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Original article

Can *Lacticaseibacillus rhamnosus* CRL1505 postbiotic improve emergency myelopoiesis in immunocompromised mice?

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ABSTRACT

We evaluated whether viable and non-viable *Lacticaseibacillus rhamnosus* CRL1505 (Lr05V or Lr05NV, respectively) was able to improve emergency myelopoiesis induced by *Streptococcus pneumoniae* (Sp) infection.

Adult Swiss-mice were orally treated with Lr05V or Lr05NV during five consecutive days. The Lr05V and Lr05NV groups and untreated control group received an intraperitoneal dose of cyclophosphamide (Cy-150 mg/kg). Then, the mice were nasally challenged with Sp (10^7 UFC/mice) on day 3 post-Cy injection. After the pneumococcal challenge, the innate and myelopoietic responses were evaluated.

The control group showed a high susceptibility to pneumococcal infection, an impaired innate immune response and a decrease of hematopoietic stem cells (HSCs: $Lin^-Sca\text{-}1^+c\text{-}Kit^+$), and myeloid multipotent precursors (MMPs: $Gr\text{-}1^+Ly6G^+Ly6C^-$) in bone marrow (BM). However, lactobacilli treatments were able to significantly increase blood neutrophils and peroxidase-positive cells, while improving cytokine production and phagocytic activity of alveolar macrophages. This, in turn, led to an early Sp lung clearance compared to the control group. Furthermore, Lr05V was more effective than Lr05NV to increase growth factors in BM, which allowed an early HSCs and MMPs recovery with respect to the control group.

Both Lr05V and Lr05NV were able to improve BM emergency myelopiesis and protection against respiratory pathogens in mice undergoing chemotherapy.

Polymorphonuclear neutrophils are the first line of defense against pathogens. Blood neutrophils number depends on the balance between bone marrow (BM) granulopoiesis and apoptosis in peripheral tissues [1]. Neutrophils are continuously generated from hematopoietic stem cells (HSC) that give rise to multipotent progenitors in BM. These progenitors can be common lymphoid and myeloid progenitors, giving rise to granulocyte-macrophage (GMP), megakaryocyte-erythrocyte, and dendritic cell progenitors. GMP, in turn, give rise to neutrophils and monocytes [2]. Neutrophil homeostasis in the steady state is regulated by the IL-23/IL-17/G-CSF axis, which has been established in several independent murine models and in human studies [3,4]. In addition, Pattern Recognition Receptors (PRR) participate in this regulation, directly linking Toll-like receptors (TLR) with granulopoiesis [3]. The response of the hematopoietic system to the demand for neutrophils, which are recruited during an infectious challenge, is known as emergency granulopoiesis [5]. During this process, patterns of pathogen-associated molecules are detected by PRR of the innate immune system. Consequently, high levels of G-CSF and GM-CSF are produced, and granulopoiesis and the release of neutrophils into the circulation are observed [5]. HSCs and myeloid progenitors can directly detect pathogens and microbiota-derived products [6,7]. Even circulating HSCs can recognize bacteria or their products in the periphery before re-entering BM [2]. Furthermore, it was shown that the neutrophil antimicrobial capacity depends on the

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recognition of peptidoglycan derived from the microbiota through Nod1 but not Nod2 or TLR4 [6]. Thus, a dysbiosis can alter granulopoiesis, deteriorate innate defenses, and reduce resistance against pathogens [6,8,9].

Many current chemotherapeutic drugs have cytotoxic side effects that impair the life quality of the patients conditioning their treatment. Cyclophosphamide (Cy) is a drug widely used as an antineoplastic alkylating agent due to its high therapeutic index and broad spectrum of activity against several types of cancer [10]. However, Cy causes marked myelosuppression and leukopenia with neutropenia [11–13]. Antineoplastic therapy can affect basal and emergency granulopoiesis and lead to an immunosuppression state with an increased susceptibility to infections [14,15]. Secondary infections in a cancer care setting are the most anticipated sequela during chemotherapy regimens. The lungs have been described as the most common site of infection in cancer patients [16].

The finding of safe immunopotentiating agents that reduce myelosuppression and improve the immune response is necessary. In recent years, scientists have emphasized the importance of functional foods for health promotion [17], where probiotics have gained special interest [18]. However, there is not enough literature on the effect of probiotic based lactic acid bacteria (BL)- on the recovery of the immune response during chemotherapy. In this context, we have demonstrated that *Lacticaseibacillus rhamnosus* CRL1505 is able to modulate the immune system by increasing resistance against infections in both immunocompetent and immunocompromised mice [19,20]. This strain is able to increase the humoral immunity against infections, accelerate the recovery of B ontogeny [20–23], and enhance basal and emergency myelopoiesis in malnutrition-immunosuppressed hosts [22,24]. Furthermore, in Cy-immunosuppressed hosts, the oral administration of CRL1505 strain improves the steady-state myelopoiesis [25]. Although the viability of BL is an important factor to achieve an optimal protective effect, it is possible to stimulate mucosal immunity and myelopoiesis in immunosuppressed hosts using non-viable BL [25–27]. Thus, we aim to assess whether the treatment with viable or non-viable *L. rhamnosus* CRL1505 may improve emergency myelopoiesis and local and systemic innate immune response induced by *Streptococcus pneumoniae* infection in Cy-immunosuppressed mice.

1. Materials and methods

1.1. Viable or non-viable BL

L. rhamnosus CRL1505 was selected because of their immunomodulatory capacity [19,20,24,25]. This strain was provided by the CERELA culture collection (Chacabuco 145, San Miguel de Tucumán, Argentina). A medium containing peptone (1.5%), tryptone (1%), meat extract (0.5%) in distilled water at pH 7 was used to rehydrate the lyophilized strain. The bacterium was cultured to the final log phase (12 h at 37 °C) in Man-Rogosa-Sharpe broth (MRS, Oxoid), washed three times with 0.01 mol/l sterile phosphate buffer saline (PBS) pH 7.2, and resuspended in sterile PBS. A non-viable *Lacticaseibacillus* was obtained by exposition to ultraviolet radiation for 2 h [25]. The use of MRS agar plates confirmed the lack of bacterial growth. Viable and non-viable forms were harvested by centrifugation, washed with sterile 0.01 mol/l PBS, pH 7.2 and the concentration was adjusted by measuring OD (590 nm). The obtained preparations were suspended in 10% skimmed milk.

1.2. Animals and feeding procedures

6-week-old Swiss-albino male mice were obtained from CERELA. Viable or non-viable *L. rhamnosus* CRL1505 were orally administered in the drinking water at the dose of 10^8 cells/mouse/day to different groups of mice (Lr05V or Lr05NV groups) for 5 consecutive days. These doses were previously selected as optimal for effects on the immune system [19]. The control group consisted of animals that did not receive viable or non-viable BL treatment (Fig. 1). Six animals per experimental group were used for each measurement. Animal protocols were approved by the Ethical Animal Protection Committee of CERELA-CONICET, Tucuman, Argentina, under the protocol number CRL-BIOT-IBT-2017/1A, and all experiments comply with the current laws of Argentina and all international organizations for experimental animal use.

1.3. Experimental pneumosepsis model in immunosuppressed hosts

At the end of the viable or non-viable BL treatments, all animals received an intraperitoneal injection of cyclophosphamide (Cy) (150 mg/kg) to induce myelosuppression as shown in Fig. 1 (day −3) [28]. On day 3 post-Cy administration, the infectious challenge was performed with a strain of *S. pneumoniae* serotype 6B (day 0, Fig. 1). The mice of the different experimental groups (Control, Lr05V and Lr05NV groups) were challenged intranasally with the pathogen by applying 25 μl of a bacterial suspension with 10^7 cells/mouse of *S. pneumoniae* in PBS [26]. Day 3 post-administration of Cy was selected because in this immunosuppression model, an extreme neutropenia and the highest degree of deterioration of the myelopoietic capacity of BM are reached at this specific point (Fig. 1) [28]. During the whole study, immunosuppressed mice were housed in plastic cages, were kept in controlled environmental conditions with light dark cycles of 12 h, and were fed balanced conventional diets and sterilized water *ad libitum*. Through CRL-BIOT-BCE-2013/2A and BIOT-CRL-10 protocols, the Institutional Animal Care and Use Committee of CERELA approved

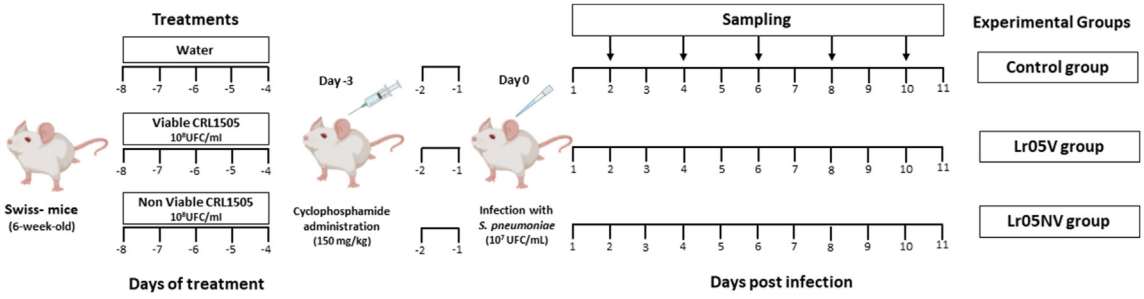

Fig. 1. Schematic of experimental model. 6-week-old Swiss mice were fed with 10^8 cells/mouse/day of viable or non-viable *Lacticaseibacillus rhamnosus* CRL1505 (Lr05V or Lr05NV groups respectively) for 5 consecutive days before intraperitoneal administration of cyclophosphamide (Cy) (150 mg/kg) (day −3). On day 3 post-Cy injection, the infectious challenge was performed intranasally with a strain of *Streptococcus pneumoniae* 6B at a dose of 10^7 CFU/ml (day 0 post-infection). The control group mice received no treatment. Sampling was carried out on days 2, 4, 6, 8 and 11 post-infection. Six animals were used per group each time.

this study. The current laws of Argentina and all international organizations for the use of experimental animals were applied in all experiments.

Before the challenge and on different post-infection days, the determinations described below were performed.

1.4. Streptococcus pneumoniae *counts in the lungs and blood*

To assess the severity of the infection, the lungs of the mice from the different experimental groups were removed aseptically. Then they were weighed and homogenized in 5 ml of sterile peptone water (0.1%). Lung homogenates were diluted appropriately, plated in duplicate on blood agar and incubated 18 h at 37 °C. Bacteremia was studied in blood samples, obtained by cardiac puncture from ketamine-xylazine-anesthetized animals, and plated on blood agar. The results were expressed as log of CFU/g lung or log of CFU/ml blood respectively [26].

1.5. *Lung injury*

For evaluation of lung tissue lesions, lungs from the different experimental groups were removed, washed out with PBS, immediately immersed in 4% (v/v) paraformaldehyde, and processed by standard histological techniques (paraffin-embedding) [26]. 5 μm sections from lungs were stained with Hematoxylin–Eosin. In addition, the albumin content and the activity of lactate dehydrogenase (LDH) in bronchoalveolar lavages (BAL) were determined to evaluate the alteration of the capillary alveolar barrier induced by the infection. Wiener Lab diagnostic kits were used and the results were expressed as units per liter of BAL fluid [26].

1.6. *Total and differential leukocyte count in blood and BM*

Blood samples were obtained by cardiac puncture in heparinized tubes from animals anesthetized with ketamine-xylazine. BM samples were obtained by flushing the femoral cavity with PBS. The blood and BM total number of leukocytes was performed by conventional hematological methods using a Neubauer counting chamber. Smears stained with May Grünwald Giemsa were analyzed under a light microscope (100×) to determine differential cell counts. The results were expressed in absolute values considering the total leukocyte count of each sample [28].

1.7. *Blood and BM myeloperoxidase activity*

While myeloperoxidase (MPO) activity was used to assess the functionality of myeloid cells in blood, it was used as a marker of myeloid cells in BM. The MPO activity was measured by the Washburn test. The results were expressed as percentages of peroxidase positive (Px+) cells [21].

1.8. *Phagocytic activity of alveolar macrophages*

Heat-killed pneumococci were labeled with fluorescein 5(6)-isothiocyanate (FITC) according to Kolling et al. [27]. Then, the FITC-labeled *S. pneumoniae* was incubated with fresh serum at 37 °C for 30 min. The bronchoalveolar lavage macrophages obtained from the different experimental groups at day 6 post-infection were incubated for 30 min with opsonized FITC-labeled *S. pneumoniae* (macrophage/bacteria ratio of 1/100). Phagocytosis was measured by flow cytometry. Cell suspensions were treated with trypan blue to block external fluorescence. The fluorescence difference between unblocked and blocked samples was calculated and plotted on an MFI plot [27].

1.9. *BM and lung cells and flow cytometry*

Single cell suspensions from BM and lungs were prepared according to Herrera et al. and Barbieri et al. [24,29]. 1×10^6 cells were treated with anti-mouse CD32/CD16 monoclonal antibody (BD Biosciences) and then stained with the following antibodies of DB Biosciences: FITC-conjugated anti-mouse Ly6G, phycoerythrin (PE)-conjugated anti-mouse Gr-1, biotinylated-conjugated anti-mouse Ly6C, FICT-conjugated anti-mouse CD34, biotinylated-conjugated anti-mouse Ly 6A/E, PE-conjugated anti-mouse CD117, FITC-conjugated anti-mouse TER-119 and allophycocyanin (APC) mouse lineage antibody cocktail. After incubation with biotinylated primary antibodies, the labeling was revealed using streptavidin-PercP. FASCalibur™ flow cytometer (BD Biosciences) was used to acquire the samples. FlowJo software (Tree Star) enabled data analysis.

1.10. *Cytokines in serum and BAL*

Tumor necrosis factor (TNF)-α and interleukin (IL)-10 were measured in serum and BAL samples at days −3, 0 and 6 post-infection using commercially available enzyme-linked immunosorbent assay (ELISA) technique kits and following the manufacturer's recommendations (ELISPOT Ready-SET-Go! eBioscience, San Diego, USA). The data were expressed in pg/ml using the standard curve of the cytokine. In addition, TNF-α/IL-10 ratio was calculated.

1.11. *Statistical analysis*

To obtain the statistical analysis of the data, GraphPad Prism software (version 6.0) was used. Results were expressed as the mean ± SD of three independent tests. The data presented a normal distribution and a 2-way ANOVA was applied. Tukey's test was used to study the differences between the two groups by comparing the means by pairs. Differences were considered significant at $P < 0.05$.

2. Results

2.1. *Viable and non-viable* L. rhamnosus *CRL1505 significantly increase the resistance of Cy-immunocompromised mice against pneumococcal infection*

The effect of Cy and treatments with viable and non-viable *L. rhamnosus* CRL1505 on the resistance against *S. pneumoniae* infection was assessed by monitoring the bacterial count curves of the different groups of mice intranasal challenged with 10^7 cells/mouse. This infective dose was selected following preliminary studies evaluating the mortality rates in Cy treated mice according to the number of *S. pneumoniae* cells administered (data not shown). Under this condition, *S. pneumoniae* number in lung and blood were determined on days 2, 4, 6, 8 and 11 post-infection. The control group showed a higher colonization level in lung compared to mice treated with viable lactobacilli throughout the studied period. The Lr05NV group showed significantly lower lung bacterial counts only on days 6 and 11 compared to the control group (Fig. 2A). Moreover, control mice negativized hemocultures the last day of the study (day 11 post-infection), while the Lr05V and Lr05NV groups showed negative hemocultures on day 4 and 6 post-infection, respectively (Fig. 2B).

Next, we evaluated the lung tissue damage through histological studies and biochemical markers of BAL such as the albumin content to measure the increase in the permeability of the bronchoalveolar capillary barrier, and the activity of LDH, an indicator of general cytotoxicity (Fig. 3). Challenge with pneumococci induced important changes on lung tissue architecture. The most notable changes were observed early after infection. Control group lungs

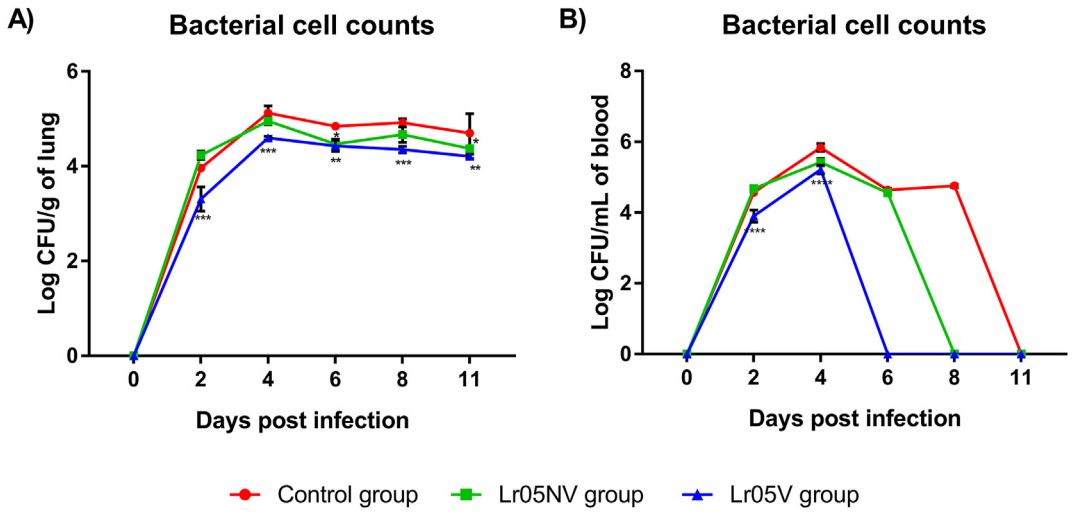

Fig. 2. Evaluation of infection resistance. A) Lung bacteria count expressed in log CFU/g of organ (*S. pneumoniae* was identified by its ability to produce red blood cell hemolysis, α-hemolysis). Heparinized blood samples obtained aseptically by cardiac puncture were used for blood cultures. Each sample was seeded on blood agar and the pathogen was identified according to hemolysis. B) Bacterial blood culture expressed in log of CFU/ml of blood. The results come from two independent experiments. Six animals per group were used in the experiments for each determination. Results are expressed as mean ± SD, n = 6. ****, ***, **, * Significant differences from the control group should be as follows ($p < 0.0001$, $p < 0.001$, $p < 0.01$, $p < 0.05$, respectively).

showed an intense inflammatory response, hemorrhage and a reduction of alveolar spaces from day 2 and more intensely on day 4 post-infection (Fig. 3A). Lung architecture of the control group showed signs of recovery from day 6 and was normal on day 10 (data not shown). In mice treated with viable and non-viable lactobacilli, lung alterations during the early days after infection were significantly lower than control. Mice receiving preventively Lr05NV or Lr05V showed a moderate inflammatory infiltrate and lower hemorrhage and no reduction of alveolar spaces (Fig. 3A). Moreover, lungs of Lr05V and Lr05NV groups showed signs of recovery from day 4 and showed a normal histology on day 6 (Fig. 3A). *S. pneumoniae* infection significantly increased albumin concentration and LDH activity in BAL of all experimental groups reaching a peak on day 4 and day 6 post-infection, respectively (Fig. 3B and C). The values of both biochemical markers of lung injury were significantly lower in the Lr05V and Lr05NV groups compared to the control group (Fig. 3B and C).

2.2. *Viable and non-viable* L. rhamnosus *CRL1505 enhanced respiratory innate immune response against pneumococcal infection in Cy-immunocompromised mice*

We next studied the respiratory innate immune response by evaluating the composition of the lung myeloid population and levels of respiratory cytokines. After *S. pneumoniae* infection, the control group showed a significant increase in pulmonary neutrophils (Gr-1^{+}Ly6G^{+}Ly6C^{-} cells) on day 6 post-infection, while the pulmonary monocytes (Gr-1^{+}Ly6G^{-}Ly6C^{+} cells) suffered a significant reduction from day 2 post-infection (Fig. 4A and B). The mice treated with viable and non-viable *L. rhamnosus* showed a significant increase in pulmonary neutrophils and monocytes on day 2 dpi with respect to the control group (Fig. 4A and B). Moreover, the preventive treatment with Lr05V induced a significant increase in pulmonary neutrophils from day 6 dpi while treatment with Lr05NV induced this increase from day 8 dpi (Fig. 4A). The Cy-immunosuppressed mice showed the presence of myeloid precursors in lungs infected with *S. pneumoniae*. Thus, the control group showed a significant increase in multipotent progenitors committed to the myeloid lineage (MPP), identified as Lin^{-}Sca-1^{+}C-kit^{+}CD34^{+} cells, and in hematopoietic stem cells called LSK cells (Lin^{-}Sca-1^{+}C-kit^{+} cells) on days 4–8 post-infection with a peak on day 6 (Fig. 4C and D). The mice that received the preventive treatments showed similar kinetics in the percentage of MPPs and LSK cells after respiratory infection. However, the preventive treatment with Lr05V and Lr05NV was able to induce a significant increase in LSK cells throughout the post-infection period. The higher number of LSK cells was accompanied by a significant increase in MPPs on day 2 dpi in both treated groups and on day 4 dpi in the Lr05V group (Fig. 4C and D). Moreover, the phagocytic activity of the alveolar macrophages was significantly higher in the mice treated preventively with the viable *L. rhamnosus* and its postbiotic compared to the control group on day 6 post-infection (Fig. 4E and F). Although we did not observe significant differences in BAL levels of IL-10 between experimental groups (Fig. 4G), the group supplemented with Lr05NV showed lower BAL concentrations of TNF-α with respect to the control group (Fig. 4H).

2.3. *Viable and non-viable* L. rhamnosus *CRL1505 enhanced systemic innate immune response against pneumococcal infection in Cy-immunocompromised mice*

Increased numbers of blood leukocytes and neutrophils as well as the percentage of blood peroxidase positive cells were observed in Lr05V and Lr05NV mice when compared to the control group before the challenge with *S. pneumoniae* (Fig. 5A–C). The respiratory infection significantly increased blood leukocytes and neutrophils in all experimental groups. However, Lr05V and Lr05NV mice showed values of blood leukocytes and neutrophils that were significantly higher than those in the control group on days 4 and 6 post-infection and on days 1, 4 and 6 post infection, respectively (Fig. 5A and B). In addition, the treatments were able to improve the percentage of blood peroxidase positive cells, which reached the maximum activity from day 2 post-infection, two days earlier than the control group (Fig. 5C). The levels of blood TNF-α and IL-10 were also increased after the challenge with *S. pneumoniae*. The preventive treatments with viable and non-viable lactobacilli induced the decrease of serum TNF-α levels compared to the control group, while only the viable lactobacilli induced an increase of serum IL-10 levels (Fig. 5D and E). Both preventive treatments were able to significantly reduce the ratio compared to the control (Fig. 5F).

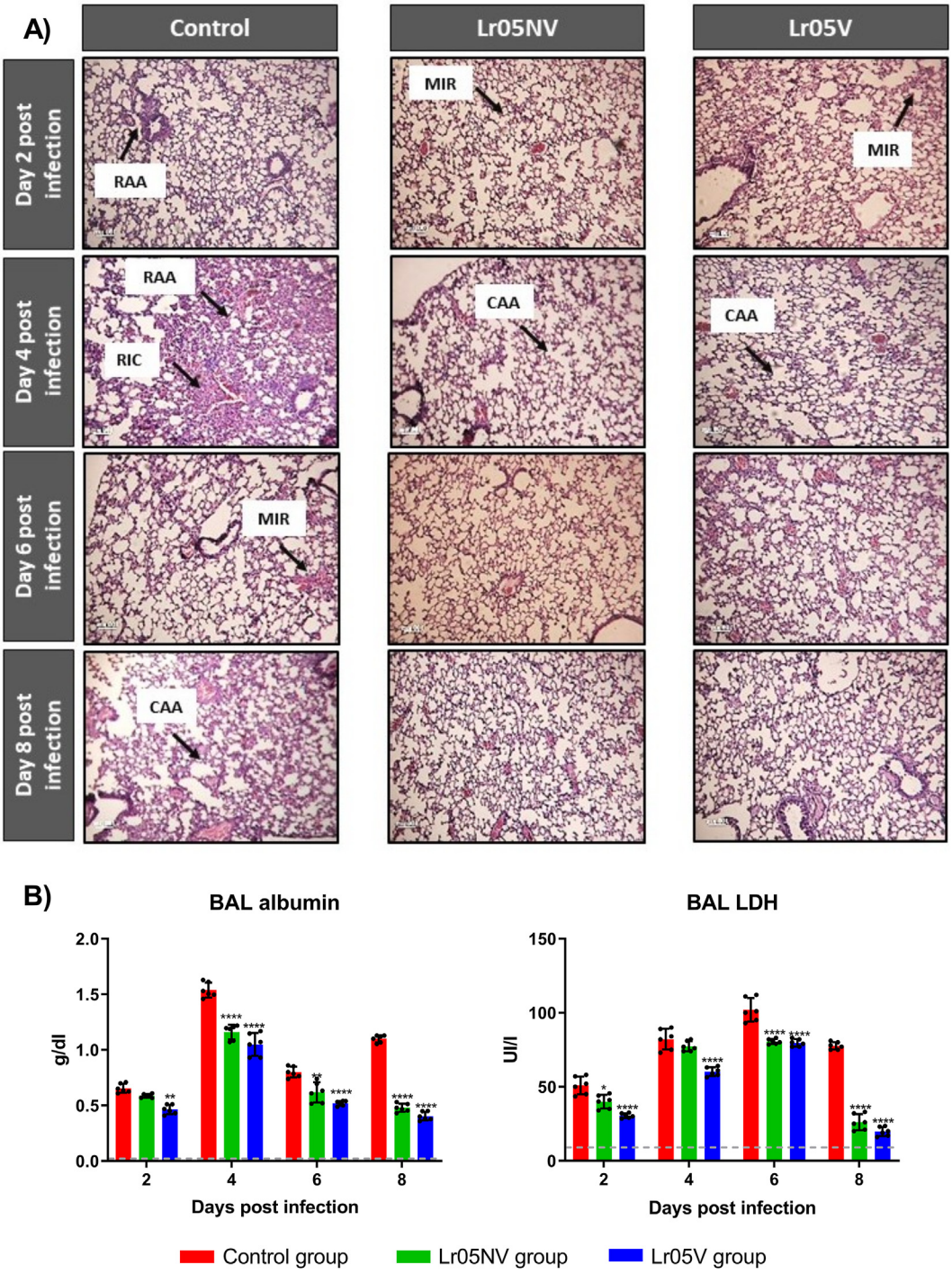

Fig. 3. Effects of viable and non-viable *Lacticaseibacillus rhamnosus* CRL1505 in lung injuries induced by pneumococcal infection. A) Light micrographs, original magnification ×10. Recruited inflammatory cells (RIC). Reduction of alveolar airspace (RAA). Moderate inflammatory reaction (MIR). Conserved alveolar airspace (CAA). B) Albumin concentration and C) Lactate dehydrogenase (LDH) activity in broncho-alveolar lavages (BAL) after the challenge with *S. pneumoniae* on days 2, 4, 6, 8 and 11 post-infection. The black dots indicate individual mouse data in the bar graph. Gray dotted transverse line represents the normal control value without any treatment. Six animals per groups were used each time. Results are expressed as mean ± SD, n = 6. ****, **, *Significant differences from the control group should be as follows ($p < 0.0001$, $p < 0.01$, $p < 0.05$, respectively).

2.4. *Viable and non-viable* L. rhamnosus *CRL1505 enhance emergency myelopyesis against pneumococcal infection in Cy-immunocompromised mice*

As it was mentioned before, the effect of preventive treatments on the emergency granulopoiesis was studied. The pneumococcal infection in the control group induced a significant increase of bone marrow leukocytes, myeloid cells and post-mitotic pool cells counts from day 1 post-infection. They oscillated between days 4 and 11 showing similar values without reaching normal values (39.5 ± 1.5 10^6 cells/femur; 17.1 ± 2.1 cells/femur; and 4.02 ± 0.6 cells/femur, respectively) (Fig. 6A, B and C). These cell counts returned to basal levels on day 21 (data not shown). Before infection, the Lr05V and Lr05NV groups showed significant higher BM leukocytes with respect to the control group. In addition, only the treatment with Lr05V showed a significant increase in myeloid cells and post-mitotic

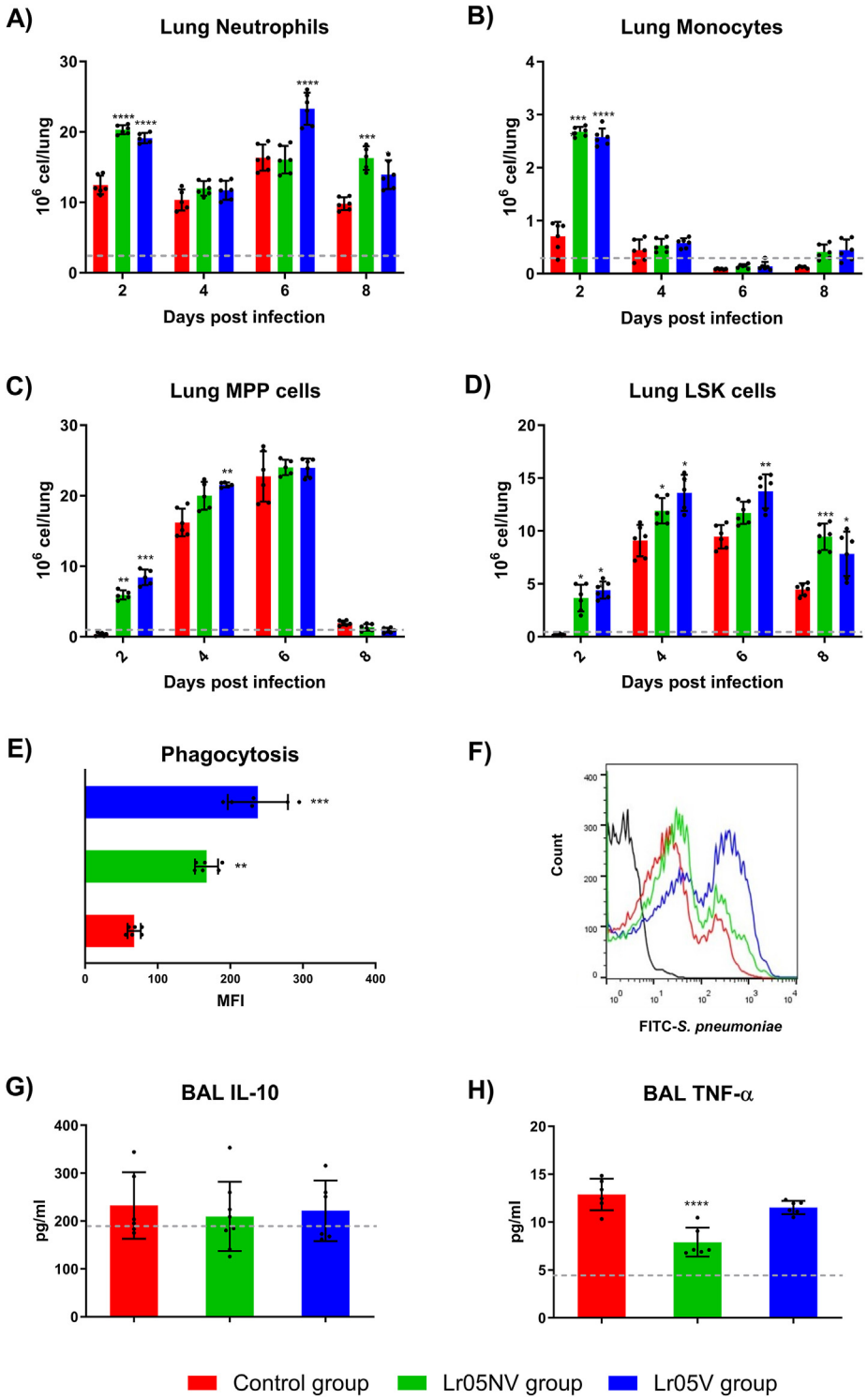

Fig. 4. Effects of viable and non-viable *Lacticaseibacillus rhamnosus* CRL1505 on respiratory immune response against pneumococcal infection. A) Lung neutrophils (Gr-1$^+$Ly6G$^+$Ly6C$^-$ cells). B) Lung monocytes (Gr-1$^+$Ly6G$^-$Ly6C$^+$ cells). C) Lung multipotent progenitors (MPP) committed to the myeloid lineage (Lin$^-$Sca-1$^+$C-kit$^+$CD34$^+$ cells). D) Lung LSK cells (Lin$^-$c-Kit$^+$SCA-1$^+$ cells). E) Phagocytic activity of the alveolar macrophages on day 6 post-infection expressed as mean fluorescence intensity (MFI). F) Comparison of *S. pneumonia*-FITC fluorescence intensity in alveolar macrophages analyzed by flow cytometry. G) BAL levels of TNF-α and H) BAL levels of IL-10 were expressed in picograms (pg)/ml using the standard curve performed with different concentrations of the corresponding cytokine. The black dots indicate individual mouse data in the bar graph. Gray dotted transverse line represents the normal control value without any treatment. Six animals per group were used each time in the experiments. Results are expressed as mean ± SD, n = 6. ****, ***, **, * The significant differences of the control group should be the following ($p < 0.0001$, $p < 0.001$, $p < 0.01$, $p < 0.5$ respectively).

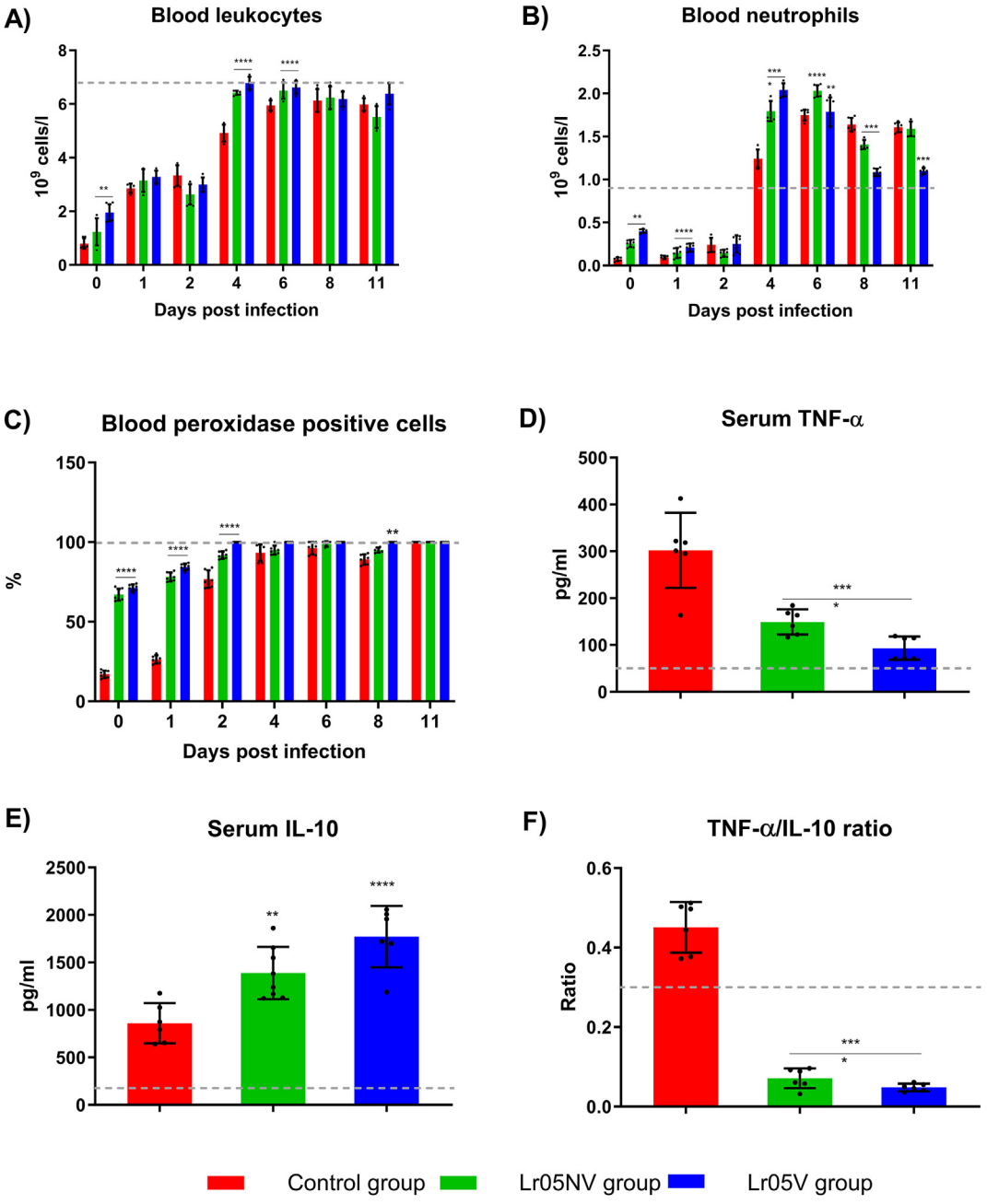

Fig. 5. Effects of viable and non-viable *Lacticaseibacillus rhamnosus* CRL1505 on innate immune response against pneumococcal infection. A) Blood leukocytes, B) neutrophils, C) and peroxidase positive (+) cells counts were performed on days 0, 2, 4, 6 and 8 post-infection in mice Cy-immunocompromised mice. D) Serum TNF-α levels and E) serum IL-10 levels were performed on day 6 post-infection and were expressed in picograms (pg)/ml. F) TNF/IL-10 ratio. The black dots indicate individual mouse data in the bar graph. Gray dotted transverse line represents the normal control value without any treatment. Six animals per group were used each time in the experiments. Results are expressed as mean ± SD, n = 6. ****, ***, **, * The significant differences of the control group should be the following ($p < 0.0001$, $p < 0.001$, $p < 0.01$, $p < 0.5$ respectively).

pool cells counts (Fig. 6A, B and C). After infection, mice treated with a viable strain showed BM leukocytes, myeloid cells, post-mitotic pool cells and peroxidase positive cells counts significantly higher than the control in all the infection period (Fig. 6A, B, C and D). Notably, the treatment with de non-viable *Lacticaseibacillus* was able to induce a similar behavior of viable form in the total cell and peroxidase positive cell counts of BM (Fig. 6A and D). In addition, the Lr05NV group showed a significant higher myeloid cell count than the control on days 6, 8 and 11 post-infection (Fig. 6B), as well as an increase in post-mitotic pool cells counts on days 4, 8 and 11 post-infection with respect to the control group (Fig. 6C).

Finally, flow cytometry studies revealed that the infectious challenge induced an increase in LSK cells, MMPs, neutrophil and monocyte precursors from day 2 post-infection in BM (Fig. 6E, F, G and H). Lactobacilli treatments were able to significantly increase the number of these progenitors after infection in BM. Lr05V and Lr05NV treatments showed a peak of LSK cells number on day 2 and 4 post-infection respectively (Fig. 6E), and a peak of monocyte progenitors count on day 2 post-infection (Fig. 6H). In addition, both *L. rhamnosus* treatments promoted an increase of neutrophil precursors from day 4 in BM when compared with the control group (Fig. 6G).

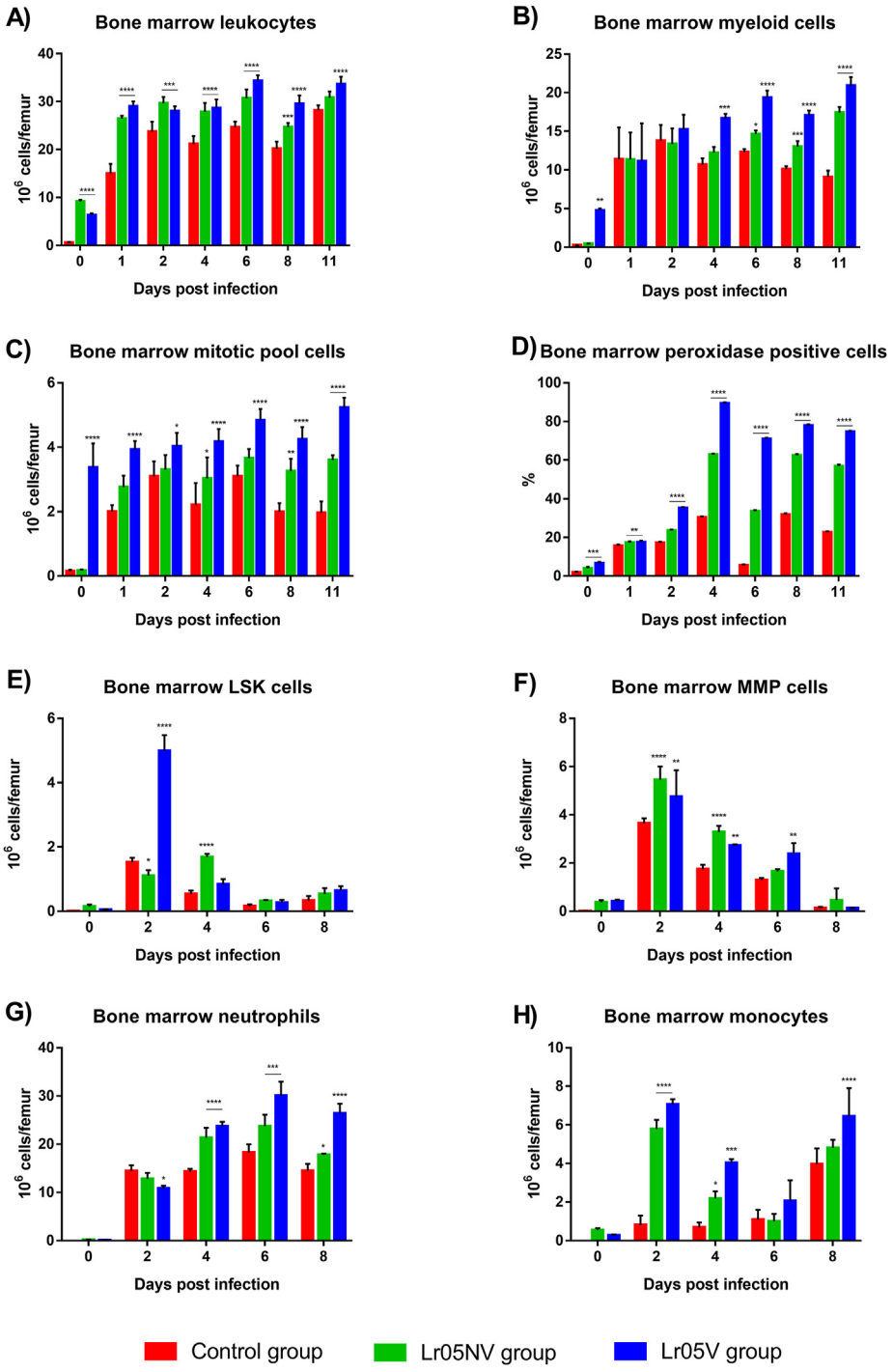

Fig. 6. Effects of viable and non-viable *Lacticaseibacillus rhamnosus* CRL1505 on emergency myelopyesis against pneumococcal infection. A) Bone marrow leukocytes B) myeloid cells, C) mitotic pool cells, and D) peroxidase positive cells were studied on days 0, 1, 2, 4, 6, 8 and 11 post-infection and expressed as 10^6 cells/femur. E) Bone marrow LSK cells ($Lin^-c\text{-}Kit^+SCA\text{-}1^+$ cells), F) multipotent progenitors (MPP) committed to the myeloid lineage ($Lin^-Sca\text{-}1^+C\text{-}kit^+CD34^+$ cells), (G) neutrophils ($Gr\text{-}1^+Ly6G^+Ly6C^-$ cells) and (H) monocytes ($Gr\text{-}1^+Ly6G^-Ly6C^+$ cells) were determined on days 0, 2, 4, 6 and 8 post-infection and expressed as percentage. The black dots indicate individual mouse data in the bar graph. Gray dotted transverse line represents the normal control value without any treatment. Six animals per group were used each time in the experiments. Results are expressed as mean ± SD, n = 6. ****, ***, **, * The significant differences of the control group should be the following ($p < 0.0001$, $p < 0.001$, $p < 0.01$, $p < 0.5$ respectively).

3. Discussion

Many researchers have demonstrated the harmful effects of chemotherapy on steady-state myelopoiesis and immune response, especially in animal models [12,13,30,31]. We have previously shown that the oral administration of CRL1505 strain improves the steady-state myelopoiesis in Cy-immunosuppressed hosts [25]. Data presented here demonstrate for the first time that both viable and non-viable *L. rhamnosus* CRL1505 were able to improve BM emergency myelopiesis and protection against a respiratory pathogen in mice undergoing chemotherapy.

S. pneumoniae is an important causative agent of diseases around the world. The invasive pneumococcal diseases are most prevalent in children under 2 years of age and adults over 65, as well as in patients with chronic pulmonary comorbidities, immunosuppressive conditions and treatment with chemotherapy or systemic glucocorticoids [32–36]. There are few studies that show the high prevalence of pneumococcal bacteremia in the population with chemotherapy treatment and the high morbimortality related to them [16]. In this regard, we found that Lr05V and Lr05NV treatments were efficient in reducing the bacterial count in the lung and, more importantly, reducing the passage time of the pathogen from 4 to 2 days, respectively, in an experimental pneumosepsis model in Cy-immunosuppressed hosts. There is no bibliographic evidence associating the immunobiotic or postbiotic consumption and the increase in respiratory infections resistance in models of immunosuppression by chemotherapeutic drugs. Even more, Lr05V and Lr05NV treatments achieved accelerated recovery from lung damage evidenced by histological changes and biochemical parameters. Similar findings were previously demonstrated with this strain in a protein deficiency immunosuppression model [26,37,38].

The defense against infection by *S. pneumoniae* requires a rapid and efficient innate immune response. Our results showed that viable and non-viable *L. rhamnosus* CRL1505 were able to enhance respiratory innate immune response against pneumococcal infection in Cy-immunocompromised mice. It is known that when the pneumococcus invades the respiratory tract, immune cells and epithelial cells secrete chemokines and cytokines promoting neutrophil migration into the lung through the pulmonary capillary walls [39]. Neutrophils phagocytize and kill the pathogen with the help of reactive oxygen species, antimicrobial proteins, and serine proteases [40]. In this regard, Lr05V and Lr05NV groups showed a significant increase in neutrophils and monocytes in the lung on day 2 dpi, which could be due to the greater reserve of these cell types as a result of preventive treatment before infection. However, the increase observed on days 6 and 8 post-infection in these groups is based on the time required for de novo maturation of myeloid cells in the bone marrow and their arrival in the lung via peripheral blood. This explains the influx of immature cells from the bone marrow to the lung, characterized in our model as LSK and MPP in the first post-infection days. This fact brings as a consequence, on the one hand, the capacity of the treatments under study to achieve the elimination of the pathogen in the blood from 6 dpi. On the other hand, despite the significant decrease in the count of *S. pneumoniae* in the lung in the treated mice, a slow decrease in this count was observed during the period studied. This may be because neutrophil depletion causes profound defects in the clearance of *S. pneumoniae* [41,42]. It is important to consider that the phagocytic function and phagolysosomal degradation of pneumococcus by neutrophils are crucial strategies for controlling pneumococcal infection [42]. In this sense, preventive treatments were not only able to improve the counts of innate immunity cells at the local level, but also to increase their functionality, evidenced in phagocytosis studies. Furthermore, *S. pneumoniae* persistence in lungs during the period studied correlates with the lower local production of the cytokines studied, the inefficient neutrophil and monocyte recruitments and the lower cell count in lungs.

At the systemic level, the increase of blood leukocytes and neutrophils coincided with the peak of *S. pneumoniae* at 4 dpi in blood cultures. These results demonstrated that preventive treatments were clearly able to improve the innate immune response by improving leukocyte counts and their functionality measured as myeloperoxidase activity [24,26,28]. Unlike what occurs at the local level, at the systemic level a balance of cytokines with increased TNF-α and IL-10 was observed. The same effect was already demonstrated in another model of immunosuppression with the viable and non-viable CRL1505 strain [23,24,26].

Hematopoietic progenitors are a critical component of innate immunity, allowing replenishment of innate immune populations in emergency myelopoiesis in the setting of systemic infection or after chemotherapy-induced myelosuppression. Emergence myelopoiesis is a tightly regulated process involving contributions from various inflammatory mediators and HSC niche factors that typically tip the balance of hematopoiesis toward a myeloid lineage [2,43]. HSCs can be activated by TLR ligands and inflammatory cytokines and chemokines that promote emergency myelopoiesis [43]. Cy administration induced a severe decrease in the total count of cells, myelopoietic progenitors and mature myeloid cells of BM at steady state, and the lowest number of cells was registered on day 3 after Cy injection [25,28]. The infectious challenge induced an evident de novo production of myeloid cells [2,43]. In this context, we demonstrated that the preventive treatments were able to enhance emergency myelopoiesis against pneumococcal infection in Cy-immunocompromised mice by cytomorphological and cytometric studies. Thus, for the first time we demonstrated that the oral administration of an immunobiotic or postbiotic (non-viable lactic acid bacteria) is capable of inducing its effects both at the systemic level (bone marrow) and at the level of another mucosa such as the respiratory mucosa.

The evidence accumulated in our previous investigations and the data discussed here allow us to suggest the hypothesis. The immunobiotic or its postbiotic can bind to PRRs in innate immune cells such as monocytes/macrophages, DCs and NK cells present in Peyer's patches, or in the lamina propria. The interaction of molecular patterns associated with pathogens with TLRs could activate several transcription factors, particularly NF-κB and MAPKs, and increase the production of pro-inflammatory cytokines [44]. In addition, viable and non-viable *L. rhamnosus* CRL1505 are able to increase the frequency of IL-17A-producing innate lymphoid cells in the lamina propria of the small intestine ($IL\text{-}17A^{+}ROR\gamma t^{+}CD4^{-}NKp46^{+}$) after injection of Cy. These results were correlated with an increase in serum IL-17A levels, which stimulates circulating levels of GM-CSF and/or G-CSF and could act distally on myelopoietic precursors in the BM cavity [25]. Based on these findings, our treatments induced a high expression of GM-CSF and a lower expression of CXCL12 in BM, compared to the control group. Thus, CRL1505 strain can beneficially activate the IL-17A/GM-CSF axis and accelerate recovery from Cy-induced immunosuppression by increasing the number of myeloid progenitors in BM [25]. Furthermore, immunobiotics and postbiotics could also exert remote effects on myelopoietic precursors by releasing bacterial products such as Nod1L that interacts with the Nod1 receptor expressed on marrow stromal cells which stimulate a wide range of cytokines that act locally within the BM microenvironment. The source of most of these circulating cytokines remains under investigation. Another possible mechanism involved is immunity trained at HSC level [44]. Tonic inflammatory signals from oral immunobiotic or postbiotic treatments could epigenetically prepare HSCs to respond to activation signals such as pneumococcal infection.

The outcomes obtained in this murine immunosuppression study provide the scientific basis for its application in immunocompromised human patients where the consumption of live microorganisms may represent a risk to their health. In addition, the patients with secondary immunodeficiencies present different degrees of dysbiosis. In these cases, the dietary supplementation with our postbiotic in the appropriate dose could provide benefits similar to those provided by a probiotic. The spectrum of applications would range from people undergoing chemotherapy,

radiotherapy, chronic consumption of antibiotics, to the physiologically immunocompromised people such as pregnant women or in the extremes of life.

We conclude that both viable and non-viable *L. rhamnosus* CRL1505 are able to improve BM emergency myelopiesis and protection against respiratory pathogens in mice undergoing chemotherapy. Importantly, they also suggest a possible avenue for therapeutic design, as enhancing the emergency myelopoietic response in chemotherapy patients that would likely not only improve the immune defense against a primary insult, but also against subsequent infections. This study suggests the preventive administration of non-viable *L. rhamnosus* CRL1505 which represents an innovative alternative to counteract the deterioration of emergency granulopoiesis that determines the treatment of patients with chemotherapy.

Author contributions

S.S. and S.A. designed the study. A.G.L., Y.K., F.G. and S.S. did the experiments. S.S. and S.A. provided financial support. B.V., F.G. and S.S. contributed to data analysis and results interpretation. B.V., S.S. and S.A. wrote the manuscript. The manuscript was revised and improved by all authors.

Declaration of competing interest

The authors declare no conflict of interest.

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