Fig. 2D-F).

***L. rhamnosus* CRL1505 enhances resistance against pneumococcal infection.** After the *S. pneumoniae* challenge, the survival of the MNC mice was 85%, whereas none of the WNC mice died during the experiment. This difference confirms that MNC mice are more susceptible to pneumococcal infection than WNC mice, as we previously demonstrated ($P = 0.02$) (18). The BCD mice had a similar survival to that found in the MNC group (88%) and it differed from the WNC mice ($P = 0.04$). The

TABLE 1 Total cell and lymphocyte numbers in spleen and lung of WNC, MNC, BCD, and BCD+Lr mice¹

Group	Weight	Spleen			Lung		
		Total cell counts	Lymphocytes	CD19 ⁺ B220 ⁺ cells	Total cell counts	Lymphocytes	CD19 ⁺ B220 ⁺ cells
	<i>mg</i>		<i>10⁶ cells/spleen</i>			<i>10⁵ cells/lung</i>	
WNC	142 ± 11.9 ^b	57.4 ± 6.34 ^a	38.4 ± 4.00 ^a	12.2 ± 2.01 ^a	21.9 ± 2.09 ^a	7.65 ± 1.10 ^a	1.59 ± 0.30 ^a
MNC	50.6 ± 10.1 ^c	14.4 ± 2.84 ^b	9.67 ± 1.42 ^c	1.82 ± 0.43 ^d	11.5 ± 1.41 ^c	3.02 ± 0.46 ^c	0.40 ± 0.05 ^c
BCD	201 ± 13.6 ^a	49.0 ± 4.44 ^a	26.2 ± 4.20 ^b	6.71 ± 0.97 ^c	19.1 ± 1.81 ^b	4.94 ± 0.63 ^b	0.71 ± 0.14 ^b
BCD+Lr	184 ± 22.0 ^a	52.3 ± 5.36 ^a	26.7 ± 4.11 ^b	8.80 ± 1.35 ^b	22.8 ± 1.82 ^a	6.85 ± 0.53 ^a	1.46 ± 0.11 ^a

¹ Values are means ± SD, *n* = 6–8. Means in a column without a common letter differ, *P* < 0.05. BCD, balanced conventional diet; BCD+Lr, balanced conventional diet plus nasally administered *Lactobacillus rhamnosus*; MNC, malnourished control mice; WNC, well-nourished control mice.

survival of BCD+Lr mice (91%) did not differ from the WNC (*P* = 0.08) or MNC (*P* = 0.38) mice (Fig. 3A). In addition, the pathogen was detected in lung samples from WNC and MNC mice throughout the 15-d period, but the MNC mice had higher bacterial cell counts. The BCD group had lower lung pathogen counts compared with the MNC group. The BCD+Lr mice had a greater resistance to the pneumococcal challenge compared with the BCD group, as they had lung pathogen counts similar to those in the WNC group (Fig. 3B). As previously described (17,18), histopathological studies have revealed significant lung injury in infected MNC mice. An intense inflammatory response and hemorrhage together with a marked reduction of alveolar spaces was observed in lungs of MNC mice. Lung tissue injuries in infected WNC mice were less severe than those observed in the MNC group. The BCD mice had histological signs intermediate to those of the WNC and MNC groups and the histological characteristics of lungs of BCD+Lr mice were similar to those of

the WNC group for the entire period assayed (Supplemental Fig. 4). Although we found differences between the BCD and BCD+Lr groups in the resistance against pneumococcal infection, no differences were observed in food intake or body weight between the 2 groups (data not shown).

L. rhamnosus CRL1505 enhances humoral immunity. Pneumococcal infection increased the number of spleen lymphocytes and CD19⁺B220⁺ cells in all the experimental groups compared with basal amounts; however, infected MNC mice had fewer lymphocytes and CD19⁺B220⁺ cells than infected WNC mice. Both the BCD and BCD+Lr treatments increased spleen lymphocytes and CD19⁺B220⁺ cells, but only the BCD+Lr group had values that were similar to those in infected WNC mice (Table 2). In addition, the MNC mice had significantly reduced numbers of all spleen B cell subpopulations. The numbers of mature, immature, T1, and T2 B cells were higher in

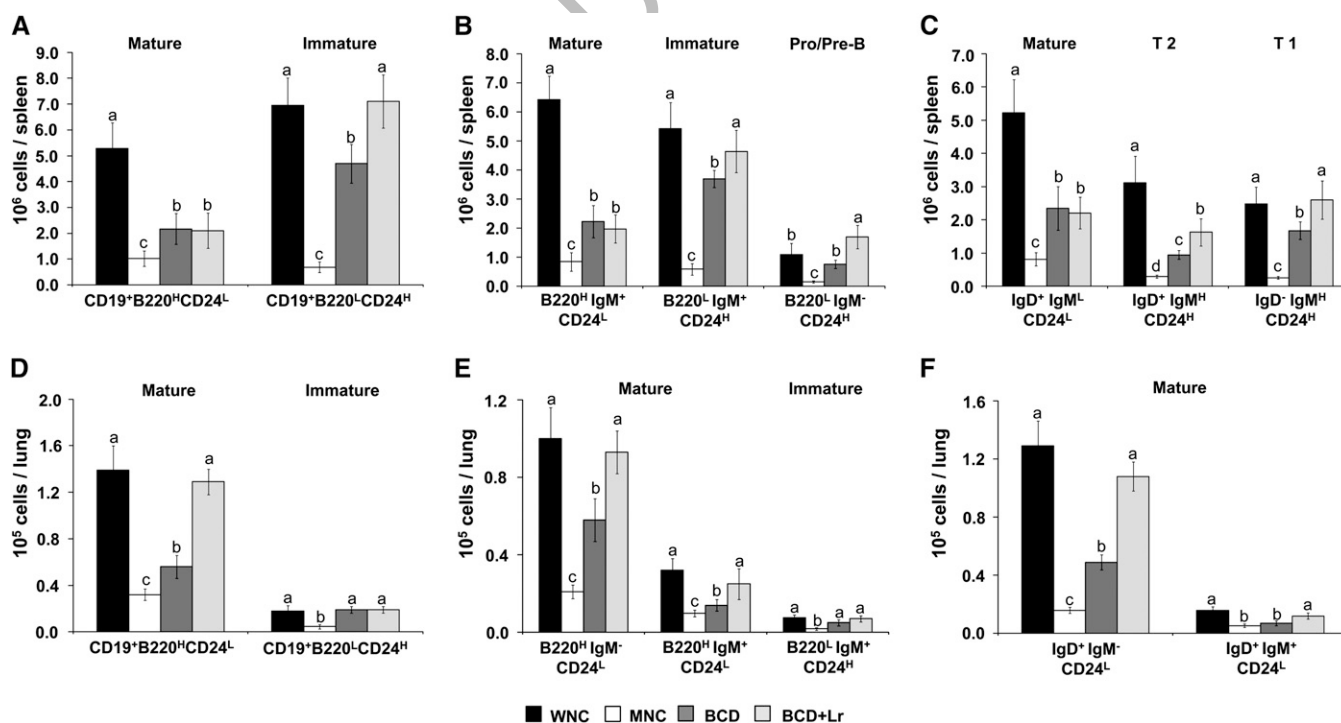


FIGURE 2 Flow cytometry study of B cell populations in spleen (A–C) and lung (D–F) of WNC, MNC, BCD, and BCD+Lr mice. Values are means ± SD, *n* = 6–8. Means without a common letter differ, *P* < 0.05. BCD, balanced conventional diet; BCD+Lr, balanced conventional diet plus nasally administered *Lactobacillus rhamnosus*; MNC, malnourished control; T1, transitional T1 B cell; T2, transitional T2 B cell; WNC, well-nourished control.

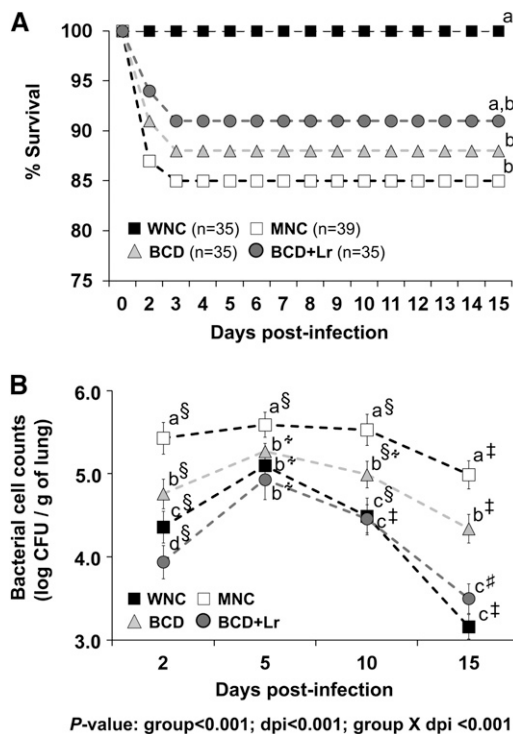


FIGURE 3 Survival (A) and bacterial cell counts on d 2, 5, 10, and 15 after challenge with *Streptococcus pneumoniae* (B) in lungs of WNC, MNC, BCD, and BCD+Lr mice. In A, the numbers of mice on d 0 are given. Percentages on d 15 without a common letter differ, $P < 0.05$. In B, values are means \pm SD, $n = 6$. Means at a time without a common letter differ, $P < 0.05$. Time points for a group without a common symbol differ, $P < 0.05$. BCD, balanced conventional diet; BCD+Lr, balanced conventional diet plus nasally administered *Lactobacillus rhamnosus*; MNC, malnourished control mice; WNC, well-nourished control mice.

BCD mice than in the MNC group; however, BCD administration did not normalize these cell populations. On the contrary, BCD+Lr mice had similar numbers of mature, immature, and transitional B cells to those found in infected WNC mice (Table 2; Supplemental Fig. 5).

Infection reduced the number of lung lymphocytes in all groups without affecting the total number of B cells (Table 3). CD19⁺B220⁺ cells in lungs of infected WNC mice were similar to those in the uninfected WNC group. However, a detailed study of lung B cell subpopulations showed that mature IgM⁻ B cells decreased after infection, whereas mature IgM⁺ B cells increased in WNC mice. The MNC group had fewer mature IgM⁻ and IgM⁺ B cells than the WNC group. The BCD group had a similar number of lung mature IgM⁻ B cells to those in WNC mice; however, there were significantly fewer mature IgM⁺ B cells than in controls. Both lung mature IgM⁻ and IgM⁺ B cells were similar to WNC mice in the BCD+Lr group (Table 3; Supplemental Fig. 6). In addition, pneumococcal infection increased the immature B cells in all the experimental groups; however, MNC mice had fewer of these cells compared with the WNC group. Both BCD and BCD+Lr treatments enhanced the numbers of immature B cells, but only the BCD+Lr group had values similar to those found in WNC mice (Table 3; Supplemental Fig. 6).

Antibody-mediated opsonization is an important host defense mechanism against encapsulated bacteria like *S. pneumoniae* (23). Therefore, we next evaluated the amounts of IgA-, IgM-, and IgG-specific antibodies in the respiratory tract and serum. There were fewer BAL anti-pneumococcal antibodies in the MNC than in the WNC mice. The BCD treatment enhanced IgG, IgA, and IgM antibodies; the amounts of IgG were similar to those in the WNC group and IgA values were located between the amounts of the MNC and WNC groups. BCD+Lr treatment normalized IgG amounts and enhanced IgA values in BAL. In fact, the anti-pneumococcal IgA antibodies in BCD+Lr mice were higher than those in the WNC group (Table 4). MNC mice had less serum IgG and IgM compared with the WNC group. BCD treatment enhanced IgM amounts, which were similar to those in the WNC group. However, BCD treatment was did not increase serum IgG antibodies. The BCD+Lr treatment increased the production of serum IgG, but these anti-pneumococcal antibodies were not as plentiful as in the WNC group. In addition, the amounts of serum IgA in the BCD+Lr mice were significantly higher than those in the WNC group (Table 4). The opsonophagocytic activity of BAL and serum antibodies was also impaired in MNC mice. Both BCD and BCD+Lr treatments

TABLE 2 Total cell and lymphocyte numbers on d 10 after the challenge with *Streptococcus pneumoniae* in spleens of WNC, MNC, BCD, and BCD+Lr mice¹

	WNC	MNC	BCD	BCD+Lr	P value		
					Group	Time	Group \times time
	<i>10⁶ cells/spleen</i>						
Total cell counts	86.8 \pm 11.5 ^{a*}	31.2 \pm 9.72 ^{b*}	81.3 \pm 10.4 ^{a*}	88.6 \pm 13.2 ^{a*}	<0.001	<0.001	0.012
Lymphocytes	55.2 \pm 12.3 ^{a*}	16.5 \pm 4.91 ^{c*}	43.0 \pm 5.70 ^{b*}	49.4 \pm 7.14 ^{a,b*}	<0.001	<0.001	<0.001
CD19 ⁺ B220 ⁺	22.0 \pm 2.70 ^{a*}	5.40 \pm 1.61 ^{c*}	16.3 \pm 2.50 ^{b*}	21.0 \pm 2.20 ^{a*}	<0.001	<0.001	<0.001
CD19 ⁺ B220 ^H CD24 ^L	8.54 \pm 2.21 ^{a*}	3.39 \pm 1.29 ^{c*}	5.69 \pm 0.88 ^{b*}	7.03 \pm 1.00 ^{a*}	<0.001	<0.001	0.007
CD19 ⁺ B220 ^L CD24 ^H	15.4 \pm 2.12 ^{a*}	1.64 \pm 0.71 ^{c*}	11.1 \pm 1.25 ^{b*}	14.9 \pm 2.00 ^{a*}	<0.001	<0.001	<0.001
B220 ^H IgM ⁺ CD24 ^L	8.71 \pm 1.50 ^{a*}	2.56 \pm 0.60 ^{c*}	6.02 \pm 1.00 ^{b*}	8.13 \pm 0.92 ^{a*}	<0.001	<0.001	<0.001
B220 ^L IgM ⁺ CD24 ^H	10.3 \pm 1.80 ^{a*}	0.73 \pm 0.37 ^c	6.86 \pm 0.85 ^{b*}	9.36 \pm 1.13 ^{a*}	<0.001	<0.001	<0.001
B220 ^L IgM ⁻ CD24 ^H	1.52 \pm 0.49 ^b	0.25 \pm 0.11 ^c	4.92 \pm 0.88 ^{b*}	5.16 \pm 1.00 ^{a*}	<0.001	<0.001	<0.001
IgD ⁺ IgM ^L CD24 ^L	7.00 \pm 1.00 ^{a*}	2.60 \pm 0.54 ^{c*}	5.44 \pm 0.59 ^{b*}	6.80 \pm 0.88 ^{a*}	<0.001	<0.001	0.001
IgD ⁺ IgM ^H CD24 ^H	3.50 \pm 0.44 ^{ab}	0.60 \pm 0.05 ^c	2.67 \pm 0.35 ^{b*}	4.15 \pm 0.57 ^{a*}	<0.001	<0.001	<0.001
IgD ⁻ gM ^H CD24 ^H	6.52 \pm 0.76 ^{a*}	0.50 \pm 0.07 ^c	4.70 \pm 0.68 ^{b*}	7.15 \pm 1.13 ^{a*}	<0.001	<0.001	<0.001

¹ Values are means \pm SD, $n = 6-8$. Means in a row without a common letter differ, $P < 0.05$. Asterisks indicate significantly higher values compared with values of d 0 before challenge (shown in Table 1 and Fig. 2), $P < 0.05$. BCD, balanced conventional diet; BCD+Lr, balanced conventional diet plus nasally administered *Lactobacillus rhamnosus*; MNC, malnourished control mice; WNC, well-nourished control mice.

TABLE 3 Total cell and lymphocyte numbers on d 10 after the challenge with *Streptococcus pneumoniae* in lungs of WNC, MNC, BCD, and BCD+Lr mice¹

	WNC	MNC	BCD	BCD+Lr	P value		
					Group	Time	Group x time
	<i>10⁵ cells/lung</i>						
Total cell counts	23.7 ± 2.58 ^{a*}	10.7 ± 1.26 ^b	22.2 ± 2.70 ^{a*}	21.2 ± 1.79 ^a	<0.001	0.046	<0.001
Lymphocytes	6.62 ± 0.64 ^{a†}	1.90 ± 0.18 ^{d†}	3.70 ± 0.39 ^{c†}	4.80 ± 0.61 ^{b†}	<0.001	<0.001	0.06
CD19 ⁺ B220 ⁺	1.61 ± 0.21 ^a	0.30 ± 0.06 ^c	0.95 ± 0.15 ^b	1.40 ± 0.20 ^a	<0.001	0.28	0.007
CD19 ⁺ B220 ^H CD24 ^L	1.11 ± 0.13 ^{a†}	0.27 ± 0.04 ^c	0.70 ± 0.11 ^b	0.93 ± 0.11 ^{a†}	<0.001	<0.001	<0.001
CD19 ⁺ B220 ^L CD24 ^H	0.48 ± 0.10 ^{a*}	0.03 ± 0.01 ^c	0.26 ± 0.07 ^{b*}	0.50 ± 0.09 ^{a*}	<0.001	<0.001	<0.001
B220 ^H IgM ⁻ CD24 ^H	0.40 ± 0.06 ^{a†}	0.16 ± 0.04 ^b	0.34 ± 0.06 ^{a†}	0.38 ± 0.06 ^{a†}	<0.001	<0.001	<0.001
B220 ^H IgM ⁺ CD24 ^L	0.55 ± 0.09 ^{a*}	0.10 ± 0.02 ^c	0.34 ± 0.04 ^b	0.44 ± 0.09 ^{ab*}	<0.001	<0.001	0.017
B220 ^L IgM ⁺ CD24 ^H	0.40 ± 0.06 ^{a*}	0.04 ± 0.01 ^c	0.22 ± 0.05 ^{b*}	0.34 ± 0.06 ^{a*}	<0.001	<0.001	<0.001
IgD ⁺ IgM ⁻ CD24 ^L	0.50 ± 0.09 ^{a†}	0.15 ± 0.02 ^b	0.34 ± 0.09 ^{a†}	0.49 ± 0.08 ^{a†}	<0.001	<0.001	<0.001
IgD ⁺ IgM ⁺ CD24 ^L	0.44 ± 0.05 ^{a*}	0.09 ± 0.01 ^c	0.23 ± 0.06 ^{b*}	0.37 ± 0.07 ^{a*}	<0.001	<0.001	<0.001

¹ Values are means ± SD, n = 6–8. Means in a row without a common letter differ, P < 0.05. Asterisks and crosses indicate significantly higher or lower values, respectively, compared with values of d 0 before challenge (shown in Table 1 and Fig. 2), P < 0.05. BCD, balanced conventional diet; BCD+Lr, balanced conventional diet plus nasally administered *Lactobacillus rhamnosus*; MNC, malnourished control mice; WNC, well-nourished control mice.

enhanced the opsonophagocytic activity of BAL and serum antibodies; however, only the BCD+Lr group had similar values to those in WNC mice (Table 4). The avidity of anti-pneumococcal IgG, IgA, and IgM in serum and BAL was also evaluated. In all the experimental groups, antibody avidity was <60% and the groups did not differ (data not shown).

L. rhamnosus CRL1505 increases number and functionality of phagocytic cells. The number and activity of blood and BAL phagocytes were enhanced on d 2 p.i in all the experimental groups compared with basal amounts (Fig. 4). The MNC mice had significantly fewer and less activity of phagocytes than the WNC group. The number of leukocytes was enhanced in the BCD group (Fig. 4A,B); however, this treatment did not increase the phagocytes' activity (Fig. 4C,D). On the contrary, the BCD+Lr mice had more phagocytes with greater activity compared with the WNC mice (Fig. 4).

Discussion

In adults, B cells are generated in the BM and migrate to the periphery at the transitional B cell stage, when they are still short lived and functionally immature. Transitional B cells are transported to the spleen, where they develop into mature B cells that recirculate among the lymph nodes. Mature B cells

play a major role in the adaptive immune response when they produce antibodies after they have been stimulated, expanded, and selected in the germinal centers in the presence of T cell help (3). It was reported that the impairment of humoral immune response in malnourished hosts relates to the number and activity of both B and T cells (24). Lymphoid atrophy, evidenced by the reduced size and cellularity of the thymus and secondary lymphoid organs, significantly contributes to the alteration of humoral and cellular immunity in malnourished individuals. Along these lines, we reported a reduction of blood lymphocytes and BM lymphoid lineage cells in our malnutrition model (25). Moreover, we demonstrated that protein malnutrition impairs the B cell population in BM without affecting their capacity to produce Igs (7). In this study, we extend these findings by demonstrating that malnutrition decreases mature and immature B cells in spleen and lung.

Antibody responses are activated in the respiratory tract after exposure to pathogens. The concentration and type of respiratory antibodies is dependent on the site of exposure. Upper airway exposure results in IgA production, which is able to protect the host against the colonization of pathogens (26). Mucosal IgA antibody production is impaired in malnourished hosts (27,28). Malnutrition remarkably reduces the number of IgA⁺ cells associated with the lamina propria of the small intestine (28) and has the same effect on the airway mucosa,

TABLE 4 Amounts and opsonophagocytic activity of anti-pneumococcal antibodies on d 10 after the challenge with *Streptococcus pneumoniae* in BAL and serum of WNC, MNC, BCD, and BCD+Lr mice¹

Group	BAL			Serum			Opsonophagocytic activity	
	IgG	IgA	IgM	IgG	IgA	IgM	BAL	Serum
	<i>mg/L</i>						<i>titer</i>	
WNC	1.74 ± 0.19 ^a	2.66 ± 0.20 ^b	3.24 ± 0.43 ^a	25.7 ± 3.90 ^a	10.9 ± 2.31 ^b	65.7 ± 3.58 ^a	35.0 ± 8.37 ^a	140 ± 30.0 ^a
MNC	1.18 ± 0.18 ^b	1.94 ± 0.30 ^c	1.78 ± 0.31 ^b	13.5 ± 3.41 ^c	9.39 ± 3.52 ^b	61.1 ± 4.89 ^b	16.7 ± 5.16 ^b	36.0 ± 8.94 ^c
BCD	1.53 ± 0.26 ^a	2.45 ± 0.34 ^{ab}	2.62 ± 0.42 ^a	16.1 ± 3.01 ^c	12.9 ± 4.82 ^b	66.2 ± 3.19 ^a	35.0 ± 5.48 ^a	60.0 ± 16.0 ^b
BCD+Lr	1.57 ± 0.24 ^a	3.16 ± 0.40 ^a	1.67 ± 0.39 ^b	21.1 ± 3.30 ^b	18.7 ± 4.70 ^a	58.7 ± 2.01 ^b	35.0 ± 5.48 ^a	100 ± 30.0 ^{ab}

¹ Values are means ± SD, n = 6–8. Means in a column without a common letter differ (P < 0.05). BAL, bronchoalveolar lavage; BCD, balanced conventional diet; BCD+Lr, balanced conventional diet plus nasally administered *Lactobacillus rhamnosus*; MNC, malnourished control mice; WNC, well-nourished control mice.

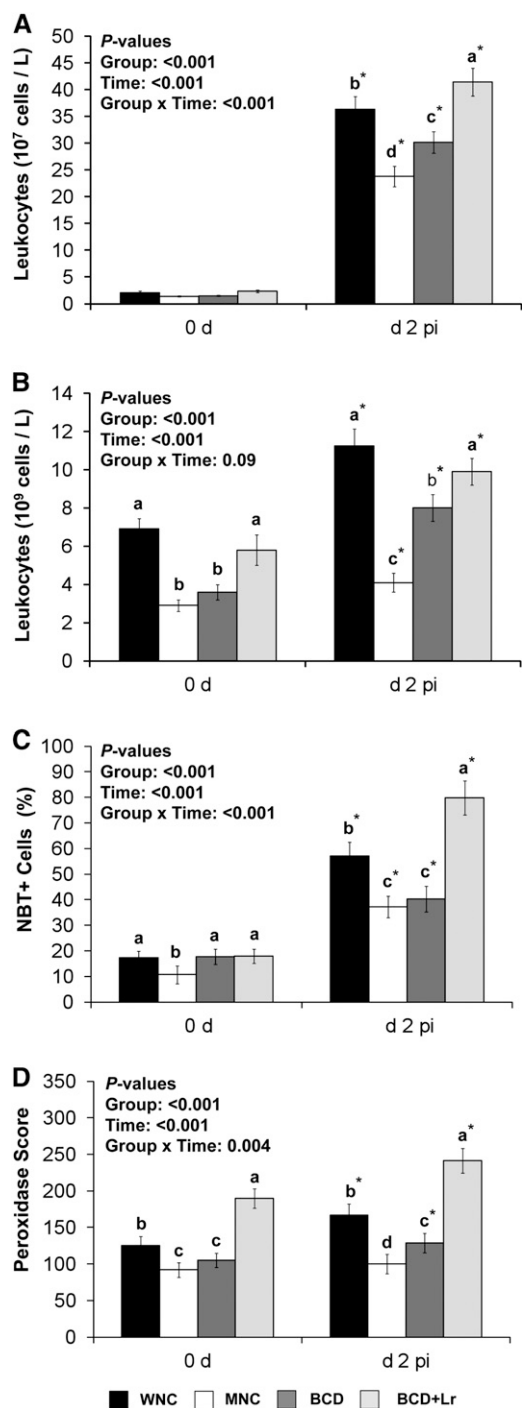


FIGURE 4 Number of BAL (A) and blood leukocytes (B), bactericidal function of BAL phagocytic cells (C) and blood neutrophils peroxidase activity (D) on d 0 (before challenge) and d 2 after the challenge with *Streptococcus pneumoniae* in WNC, MNC, BCD, and BCD+Lr mice. Values are means \pm SD, $n = 6$. Means at a time without a common letter differ, $P < 0.05$. Time points for a group without a common symbol differ, $P < 0.05$. BAL, bronchoalveolar lavage; BCD, balanced conventional diet; BCD+Lr, balanced conventional diet plus nasally administered *Lactobacillus rhamnosus*; MNC, malnourished control mice; NBT, nitroblue tetrazolium; WNC, well-nourished control mice.

because we found fewer IgA⁺ cells in the bronchus-associated lymphoid tissue of MNC mice (29). In the present and previous studies, we observed a significant impairment of the respiratory humoral immune response against *S. pneumoniae* infection in

MNC mice, which was evidenced by the lower amounts of BAL IgA anti-pneumococcal antibodies (18,30). On the other hand, when *S. pneumoniae* reaches the alveolar space in the deep lung, there is a differentiation and expansion of IgG antibody-secreting plasma cells (31,32). These antibodies are important in the protection against pneumococcal infection, because opsonizing IgG antibodies allow complement fixation and enhance macrophage microbicidal activity. Humoral immune activation in the lung also induces the production of antibodies at the systemic level that are responsible for preventing the passage of *S. pneumoniae* into the blood (33). Here, we demonstrated that malnutrition reduced the production of serum and BAL antipneumococcal IgG antibodies. Moreover, the opsonophagocytic activity of IgG antibodies was significantly reduced in MNC mice. These findings indicate that protein malnutrition significantly affects B cell populations in BM, spleen, and lungs, inducing an impairment of the humoral immune response and increasing the susceptibility of malnourished hosts to respiratory pathogens such as *S. pneumoniae*.

Considering the immunomodulatory capacity of certain LAB, our laboratory has proposed their use as supplements in treatments aimed to recover the immunosuppression associated with malnutrition. Our previous studies in immunocompetent mice demonstrated that nasally administered LAB induce systemic and respiratory immune responses superior to those obtained using oral stimulation (13). Therefore, in this work, we evaluated the effect of nasally administered *L. rhamnosus* CRL1505 to improve respiratory defenses during recovery in malnourished mice. The use of nongenetically modified LAB nasally given to stimulate respiratory immunity and prevent lung infections has scarcely been studied. Hori et al. (14) reported that nasal treatment of immunocompetent adult mice with heat-killed *L. casei* Shirota stimulated respiratory tract cellular immunity and increased the resistance against influenza virus infection. Our laboratory studied the effect of *L. lactis* NZ9000 nasal administration to adult mice and showed that this treatment enhanced the resistance against *S. pneumoniae* by upregulating innate and specific immune responses in both respiratory and systemic compartments (15). More recently, it was shown that nasally administered LAB are highly effective at reducing inflammation induced by viral infection and protecting against lethal disease. Specifically, adult mice stimulated via intranasal inoculation with heat-killed or live *L. reuteri* or *L. plantarum* were protected against the infection with the virulent rodent pathogen pneumonia virus. Lactobacilli administration resulted in reduced virus recovery and prolonged survival (16). To our knowledge, no other reports on protection against respiratory pathogens induced by nasally administered LAB have been published. In the 3 studies mentioned above, adult immunocompetent mice were used; therefore, the effect of a nasal treatment with LAB in immunocompromised hosts has been less extensively studied. The first report, to our knowledge, suggesting an improvement of respiratory immunity in immunocompromised hosts induced by nasally administered LAB was developed in our laboratory (17). We demonstrated that the nasal treatment of malnourished mice with heat-killed *L. casei* CRL431 increased their resistance to pneumococcal infection. In the present work, we demonstrated that nasal administration of *L. rhamnosus* CRL1505 induces the recovery of splenic immature B cells and lung mature B lymphocytes. Moreover, malnourished mice treated with *L. rhamnosus* CRL1505 were able to mount a normal immune response against the respiratory infection. In fact, higher amounts of respiratory and serum IgA and IgG were found in the BCD+Lr mice compared with the

MNC and BCD groups. Our results also showed that BCD+Lr treatment had no effect on IgM production. The probable cause for not observing an effect on IgM amounts is that the determinations of antibodies were performed on d 10 p.i. At this time point, the amounts of serum IgG and BAL IgA are more relevant.

In adult mice, 2 distinct types of cells constitute the B cell defense system against infection: B1 and mature B cells. Mature B cells produce high-affinity antibodies in collaboration with T cells and are able to control and clear bacterial and viral infections. On the other hand, B1 B cells represent the first-line defense against infections produced by encapsulated bacteria through their capacity to produce antibodies without T cell help (3). The early recovery of B cell populations would be of great importance for preventing infections in an immunocompromised, malnourished host. Our previous work showed that the oral administration of *L. rhamnosus* CRL1505 for 5 d to malnourished mice induced the recovery of B cells in BM and spleen (7). However, only 2 d of nasal administration of this lactobacillus was enough to achieve the normalization of B cells in spleen. Moreover, *L. rhamnosus* CRL1505 nasal treatment increased the number and functionality of respiratory B cells, which would be of great importance considering the high incidence of respiratory infections in immunocompromised hosts, including those associated to malnutrition states. Some works evaluated the effect of probiotics on the gut-associated lymphoid tissue and the stimulation of the common mucosal immune system to improve immunity against respiratory pathogens and for the recovery of lung immunity in malnourished hosts (7,13). We showed that other important inductive sites for mucosal B cell recovery using immunobiotics are bronchus-associated lymphoid tissue and naso-pharynx-associated lymphoid tissue. According to our results, it is conceivable that the oral route of administration is not optimal for regulating local responses to infection with respiratory pathogens. As such, we and others considered the possibility that targeting live LAB to the respiratory mucosa might result in a more effective immunomodulatory response, similar to the benefits observed when the intestinal mucosa is exposed directly to immunobiotics (15–17).

The improvement of B cells and antibody production induced by *L. rhamnosus* CRL1505 administration could explain the reduction of lung pathogen counts and the inflammatory damage of lung tissue after d 10 p.i. However, differences in survival and lung tissue damage observed between the groups during the early days after pneumococcal challenge would be explained by the effect of *L. rhamnosus* CRL1505 on innate immunity. It is known that both innate and adaptive immune responses are essential to avoid the invasive pneumococcal disease (34). Alveolar macrophages and recruited neutrophils represent the first phagocytic defense in the lungs against pneumococci (35). Our experiments showed that BCD+Lr mice had an improved local and systemic innate immune response evidenced by the greater number and activity of BAL and blood leukocytes even when compared with the WNC group. Although the recovery of innate immunity induced by *L. rhamnosus* CRL1505 is important during the early days of pneumococcal infection, once the pathogen overcomes this barrier, adaptive immunity is key for the resolution of infection. In this sense, the recovery of B cells is important for the protection of the host through the production of specific antibodies and their antiinflammatory capacity.

In conclusion, we demonstrated that malnutrition induces a marked hypoplasia of lymphoid organs and prominent

lymphopenia, which also induces a significant reduction of lymphocytes in other organs such as the lung. The impairment of lymphocyte populations explains at least in part the higher susceptibility to infections found in malnourished hosts. In addition, this study demonstrates that priming of the respiratory mucosa of immunocompromised malnourished mice with *L. rhamnosus* CRL1505 significantly improves the number and activity of phagocytic cells and the number of B cells in spleen and lungs. Moreover, administration of live lactobacilli directly to the respiratory mucosa resulted in a diminished susceptibility to *S. pneumoniae* infection and an improved innate and humoral immune response against the respiratory pathogen. Although further studies are necessary to clarify the effect of malnutrition and immunobiotics in other immune cell populations involved in the protection against respiratory pathogens, this work gives evidence of the importance of using nasal immunobiotics to accelerate the recovery of respiratory immunity of immunocompromised malnourished hosts.

Acknowledgments

N.B., J.V., M.H., S.S., and S.A. conceived and designed the experiments; N.B., M.H., and S.S. performed the experiments; N.B., J.V., and S.S. analyzed the data; N.B., J.V., S.S., and S.A. wrote the paper and had primary responsibility for the final content. All authors read and approved the final manuscript.

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