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Article *in* Beneficial Microbes · May 2017

DOI: 10.3920/BM2016.0152

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Publication information

Beneficial Microbes: ISSN 1876-2883 (paper edition); ISSN 1876-2891 (online edition)

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Beneficial Microbes, 2017; 8(3): 393-405

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Lactobacillus rhamnosus CRL1505 nasal administration improves recovery of T-cell mediated immunity against pneumococcal infection in malnourished mice

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Received: 26 August 2016 / Accepted: 11 December 2016

RESEARCH ARTICLE

Abstract

Immunobiotic lactic acid bacteria have become an interesting alternative for the prevention of respiratory infections. Previously, we demonstrated that the nasal administration of *Lactobacillus rhamnosus* CRL1505, during repletion of malnourished mice, resulted in diminished susceptibility to the challenge with the respiratory pathogen *Streptococcus pneumoniae*. Considering the known alterations induced by malnutrition on T lymphocytes and the importance of this cell population on the protection against respiratory pathogens, we aimed to study the effect of *L. rhamnosus* CRL1505 nasal administration on the recovery of T cell-mediated defences against pneumococcal infection in malnourished mice under nutritional recovery. Malnourished mice received a balanced conventional diet (BCD) for seven days or BCD for seven days with nasal *L. rhamnosus* CRL1505 supplementation during last two days of the treatment. After the treatments mice were infected with *S. pneumoniae*. Flow cytometry studies were carried out in bone marrow, thymus, spleen and lung to study T cells, and Th_1/Th_2 cytokine profiles were determined in broncho-alveolar lavages and serum. The administration of CRL1505 strain to malnourished mice under recovery reduced quantitative and qualitative alterations of $CD4^+$ T cells in the bone marrow, thymus, spleen and lung induced by malnutrition. In addition, CRL1505 treatment augmented Th_2-cytokines (interleukin 10 and 4) in respiratory and systemic compartments after pneumococcal infection. These results show that modulation of $CD4^+$ T lymphocytes induced by *L. rhamnosus* CRL1505 has an important role in the beneficial effect induced by this strain on the recovery of malnourished mice. These data also indicate that nasally administered *L. rhamnosus* CRL1505 may represent a non-invasive alternative to modulate and improve the T cell-mediated immunity against respiratory pathogens in immunocompromised malnourished hosts.

Keywords: lactic acid bacteria, immunobiotics, immunocompromised hosts, T lymphocytes, *Streptococcus pneumoniae*

1. Introduction

The differentiation of T cells is a complex and dynamic process that leads to the production of functionally distinct populations within the thymus: γδ T cell and different αβ T cell subsets including helper CD4 T cells, cytotoxic CD8 T cells, Treg cells and natural killer T (NKT) cells. The bone marrow progenitor cells enter in the thymus as $CD4^-CD8^-$ double-negative (DN) cells and proceed through successive steps of maturation (Kastner *et al.*, 2010). DN thymocytes are further divided into at least four developmental stages based on the differential expression of CD44 and CD25: $CD44^+CD25^-$ (DN1), $CD44^+CD25^+$ (DN2), $CD44^-CD25^+$ (DN3), and $CD44^-CD25^-$ (DN4). The αβ T cell population develop from DN4 thymocytes that further differentiate into $CD4^+CD8^+$ double-positive (DP) $CD3^{-/low}\alpha\beta TCR^{low}$ thymocytes, which are the most abundant cells within the thymus. Positive selection events between the T cell receptor (TCR) expressed by DP cells and major histocompatibility complex molecules expressed by thymic stromal cells lead to the appearance of $CD4^+$ and $CD8^+$ single-positive (SP) $CD3^{high}TCR^{high}$ immunocompetent T cells that leave the thymus to the periphery (Bhandoola *et al.*, 2007; Kastner *et al.*, 2010). It is well known that CD4 T cells play an integral

role in adaptive immune responses. Following activation, naive CD4 T cells differentiate into one of several lineages of T helper cells (Th1, Th2, Th17, or Treg), depending primarily on the antigen, the strength of the TCR signal, and the cytokines present in the surrounding extracellular environment (Fietta and Delsante, 2009).

Malnutrition is globally the most important risk factor for illness and death. Nutritional deficiencies may be one of the most common causes of secondary immunodeficiency in humans (Black *et al.*, 2008; Caulfield *et al.*, 2004). Diverse studies have demonstrated that malnourished individuals frequently possess a great susceptibility to infections with high mortality rates (Barbieri *et al.*, 2013; Pelletier and Frongillo, 2003; Rodríguez *et al.*, 2011; Villena *et al.*, 2005). Malnutrition impairs lympho-haematopoietic organs and alters immune responses (Borelli *et al.*, 2004; Salva *et al.*, 2012). Lymphoid atrophy is a well-recognised consequence of nutritional deprivation as demonstrated in animal models and human trials (Savino, 2002; Savino and Dardenne, 2010). As T cells are a crucial component of the adaptive immune response, several studies have examined the effect of malnutrition on T cell number and function. Mice fed a protein-deficient diet had spleen atrophy and decreased T cell numbers compared to control mice (Gerriets and MacIver, 2014; Taylor *et al.*, 2013). Decreased T cell numbers observed in malnourished mice were also reported in humans. Malnourished children had decreased CD4 and CD8 T cell numbers in blood, compared to well-nourished children (Nájera *et al.*, 2004). Several studies have also demonstrated the impairment of thymic T cell production during malnutrition (Ortiz *et al.*, 2009; Savino, 2002; Savino and Dardenne, 2010).

Up to two-thirds of hospitalised malnourished children are diagnosed with pneumonia; being *Streptococcus pneumoniae* the most important etiologic agent (Rodríguez *et al.*, 2011). Pneumococcal infection can lead to the generation of both T cell and B cell immune responses to polysaccharide and protein antigens (Aslam *et al.*, 2011; Goldblatt *et al.*, 2005). T cells play an important role in the development and maintenance of class switched antibody responses, although T cell independent B cell class switching also occurs. Indeed, anti-pneumococcal protein antibody responses are T cell dependent and T cell responses to both pneumococcal proteins and whole pneumococcus have been demonstrated by measuring cytokine secretion and T cell proliferation (Aslam *et al.*, 2011). In addition to influencing antibody production by B cells, T cells can activate cell mediated immunity via the secretion of interferon (IFN)-γ, interleukin (IL)-17 and IL-22. It is likely that these responses are important in clearing mucosal colonisation in children and maintaining protective immunity in adults (Aslam *et al.*, 2011).

In the last years, the use of lactic acid bacteria (LAB) with immunomodulatory capabilities (immunobiotics) has become an interesting alternative for the prevention of respiratory infections (Cangemi de Gutierrez *et al.*, 2001; Gabryszewski *et al.*, 2011; Kitazawa and Villena, 2014; Racedo *et al.*, 2006). It has been demonstrated that certain immunobiotic LAB strains are able to exert beneficial effect on the host´s respiratory immunity and improve the resistance against bacterial and viral pathogens (Villena *et al.*, 2013). Moreover, immunobiotics can be helpful for the prevention of respiratory infections when used as supplements in treatments aimed to recover malnourished hosts (Barbieri *et al.*, 2013; Herrera *et al.*, 2014). In this regard, we demonstrated that it is possible to accelerate the recovery of mucosal and systemic defences when immunobiotic strains, such as *Lactobacillus rhamnosus* CRL1505, are administered together with the repletion diet. We showed that nasal administration of *L. rhamnosus* CRL1505 was able to protect recovering malnourished mice from respiratory *S. pneumoniae* challenge (Barbieri *et al.*, 2013; Herrera *et al.*, 2014). Moreover, we reported that the treatment of malnourished mice with balanced conventional diet (BCD) supplemented with the CRL1505 strain was able to normalise spleen and lung B cells numbers, increase the levels of broncho-alveolar and serum anti-pneumococcal antibodies, improve respiratory innate immunity and accelerate the recovery of granulopoiesis (Barbieri *et al.*, 2013; Herrera *et al.*, 2014).

In this work we aimed to evaluate the alterations induced by malnutrition on T lymphocytes and, the impact on T cell-mediated immunity against pneumococcal infection. In addition, we studied the effect of *L. rhamnosus* CRL1505 nasal administration on the recovery of T cell populations on bone marrow, thymus, spleen and lung, and its potential beneficial effect during the generation of the immune response against the respiratory pathogen in malnourished mice under nutritional recovery.

2. Materials and methods

Microorganisms

L. rhamnosus CRL1505 was obtained from the CERELA culture collection. Lactobacilli (stored at -70 °C) were activated and cultured for 12 h at 37 °C in Man-Rogosa-Sharpe (MRS; Oxoid, Basingstoke, UK) broth. The bacteria were harvested by centrifugation and washed with sterile 0.01 mol/l phosphate buffered saline (PBS), pH 7.2 (Salva *et al.*, 2012). *S. pneumoniae* serotype 14 (ANLIS, Buenos Aires, Argentina) was obtained from the respiratory tract of a patient from the Children's Hospital, Tucuman, Argentina.

Animals and feeding procedures

Male three weeks old Swiss-albino mice were obtained from CERELA. Weaned mice were malnourished with a protein-free diet (PFD) for 21 days, and the mice that weighed 45-50% less than well-nourished mice were selected for the experiments. Malnourished mice were divided in two groups for treatments: mice were fed for seven days with a BCD (BCD group) or BCD for seven days with nasal *L. rhamnosus* CRL1505 (10^8 cells/mouse/day) supplementation during last two days of the treatment (BCD+Lr group) (Barbieri *et al.*, 2013). A third group of malnourished mice was used as the malnourished control group (MNC). MNC mice received only a PFD during experiments. Normal mice were used as the well-nourished control (WNC) group. WNC mice consumed only the BCD *ad libitum* during experiments. The compositions of the BCD and PDF diets were previously described (Villena *et al.*, 2005). Animal studies and protocols were approved by the CERELA Ethical Committee of Animal Care.

Pneumococcal infection

Challenge with *S. pneumoniae* was carried out on the day after the end of each repletion treatment (day eight) by dropping 25 µl of the inoculum containing 10^5 log-phase cells of *S. pneumoniae* in PBS into each nostril as described previously (Barbieri *et al.*, 2013; Villena *et al.*, 2005).

Cell preparation

Thymuses were collected and mechanically disaggregated. A single-cell suspension from the thymus was obtained by gently passing the collected tissue through a tissue strainer with PBS with 2% foetal bovine serum (FBS) (FACS buffer). Single bone marrow, spleen and lung cells from mice were prepared using the method described previously (Barbieri *et al.*, 2013; Salva *et al.*, 2012). In brief, bone marrow cells were isolated by flushing femurs of mice with RPMI-1640. Spleens were homogenised through a tissue strainer with RPMI 1640 with 2% FBS. Lungs were minced and incubated in digestion medium (RPMI-1640 with 5% FBS and 140 kU/l collagenase type I; Sigma, St. Louis, MO, USA) and then, the samples were homogenised through a tissue strainer with RPMI 1640 with 5% FBS. Finally, samples were subjected to red blood cells lysis (Tris-ammonium chloride, BD PharMingen, Franklin Lakes, NJ, USA) and counted on a haemocytometer. Viability of cells was assessed by trypan blue exclusion.

Flow cytometry

Flow cytometry was performed using a BD FACSCalibur™ flow cytometer (BD Biosciences, San Diego, CA, USA) and data were analysed using FlowJo software (TreeStar, Ashland, OR, USA). Thymus, bone marrow, spleen or lung cells were pre-incubated with anti-mouse CD32/CD16 monoclonal antibody (Fc block) and stained with the following antibodies from BD PharMingen: fluorescein isothiocyanate (FITC)-labelled anti-mouse CD3, FITC-labelled anti-mouse CD25, PE-labelled anti-mouse CD8, biotinylated anti-mouse CD4 and biotinylated anti-mouse TCRαβ antibodies. Streptavidin-PerCP (BD PahrMingen) was used as a second-step reagent. 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.

Cytokine detection by ELISA

Blood samples were obtained through cardiac puncture and broncho-alveolar lavage (BAL) samples were obtained as follows: the trachea was exposed and intubated with a catheter, and two sequential broncho-alveolar lavages were performed in each mouse by injecting PBS; the recovered fluid was centrifuged and the supernatant was kept frozen until use. Cytokine concentrations in BAL and serum were measured by mouse Th1/Th2 ELISA Ready SET Go! Kit (BD Bioscience), including IL-2 and IFN-γ as Th1-type, IL-4 and IL-10 as Th2-type cytokines. The sensitivity of assays for each cytokine was as follows: 4 pg/ml for IL-2 and IFN-γ, and 2 pg/ml for IL-4 and IL-10.

Statistical analysis

Experiments were performed in duplicate and results were expressed as means ± standard deviation. Statistical analysis was conducted using MINITAB software (version 15 for Windows). Two-factor ANOVA was used to test the effects of experimental group, time and their interaction. Tukey's post hoc test was used to test for differences between the mean values. Significance was set at $P<0.05$.

3. Results

Effect of malnutrition and *Lactobacillus rhamnosus* treatment on thymopoietic stages

Thymuses from MNC mice were smaller and contained lower numbers of thymocytes compared with WNC mice (Table 1). BCD and BCD+Lr treatments induced increases of thymus weight together with a significant enhancement of thymocytes numbers (Table 1). Phenotypic subpopulation analysis was performed on thymus cells using flow cytometry. The number of $CD3^-CD4^-CD8^-$ DN thymocytes as well as DN subpopulations ($CD25^+$ or $CD25^-$) in MNC mice was lower than those observed in the WNC group (Table 1). T cells actively undergoing thymopoiesis show moderate CD3 expression while high CD3 expression is a hallmark of mature T cells. Another measure of thymopoiesis is the frequency of $CD4^+CD8^+$

Table 1. Effect of *Lactobacillus rhamnosus* CRL1505 on thymopoietic stages in replete malnourished mice.[1]

		Group			
Thymus		**WNC**	**MNC**	**BCD**	**BCD+Lr**
Weight	10^{-6} kg	91.0±5.8^{a}	20.5±2.0^{c}	63.9±5.2^{b}	67.9±5.3^{b}
Total cell counts	10^{6} cells/thymus	236.6±37.6^{b}	33.3±6.0^{c}	389.3±44.0^{a}	384.4±45.9^{a}
Lymphocytes	10^{6} cells/thymus	196.6±33.2^{b}	27.6±5.0^{c}	318.7±34.9^{a}	315.5±40.7^{a}
DN	10^{6} cells/thymus	3.32±0.84^{a}	0.46±0.21^{c}	2.51±0.48^{b}	2.54±0.46^{b}
CD25^{-} DN	10^{6} cells/thymus	2.19±0.49^{a}	0.17±0.01^{c}	1.44±0.35^{b}	1.22±0.15^{b}
CD25^{+} DN	10^{6} cells/thymus	1.04±0.38^{a}	0.16±0.03^{b}	0.94±0.14^{a}	1.07±0.20^{a}
DP	10^{6} cells/thymus	169.26±36.06^{b}	22.58±6.13^{c}	279.58±34.41^{a}	264.00±38.44^{a}
CD3^{-} DP	10^{6} cells/thymus	147.82±34.61^{b}	21.80±3.69^{c}	251.82±36.90^{a}	224.65±30.88^{a}
CD3^{Low} DP	10^{6} cells/thymus	15.44±3.88^{a}	2.25±0.56^{b}	20.12±5.09^{a}	22.71±3.00^{a}
CD4 SP	10^{6} cells/thymus	26.03±4.58^{b}	3.86±0.88^{c}	25.17±5.34^{b}	35.39±6.68^{a}
CD25^{+} CD4^{+} SP	10^{6} cells/thymus	0.62±0.15^{a}	0.08±0.02^{b}	0.50±0.09^{a}	0.63±0.14^{a}
CD8^{+} SP	10^{6} cells/thymus	6.15±2.39^{a}	0.97±0.37^{c}	4.55±2.36^{a}	5.60±2.23^{a}

[1] Malnourished mice were replete for seven days with a balanced conventional diet (BCD) or BCD supplemented with nasally administered *L. rhamnosus* CRL1505 (BCD+Lr). Malnourished (MNC) and well-nourished (WNC) mice were used as controls. DN = double-negative thymocytes; DP = double-positive thymocyte; SP = simple positive thymocytes. Values are means ± standard deviation, n=6-8. Means in a row without a common superscript letter differ (P<0.05).

DP T cells (Bandera *et al.*, 2010). Compared to WNC mice, MNC group had lower numbers of DP T cells (Table 1). When SP cells were evaluated, we found lower numbers of CD4 SP (CD4^{+}CD8^{-}CD3^{High}), CD25^{+} CD4 SP (putative natural Treg) and CD8 SP (CD8^{+}CD4^{-}CD3^{High}) cells in MNC mice (Table 1).

Both BCD and BCD+Lr treatments augmented the number of DN thymocytes and were able to normalise CD25^{+} DN population (Table 1). The DP T cells of the repleted groups showed higher numbers when compared with the WNC group. In addition, both treatments increased CD8 SP and CD4 SP numbers; however, BCD+Lr group reached values of CD4 SP that were higher than those in the other experimental groups (Table 1).

These data indicate that the repletion treatments induced an active thymopoiesis and, that the effect of *L. rhamnosus* CRL1505 nasal treatment was more remarkable in the CD4 SP population.

Effect of malnutrition and *Lactobacillus rhamnosus* treatment on bone marrow, spleen and lung T cells

Malnutrition induced a significant reduction of bone marrow, spleen and lung lymphocytes numbers and only mice treated with BCD+Lr were able to normalise this parameter in bone marrow and lung (Table 2).

Surface expression of CD3, CD8 and CD4 was examined in bone marrow, spleen and lung lymphocytes of all experimental groups (Figure 1). Protein deprivation increased bone marrow CD3^{+}CD4^{+} and CD3^{+}CD8^{+} cell numbers. Mice treated with BCD and BCD+Lr showed lower numbers of both CD3^{+}CD4^{+} and CD3^{+}CD8^{+} cells than the WNC group. However, the number of CD4 T cells in the group treated with *L. rhamnosus* was higher than those of BCD mice (Figure 1A). In spleen and lung, we observed that CD3^{+}CD4^{+} and CD3^{+}CD8^{+} cell numbers decreased in MNC mice in comparison with WNC mice. The treatment with BCD+Lr, unlike the BCD treatment, was able to induce a significant increase of CD4 T cell numbers in spleen and lung (Figure 1B, C). Both BCD and BCD+Lr treatments were able to normalise the values of CD8 T cells in lung (Figure 1C).

These results demonstrate that the treatment of malnourished mice with *L. rhamnosus* was able to attenuate the impact of malnutrition in the bone marrow CD4 T cells and significantly improved the number of these cells in spleen and lung. Taking into account that nasal administration of *L. rhamnosus* CRL1505 was able to enhance the immune response of recovering malnourished mice against respiratory *S. pneumoniae* challenge (Barbieri *et al.*, 2013; Herrera *et al.*, 2014), we studied the relationship of the influence of *L. rhamnosus* on T cells and the improvement of the immune response against pneumococcus.

Table 2. Effect of *Lactobacillus rhamnosus* CRL1505 supplementation on lymphocytes number in bone marrow, spleen and lungs before and after the challenge with *Streptococcus pneumoniae*.[1]

		Group			
		WNC	MNC	BCD	BCD+Lr
Before the challenge					
Bone marrow					
Total cell counts	10^6 cells	42.1±2.5^a	17.9±2.0^c	32.3±1.9^b	39.1±3.4^a
Lymphocytes	10^6 cells	13.0±1.8^a	3.6±1.5^c	9.0±1.2^b	13.4±2.0^a
Spleen					
Total cell counts	10^6 cells	57.4±6.3^a	14.4±2.8^b	49.0±4.4^a	52.3±5.4^a
Lymphocytes	10^6 cells	38.4±4.0^a	9.7±1.4^c	26.2±4.2^b	26.7±4.1^b
Weight	10^{-6} kg	142.6±12.0^b	50.7±10.1^c	201.1±15.6^a	190.8±19.0^a
Lung					
Total cell counts	10^5 cells	21.9±2.09^a	11.5±1.41^c	19.0±1.81^b	22.8±1.82^a
Lymphocytes	10^5 cells	7.65±1.10^a	3.02±0.46^c	4.94±0.63^b	6.85±0.53^a
After the challenge (day 10 post infection)					
Bone marrow					
Total cell counts	10^6 cells	41.1±4.5^a	20.4±2.9^b	36.8±4.9^a	38.7±4.6^a
Lymphocytes	10^6 cells	10.0±2.2$^{a\#}$	2.8±0.8^b	9.0±2.0^a	9.9±1.2$^{a\#}$
Spleen					
Total cell counts	10^6 cells	86.8±11.5^{a*}	31.2±9.7^{b*}	81.3±10.4^{a*}	88.6±13.2^{a*}
Lymphocytes	10^6 cells	55.2±12.3^{a*}	16.5±4.9^{c*}	43.0±5.7^{b*}	49.4±7.1^{a*}
Lung					
Total cell counts	10^5 cells	23.7±2.5^{a*}	10.7±1.2^b	22.2±2.7^{a*}	21.2±1.8^a
Lymphocytes	10^5 cells	6.6±0.6$^{a\#}$	1.9±0.2$^{d\#}$	3.7±0.4$^{c\#}$	4.8±0.6$^{b\#}$

[1] Malnourished mice were replete for seven days with a balanced conventional diet (BCD) or BCD supplemented with nasally administered *L. rhamnosus* CRL1505 (BCD+Lr). Malnourished (MNC) and well-nourished (WNC) mice were used as controls. Values are means ± standard deviation, n=6-8. Means in a row without a common superscript letter differ (P<0.05).

* Significantly higher than the basal values (before challenge). # Significantly lower than the basal values (P<0.05).

Effect of malnutrition and *Lactobacillus rhamnosus* treatment on T cells after *Streptococcus pneumoniae* challenge

Infected MNC mice showed lower numbers of thymus lymphocyte together with a reduction in the number of all T cell populations with respect to the infected WNC group (Table 3). In the BCD and BCD+Lr groups, infection increased the number of DN thymocytes that reached values similar to WNC mice. In the BCD group, this increase was produced mainly by the $CD25^+$ DN population while in BCD+Lr group the increment was caused by $CD25^-$ DN cells (Table 3). DP thymocytes in BCD and BCD+Lr groups were higher than those in WNC mice. In addition, pneumococcal infection increased the number of CD4 and CD8 SP cells in the thymus of repleted groups. However, the group that received *L. rhamnosus* CRL1505 showed higher numbers of CD4 SP cells than those observed in the other experimental groups (Table 3).

The respiratory infection reduced the number of bone marrow $CD3^+CD4^+$ and $CD3^+CD8^+$ cells in all the experimental groups (Figure 2A). Compared to infected WNC mice, infected MNC group had similar values of bone marrow CD4 and CD8 T cells while in the BCD group the value of these populations were lower than the controls. The group treated with *L. rhamnosus* showed normal numbers of CD4 T cells and values of CD8 T cells higher than BCD mice (Figure 2A).

In spleen, the infection with *S. pneumoniae* increased the total number of lymphocytes as well as $CD3^+CD4^+$ and $CD3^+CD8^+$ cells in all the experimental groups when compared with basal levels (Table 2, Figure 2B). Infected MNC mice showed the lowest values of these spleen populations. Both BCD and BCD+Lr repletion treatments increased spleen CD4 and CD8 T cells, but only the group treated with *L. rhamnosus* CRL1505 was able to normalised the values of CD4 T cells after the challenge (Figure 2B).

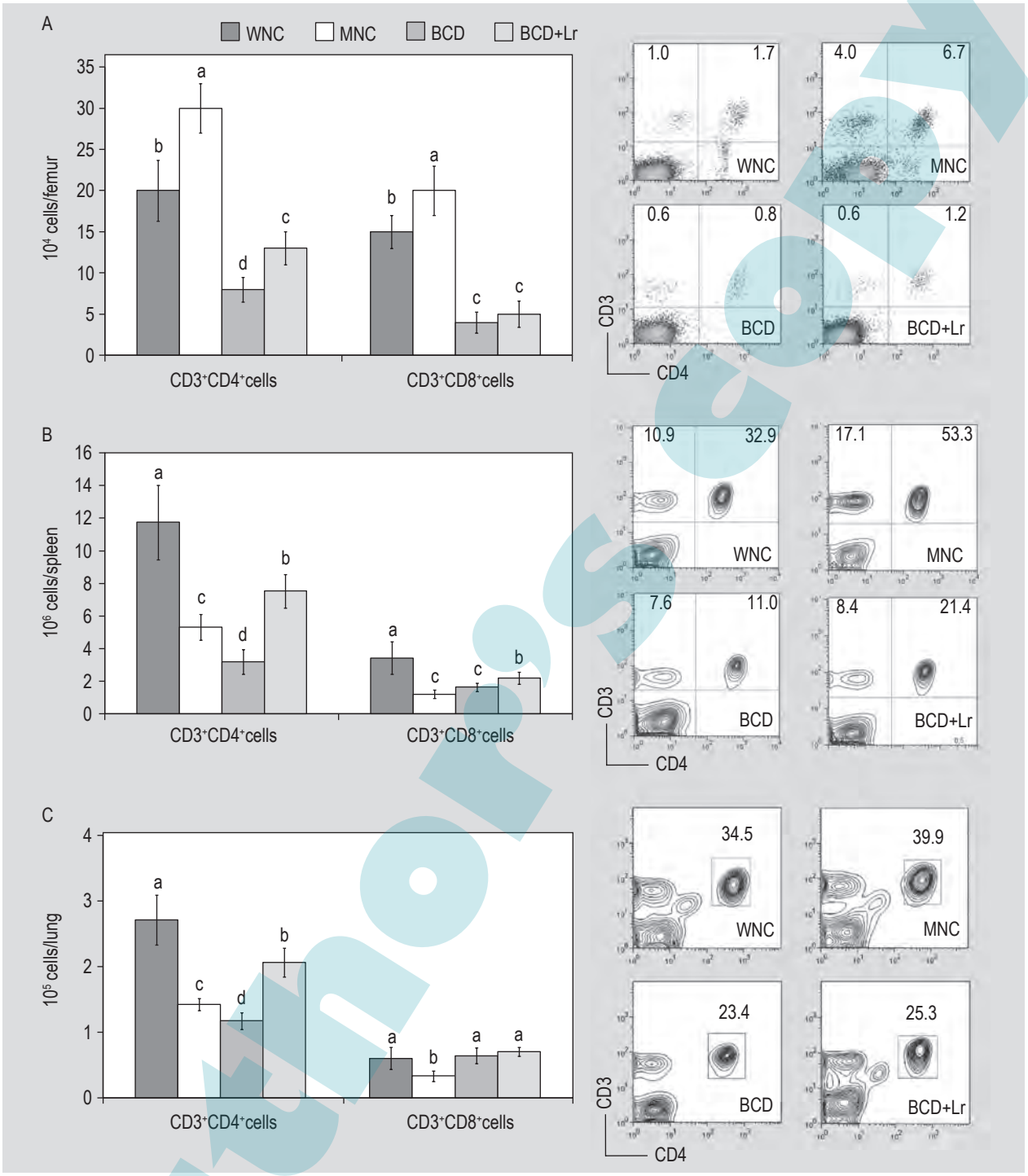

Figure 1. Bone marrow (A), spleen (B) and lung (C) $CD3^+CD4^+$and $CD3^+CD8^+$ T cells. Malnourished mice were replete for seven days with a balanced conventional diet (BCD) or BCD supplemented with nasally administered viable *Lactobacillus rhamnosus* CRL1505 (BCD+Lr). Well-nourished control (WNC) and malnourished control (MNC) mice were used as controls. Values are means ± standard deviation, n=6-8. Means without a common letter (a,b,c,d) differ (P<0.05).

Pneumococcal challenge reduced the number of lung lymphocytes and CD4 T cells in all groups (Table 2, Figure 2C). BCD treatment induced no modifications in the values of lung $CD3^+CD4^+$ or $CD3^+CD8^+$ cells after the infection, which were similar to those of the infected MNC mice (Figure 2C). Interestingly, the diet supplemented with *L. rhamnosus* was able to improve the number of lung CD4 and CD8 T cells reaching values that were higher to those of MNC mice (Figure 2C).

Table 3. Effect of *Lactobacillus rhamnosus* CRL1505 supplementation on thymopoietic stages after the challenge with *Streptococcus pneumoniae*.[1]

		Group			
Thymus		**WNC**	**MNC**	**BCD**	**BCD+Lr**
Total cell counts	10^6 cells/thymus	281.0±45.1^{b}	66.7±8.2^{c}	423.1±52.7^{a}	447.8±55.9^{a*}
Lymphocytes	10^6 cells/thymus	235.6±33.1^{b}	56.8±7.9^{c}	318.7±34.9^{a}	370.8±42.5^{a*}
DN	10^6 cells/thymus	3.49±0.69^{a}	1.32±0.30^{b}	4.12±0.44^{a*}	3.73±0.62^{a*}
CD25$^-$ DN	10^6 cells/thymus	1.72±0.30^{b}	0.73±0.20^{c}	1.89±0.22^{b}	2.48±0.33^{a*}
CD25$^+$ DN	10^6 cells/thymus	1.96±0.32^{a,b*}	0.58±0.10^{c}	2.06±0.26^{a*}	1.47±0.29^{b}
DP	10^6 cells/thymus	196.91±34.81^{b}	46.22±7.28^{c}	299.08±49.76^{a}	299.97±47.28^{a}
CD3$^-$ DP	10^6 cells/thymus	168.60±33.83^{b}	42.54±6.58^{c}	260.65±50.03^{a}	269.95±45.51^{a}
CD3^{Low} DP	10^6 cells/thymus	19.35±3.17^{b}	3.37±0.90^{c}	21.16±6.00^{b}	27.74±5.20^{a*}
CD4 SP	10^6 cells/thymus	25.40±5.79^{c}	6.52±2.25^{d}	43.61±9.78^{b*}	56.31±7.47^{a*}
CD25$^+$ CD4$^+$ SP	10^6 cells/thymus	0.77±0.09^{b}	0.15±0.03^{c}	1.13±0.19^{a*}	1.14±0.13^{a*}
CD8$^+$ SP	10^6 cells/thymus	7.30±2.15^{b}	1.79±0.65^{c}	9.76±2.16^{a*}	9.12±2.61^{a*}

[1] Malnourished mice were replete for seven days with a balanced conventional diet (BCD) or BCD supplemented with nasally administered *L. rhamnosus* CRL1505 (BCD+Lr) and then challenged with *S. pneumoniae*. Malnourished (MNC) and well-nourished (WNC) mice were used as controls. DN = double-negative thymocytes, DP = double-positive thymocytes, SP = simple positive thymocytes. Values are means ± standard deviation, n=6-8. Means in a column without a common superscript letter differ (P<0.05). * Significantly higher than the basal values (before challenge). P<0.05.

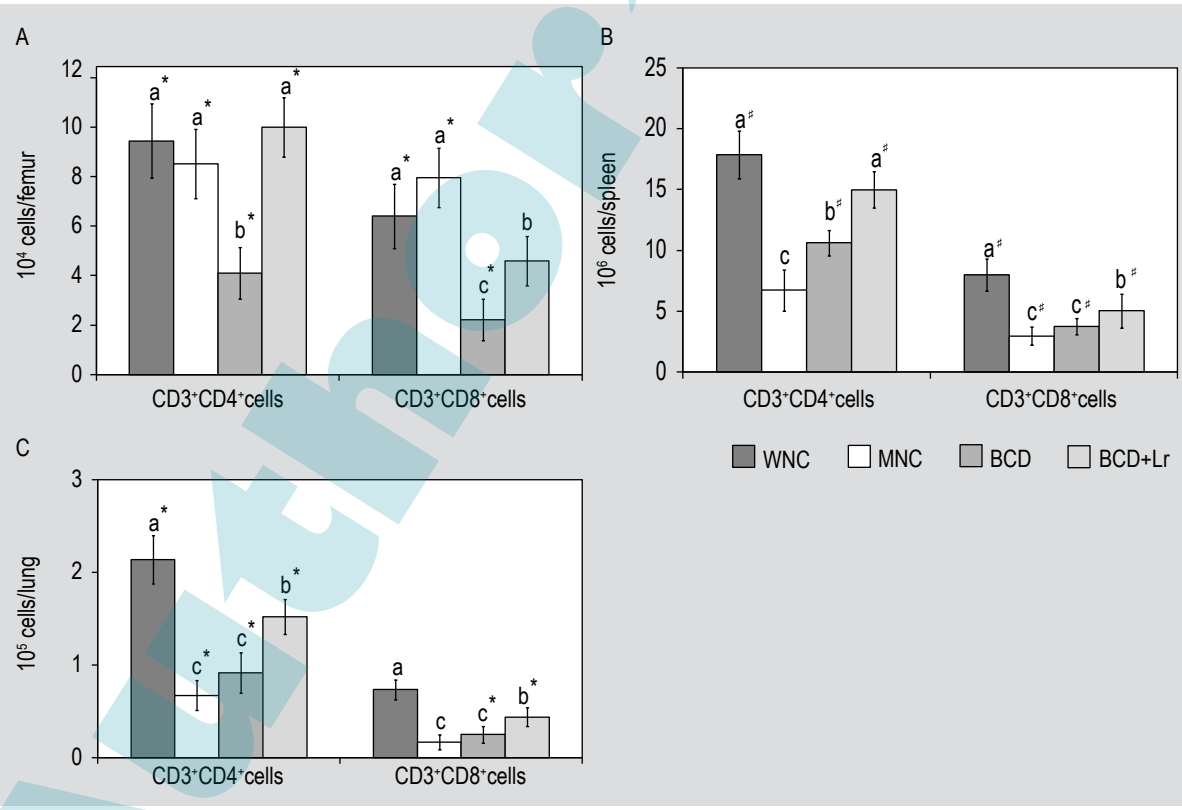

Figure 2. Bone marrow (A), spleen (B) and lung (C) T cells on day ten after the challenge with *Streptococcus pneumoniae*. Malnourished mice were replete for seven days with a balanced conventional diet (BCD) or BCD supplemented with nasally administered viable *Lactobacillus rhamnosus* CRL1505 (BCD+Lr). Well-nourished control (WNC) and malnourished control (MNC) mice were used as controls. Values are means ± standard deviation, n=6-8. Means without a common letter (a,b,c,d) differ (P<0.05). # Significantly higher than the basal values (before the challenge). * Significantly lower than the basal values (P<0.05).

Effect of malnutrition and *Lactobacillus rhamnosus* treatment on cytokine profiles

CD4 T cells produce and secrete a variety of cytokines that control and co-ordinate effector mechanisms involved in pathogen clearance. Therefore, Th1 and Th2 cytokine profiles were studied in pulmonary and systemic compartments before the challenge (basal values) and on day five after pneumococcal infection (Figure 3).

Before the challenge with *S. pneumoniae*, we observed differences between the groups when analysing the levels of BAL IL-10. The group that received *L. rhamnosus* CRL1505 was the only group able to induce a significant increase in the levels of this cytokine (Figure 3D). After the pneumococcal challenge, BAL IL-2 and IFN-γ increased significantly in all the experimental groups. BAL IL-2 production did not differ between the groups while the levels of IFN-γ were lower in BCD and BCD+Lr groups than control groups. In addition, BCD+Lr mice showed levels of IFN-γ that were higher to those of BCD mice (Figure 3C). The infection also increased BAL IL-4 values in all experimental groups. Repletion with BCD was capable of normalising the IL-4 response to infection, while BCD+Lr group significantly improved the production of IL-4, which showed higher levels than those of the WNC group (Figure 3C). We also observed a significant production of IL-10 in the respiratory tract of WNC, BCD and BCD+Lr groups after the infection. On the contrary, MNC mice were not able to increase the production of this cytokine in response to pathogen. BCD+Lr group showed higher BAL IL-10 levels than BCD and WNC groups (Figure 3D).

In serum, the infection with *S. pneumoniae* increased the levels of IL-2 and IFN-γ in all the experimental groups. However, mice in the BCD and BCD+Lr groups showed levels of these cytokines lower to those of the control groups (Figure 4A, B). The concentrations of serum IL-4 increased

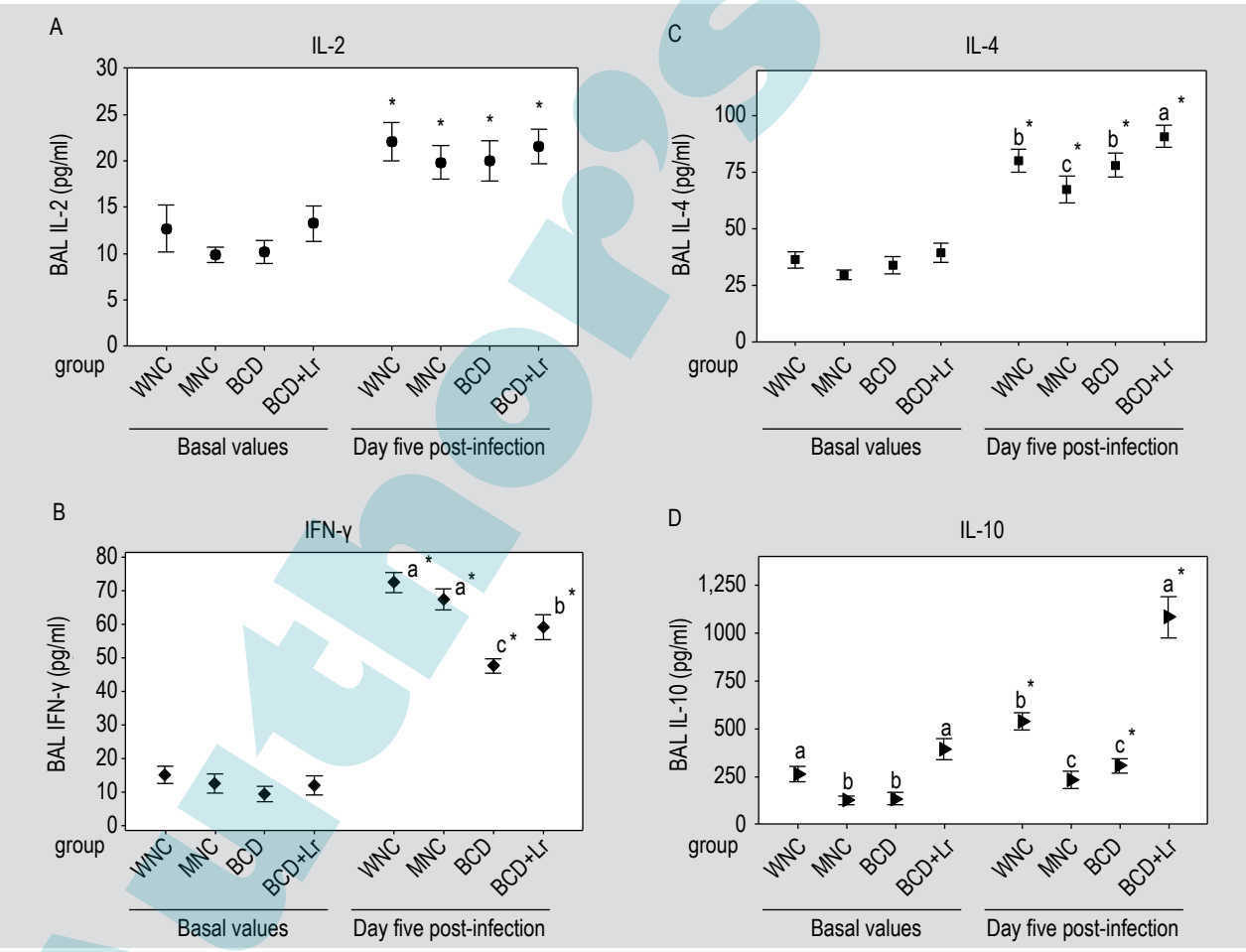

Figure 3. T helper type 1 (Th1) (A, B) and type 2 (Th2) (C, D) cytokines concentration in BAL before (basal values) and after the challenge (day five post-infection) with *Streptococcus pneumoniae*. Malnourished mice were replete for seven days with a balanced conventional diet (BCD) or BCD supplemented with nasally administered viable *Lactobacillus rhamnosus* CRL1505 (BCD+Lr). Well-nourished control (WNC) and malnourished control (MNC) mice were used as controls. Values are means ± standard deviation, n=6-8. Means without a common letter (a,b,c,d) differ ($P<0.05$). * Significantly higher than the basal values ($P<0.05$).

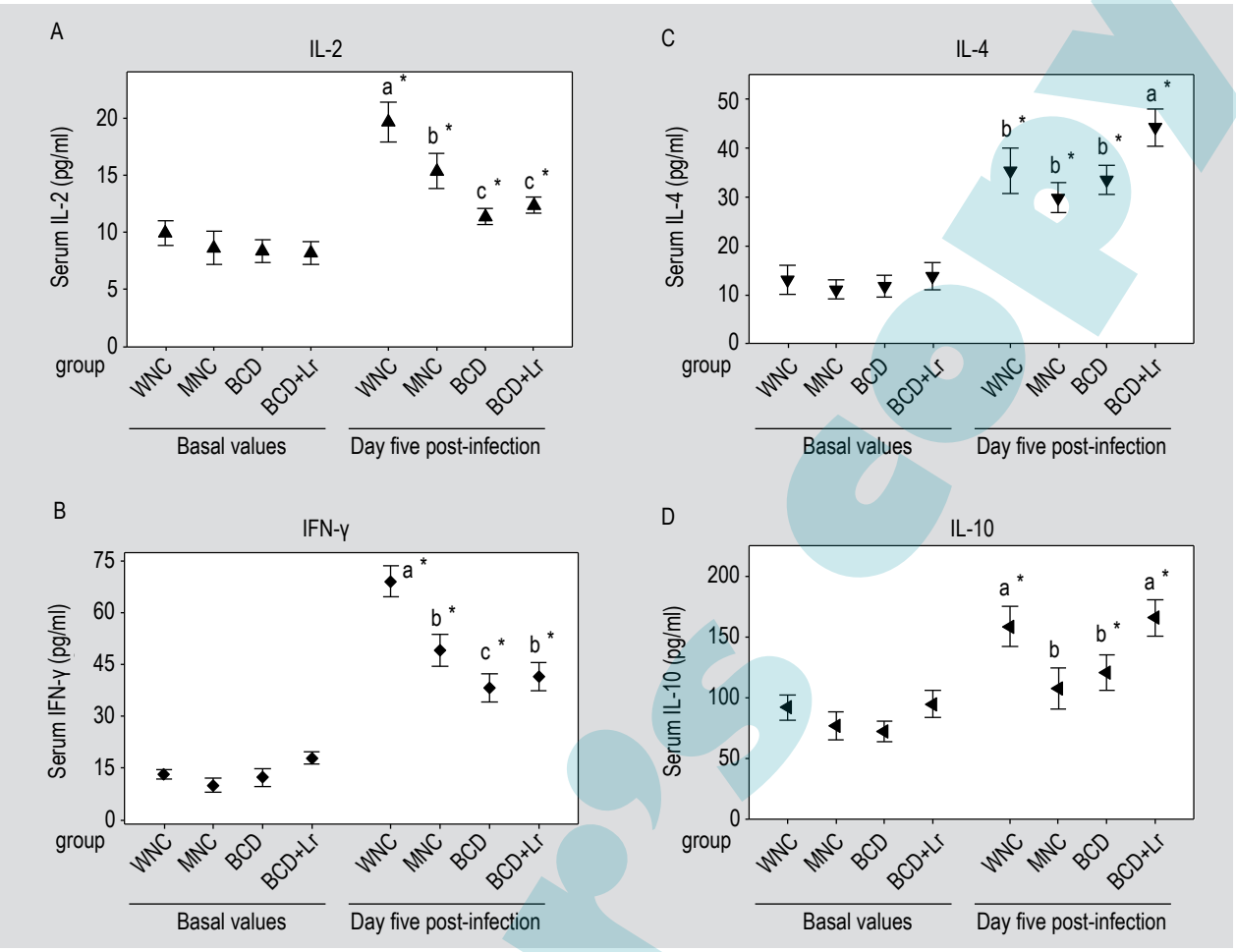

Figure 4. T helper type 1 (Th1) (A,B) and type 2 (Th2) (C,D) cytokines concentration in serum before (basal values) and after the challenge (day five post-infection) with *Streptococcus pneumoniae*. Malnourished mice were replete for seven days with a balanced conventional diet (BCD) or BCD supplemented with nasally administered viable *Lactobacillus rhamnosus* CRL1505 (BCD+Lr). Well-nourished control (WNC) and malnourished control (MNC) mice were used as controls. Values are means ± standard deviation, n=6-8. Means without a common letter (a,b,c,d) differ ($P<0.05$). * Significantly higher than the basal values ($P<0.05$).

in all the experimental groups after the challenge. The levels of serum IL-10 also increased in WNC, BCD and BCD+Lr groups while the MNC group failed to induce modifications in this cytokine after the infection (Figure 4D). Serum IL-4 production in BDC+Lr group was higher than those in the other experimental groups and only this group was able to reach IL-10 values similar to those of the WNC mice after the pneumococcal infection (Figure 4D).

4. Discussion

Previous reports showed that nasally administered specific *Lactobacillus* and *Lactococcus* strains were able to improve the protective immune response against *S. pneumoniae* (Cangemi de Gutierrez *et al.*, 2001; Medina *et al.*, 2008; Villena *et al.*, 2009). In this regard, we demonstrated that the nasal administration of *L. rhamnosus* CRL1505 together with the repletion diet (BCD) was able to increase the clearance rate of *S. pneumoniae* from lung when compared with the BCD group (Barbieri *et al.*, 2013; Herrera *et al.*, 2014). These differences between the groups were explained by the improvement of immune responses mediated by myeloid cells and B lymphocytes in the BCD+Lr group (Barbieri *et al.*, 2013; Herrera *et al.*, 2014). This study extends those previous findings by demonstrating that malnutrition severely affects T population in the bone marrow, thymus, spleen and lung, and that the nasal administration of the CRL1505 strain reduces the quantitative and qualitative alterations of CD4 T cells in malnourished mice under recovery.

The thymus is the primary organ of thymopoiesis and is a key target organ in malnutrition. A severe thymic atrophy was described in malnourished hosts as a result of massive thymocyte apoptosis (particularly affecting the immature DP cell subset) and the decrease in cell proliferation (Savino and Dardenne, 2010). In addition, Ortiz *et al.* (2009) have demonstrated that malnutrition induces an increase of

apoptosis not only in the thymus but also in the spleen. Moreover, it was reported that nutritional deficiencies enhanced apoptosis in peripheral blood lymphocytes from malnourished children (El-Hodhod *et al.*, 2005). Therefore, it seems probable that apoptosis might play a key role in the disruption of lymphopoiesis and the lymphoid tissue atrophy induced by malnutrition. These changes are related to deficiencies in T cell function and the impaired peripheral immune response seen in malnourished individuals (Savino and Dardenne, 2010). In our experimental model, the reduction of lymphocytes in thymus, spleen and bone marrow observed in MNC mice clearly shows that malnutrition induced a loss of lymphoid tissues. Then, our findings agree with previous observations which determined that thymocyte and splenocyte depletion is a common outcome of malnutrition (Ortiz *et al.*, 2009;Savino, 2002; Savino and Dardenne, 2010), which is associated with bone marrow atrophy (Borelli *et al.*, 2007; Salva *et al.*, 2012).

As mentioned before, active thymopoiesis is characterised by high CD3 expression and increased frequency of DP T cells (Bandera *et al.*, 2010). Our results showed active thymopoiesis in BCD and BCD+Lr mice, with numbers of DP T cells even higher than those of WNC mice. Although both repletion treatments increased mature T cells, *L. rhamnosus* nasal treatment showed a more remarkable effect on CD4 SP population. In addition, only the treatment with BCD plus *L. rhamnosus* significantly increased the number of $CD3^+CD4^+$ cells in spleen and lung of malnourished mice. These results indicate that lactobacilli administration would induce an accelerated rate of CD4 SP T cells development in the thymus and their migration to the peripheral tissues.

Considering that T cell mediated immune responses are important in the defence against *S. pneumoniae*, we performed quantitative and qualitative studies of the different T cells populations of thymus, bone marrow, spleen and lung after the pneumococcal infection.

In the thymus, both BCD and BCD+Lr mice showed increased numbers of DN T cells after infection when compared with the WNC group. In the BCD group the increase was achieved at expense of the $CD25^+$ DN population, which include DN2 and DN3 stages. On the other hand, the increase in BCD+Lr mice was achieved at expense of $CD25^-$ DN cells, which include DN1 and DN4 stages of maturation. Further studies are needed to explain the significance of the differences found between both repletion treatments. However, considering that we previously demonstrated that *L. rhamnosus* CRL1505 influences the recovery of the haematopoiesis affected in malnourished hosts (Salva *et al.*, 2012) and, that previous works reported the increase of DN1 population involved T progenitors coming from bone marrow (Bhandoola *et al.*, 2007; Kastner *et al.*, 2010); we can speculate that the increase of thymic $CD25^-$ DN cells in *L. rhamnosus* treated mice, could be at least in part, explained by a greater influx of T precursor from bone marrow to the thymus (DN1).

An increase in pre-DP or DN4 population represents an increase of thymocytes that have successfully completed the rearrangement of the TCR β genes that give rise to the DP cells. In this study, we observed that in the repleted groups, the number of DP cells after infection remained higher than the WNC group, indicating that there was still an active thymopoiesis. However, the group of mice treated with lactobacilli presented values of $CD3^{low}$ DP cells that were higher than the other groups, which is probably related to the increase of CD4 SP cells, observed in this group. In addition to promoting the development of CD4 T cells in the thymus after the pneumococcal challenge, the treatment with *L. rhamnosus* managed to normalise the spleen and bone marrow $CD3^+CD4^+$ population, and maintain the values of lung $CD3^+CD4^+$cells higher than the BCD group. This effect of *L. rhamnosus* CRL1505 could be a key factor for the protection against *S. pneumoniae* infection since it was described the importance of the CD4 T cells present at the time of challenge for the immunity to pneumococcal colonisation (Malley *et al.*, 2005).

Mucosal Th2 cells are not frequently found in the respiratory tract probably due to the limited IL-4 production in the normal mucosa (Hooper and Macpherson, 2010). However, under inflammatory conditions, cytokines in the airways environment change dramatically. When a Th2 response is needed, there is a production of IL-4, IL-5, IL-6 and IL-10, which contributes to stimulate B cells to proliferate and mature into antibody producing cells (Kiyono and Fukuyama, 2004). Th2 cells can help B cells with IgG, IgM, and IgA production. Particularly, they are essential in determining B cell antibody class switching (Fietta and Delsante, 2009; Shimosato *et al.*, 2011). In our experimental model of malnutrition, the low levels of IL-4 could be related to an impaired Th2 response which would explain the low concentration of anti-pneumococcal antibodies found in MNC mice in previous works (Barbieri *et al.*, 2013; Villena *et al.*, 2005). In the current study, we found a significant up-regulation in BAL and serum Th2-cytokine IL-4 in malnourished mice fed with BCD plus *L. rhamnosus* CRL1505 after pneumococcal infection, unlike BCD treatment. Moreover, we have previously reported that BCD+Lr treatment enhances BAL IgA and serum IgG anti-pneumococcal production (Barbieri *et al.*, 2013). In this sense, the effect of *L. rhamnosus* on the humoral immune response is coincident with the capacity of this strain to induce Th2-cytokines. These results are in line with previous reports of Shimosato *et al.* (2011) demonstrating the ability of *L. pentosus* ONRICb0240 to improve IgA production and the recovery of the intestinal immune system in protein-energy malnurished mice by augmentation of Th2 cytokines.

IL-10 plays a key role in the modulation of the immune response induced after pneumococcal infection, limiting the inflammatory damage and stimulating antibody production. In this sense, Zhang *et al.* (2006) demonstrated the regulatory role of IL-10 in B cell antibody responses against pneumococcal proteins. Authors suggested that IL-10 and IFN-γ are key regulators of mucosal anti-pneumococcal protein antibody production and are important in local protection against pneumococci. On the other hand, in a previous report, we showed that oral administration of *Lactobacillus casei* beneficially regulates the balance between TNF-α and IL-10, allowing a more effective immune response against infection and modulating the inflammatory response, with less damage to the lung (Racedo *et al.*, 2006). Here we showed that *L. rhamnosus* CRL1505 treatment induced the greatest increase of IL-10 in BAL after pneumococcal infection, which could be critical to the control of inflammation and tissue damage, improving the protection against infection in this group of mice. It was recently described the importance of IL-10 for the protection of lung tissue during *S. pneumoniae* infection, by controlling the recruitment of neutrophils to the airways (Peñaloza *et al.*, 2015). Thus, the IL-10 induction by *L. rhamnosus* would favour the humoral specific immune response and would regulate the inflammatory response after pneumococcal infection. Low IL-10 levels, observed in malnourished mice, would compromise the regulation of the host defence response against infectious challenge.

There are contradictory reports on the role of the Th1 cytokine IFN-γ in protection against pneumococcal infection. Some works reported that IFN-γ is not required for an effective pulmonary defence in pneumonia due to *S. pneumoniae* (Rijneveld *et al.*, 2002; Sun and Metzger, 2008). However, IFN-γ is considered to enhance chemokine expression and promote pulmonary phagocytic cells recruitment into the infected lung, playing an important role in the phagocytic-mediated host protective responses against pneumococcal infection (Nakamatsu *et al.*, 2007; Sun *et al.*, 2007). In addition, it seems that the lack of IL-10 leads to the higher levels of IFN-γ, which might lead to an exacerbated innate inflammation (Peñaloza *et al.*, 2015). These data exemplify the complex role of IFN-γ in immunity during pulmonary infection. Interestingly, the MNC group showed values of BAL IFN-γ that were similar to the WNC mice. It is possible that this imbalance between high IFN-γ and low IL-10 levels in the respiratory tract, could explain the inflammatory tissue damage observed in MNC mice as well as the difficulties of this group to eradicate the pathogen from the lungs (Barbieri *et al.*, 2013). After infection, the BCD+Lr group showed different pattern of cytokines (IFN-γ, IL-4 and IL-10) in BAL than those of the BCD mice. The differences between both repleted groups, could explain the higher capacity of lactobacilli-treated mice to eliminate the respiratory pathogen (Barbieri *et al.*, 2013). Long and Nanthakumar (2004) showed that different nutrient deficiencies could influence the natural history of different pathogen infections where the protective roles of the Th1-Th2 responses are distinct. Then, the nutritional status of the different experimental groups could influence the type of Th response induced.

The induction and maintenance of antigen specific T cell responses is an essential feature in the protection against *S. pneumoniae.* In this work we demonstrate that the nasal administration of *L. rhamnosus* CRL1505 as a supplement in a repletion diet accelerates the recovery of CD4 T cell populations in the thymus, bone marrow, spleen and lung which are severely affected by malnutrition. Additionally, *L rhamnosus* CRL1505 treatment is able to beneficially modulate cytokine production in response to *S. pneumoniae* infection. Then, nasal administration of *L. rhamnosus* CRL1505 may represent a non-invasive means to modulate and improve T cell-mediated immunity against respiratory pathogens in immunocompromised malnourished hosts. Further studies are needed to elucidate the subtypes of CD4 T cells involved in the improvement of the defence against pneumococcal infection.

Acknowledgements

This work was supported by grants from CIUNT 26D/403, PICT 2010 no. 1381 and PICT 2013 no. 3209. The authors declare no conflict of interest.

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