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Probiotic *Lactobacillus* strains protect against myelosuppression and immunosuppression in cyclophosphamide-treated mice

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ABSTRACT

This work evaluated the capacity of two probiotic strains, *Lactobacillus casei* CRL431 and *Lactobacillus rhamnosus* CRL1506, to protect against myelosuppression and immunosuppression in cyclophosphamide (Cy)-treated mice. Changes in mature granulocytes and progenitor cells in bone marrow (BM) and blood were studied. In addition, the ability of probiotics to accelerate the recovery of the immune response against the opportunistic pathogen *Candida albicans* was evaluated. We demonstrated for the first time that the preventive treatment with immunomodulatory lactobacilli such as *L. casei* CRL431 or *L. rhamnosus* CRL1506 was able to increase immature myeloid progenitors in the BM, allowing an early recovery of myeloid cells after Cy administration. Probiotic lactobacilli were also capable to induce an early recovery of neutrophils in blood, improve phagocytic cells recruitment to infectious sites and increase the resistance against the opportunistic pathogen *C. albicans*. Although deeper studies regarding the cellular and molecular mechanisms of probiotic actions are needed, these findings support the idea that strains like CRL431 and CRL1506 may accelerate the recovery of Cy-caused immunosuppression by immunopotentiating myeloid cells. Then, probiotic lactobacilli have the potential to be used as alternatives for lessening chemotherapy-induced immunosuppression in cancer patients.

1. Introduction

Cyclophosphamide (Cy) is one of the most widely used alkylating agent and a major constituent of combined chemotherapy regimens [1]. Cy has a high cytotoxicity on tumor cells and therefore it is used in the treatment of acute and chronic leukemias [2,3], multiple myeloma [4], lymphoma [5,6], autoimmune diseases [7] and patient preparation for bone marrow transplant [1,8]. However, Cy has low specificity and it has a broad spectrum of cytotoxic effects on normal cells [9]. Among the side effects caused by Cy highlights the induction of myelosuppression, since this drug interferes with the proliferation and differentiation of cells in the bone marrow (BM) [10–12]. These changes in BM are associated to a marked leukopenia and neutropenia [13,14] and to an increase in the susceptibility to infections [15,16].

Management of immunocompromised patients under chemotherapy treatments is complex and infectious diseases are among the most important problems in this population. In these patients, the occurrence of bacterial and fungal infections is usually very serious and can be rapidly fatal if untreated [17,18]. Early hospitalization and intravenous treatment with colony stimulating factors and broad-spectrum antibiotics have achieved a significant reduction in the number of infections in these patients. However, there are obvious problems resulting from hospitalization as well as the toxicity of these drugs, psychological and economic decline. For these reasons, it is essential to avoid damage of non-malignant cells during the clinical application of Cy, in order to reduce morbidity and mortality of infections in immunocompromised patients.

Many attempts are being investigated to find safe immune-potentiating agents able to reduce myelosuppression and improve immune response in chemotherapy-treated patients. In this regard, scientists have emphasized the importance of functional foods and dietary supplements for health promotion [19]. The use of probiotic foods for improving immune health status in immunocompromised patients has gained a special interest in recent years [20]. In this sense, our laboratory and others conducted studies in different experimental models of immunosuppression in order to evaluate the capacity of probiotic lactic acid bacteria (LAB) to improve immunity. The results of these investigations showed that some LAB strains are able to improve the resistance against various pathogens such as *Pseudomonas aeruginosa, Gardnerella vaginalis, Streptococcus pneumoniae, Candida albicans* and *Salmonella*

Abbreviations: BM, bone marrow; CFU, colony forming unit; Cy, cyclophosphamide; DCs, dendritic cells; LAB, lactic acid bacteria; Lc431, *Lactobacillus casei* CRL431; Lr1506, *Lactobacillus rhamnosus* CRL1506; MPO, myeloperoxidase; PBS, phosphate buffer saline; Px+, peroxidase positive.

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aeruginosa in immunocompromised hosts [21]. We demonstrated in an experimental model of malnourished immunocompromised mice that a repletion diet supplemented with *Lactobacillus casei* CRL431 or *L. rhamnosus* CRL1506 accelerated the normalization of innate and specific immune responses against pneumococcal infection [22–24]. Moreover, taking into account the relationships between the hematopoietic and the immune systems, and the immunomodulatory effect of these probiotic LAB strains, we also demonstrated that the addition of probiotic microorganisms to repletion diets improved innate and adaptive immunity in immunocompromised hosts and that this effect is mediated in part by a beneficial influence on hematopoiesis [22,23,25,26]. These results provide the scientific basis for proposing probiotic LAB strains as potential adjuvant to minimize the deleterious effects associated with antineoplastic therapy, especially those related to myelotoxicity and immunosuppression.

Therefore, the aim of this work was to evaluate the capacity of *L. casei* CRL431 and *L. rhamnosus* CRL1506 to protect against myelosuppression and immunosuppression in Cy-treated mice. For this purpose, we determined the daily changes in mature granulocytes and progenitor cells in blood and BM after chemotherapy. Furthermore, we measured the effect of preventive treatments with probiotic LAB on blood leucocytes recovery as well as progenitor and stem cell mobilization in the BM. In addition, we evaluated the ability of probiotics to accelerate the recovery of the immune response against the opportunistic pathogen *Candida albicans*.

2. Materials and methods

2.1. Probiotic microorganisms

Lactobacillus casei CRL431 and *L. rhamnosus* CRL1506 were obtained from the CERELA culture collection. Lactic acid bacteria (LAB) (stored at −70 °C) were activated and cultured for 18 h at 37 °C (final log phase) in Man–Rogosa–Sharpe broth. The microorganisms were harvested by centrifugation and washed three times with sterile 0.01 mol/l phosphate buffer saline (PBS), pH 7.2. Finally, bacteria were suspended in 10% non-fat milk to be administered to mice. Both strains were selected from several LAB strains in preliminary experiments in Cy-treated mice (data not shown).

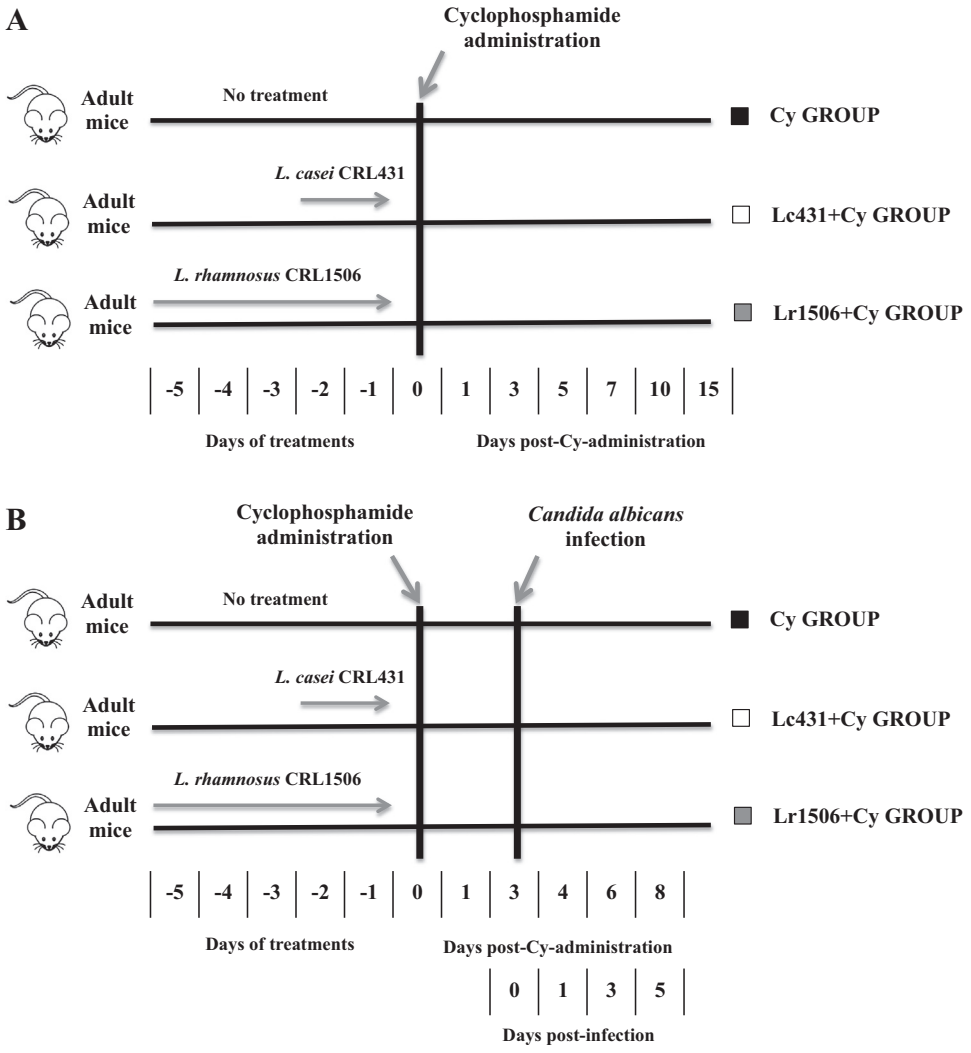

Fig. 1. Experimental protocols used in this work. (A) Male 6-week-old Swiss mice were fed with *Lactobacillus casei* CRL431 (Lc431) or *Lactobacillus rhamnosus* CRL1506 (Lr1506) for 2 consecutive days at a dose of 10^9 cells/mouse/day or 5 consecutive days at a dose of 10^8 cells/mouse/day respectively. The treated groups and the untreated control mice received one dose cyclophosphamide (Cy, 150 mg/kg) intraperitoneally. Determinations were performed on day 0 (before Cy administration) and in different time points after Cy administration, during 15 days. Six animals per each time point per groups were used in the experiments. (B) Male 6-week-old Swiss mice were fed Lc431 or Lr1506 as described before. The treated groups and the untreated control mice received one dose of Cy (150 mg/kg) intraperitoneally. Challenge with *Candida albicans* (intraperitoneal injection of 10^7 cells) was performed on the day 3 after Cy administration, which was considered day 0. Determinations were performed on day 0 (before infection) and on days 1, 3 and 5 post-infection (dpi). Six animals per each time point per groups were used in the experiments.

2.2. Experimental model and feeding procedures

Male 6-week-old Swiss mice were obtained from the closed colony kept at CERELA and they were housed individually in plastic cages at 25 °C. The animals received one dose of 150 mg/kg Cy intraperitoneally on day 0. The dose of Cy administration was selected following preliminary experiments (data not shown). During the 15 days of study, immunosuppressed mice were kept in controlled environmental conditions with light dark cycles of 12 h, isolated in plastic cages coated with metal mesh, in order to minimize contact with ambient particles. Mice were fed balanced conventional diet and sterilized water *ad libitium*.

Different groups of mice were fed with 10^9 cells/mouse/day of *L. casei* CRL431, or 10^8 cells/mouse/day of *L. rhamnosus* CRL1506 for 2 and 5 consecutive days respectively, and before injection of Cy (Lc431 + Cy and Lr1506 + Cy groups). These doses were previously selected as optimal for effects on the immune system [27,28]. Animals without probiotic treatment were used as a control group (Cy group) (Fig. 1A). Six animals per each time point per groups were used in the experiments. All experiments were carried out in compliance with the Guide for Care and Use of Laboratory Animals and approved by the Ethical Committee of Animal Care at CERELA. Determinations described below were performed on day 0 (before Cy administration) and in different time points after Cy administration, during 15 days.

2.3. Histological studies

The femoral bone from the different experimental groups was removed and immediately immersed in 4% paraformaldehyde, decalcified in 50% formic acid and 15% sodium citrate, and processed by standard histological techniques (paraffin-embedding) [26]. 5 μm sections from femurs were stained with Haematoxylin–Eosin (HE).

2.4. Total and differential number of blood and BM leukocytes

Blood samples were obtained through cardiac puncture. BM samples were obtained by flushing the femoral cavity with PBS. Blood and BM total number of leukocytes was performed using a Neubauer counting chamber. The differential cell counts were carried out with smears stained with May Grünwald Giemsa under a light microscope (100×), and absolute numbers were calculated as described previously [23].

2.5. Blood and BM myeloperoxidase activity

The myeloperoxidase (MPO) activity was used to as marker of myeloid cells in BM, and to evaluate functionality of myeloid cells in blood. The measurement of MPO activity was determined using Washburn test. The results were expressed as percentages and score of peroxidase positive (Px+) cells [22].

2.6. Phagocityc and microbicidal activities

Peritoneal macrophages were collected on day 0, and on days 3, 7 and 10 post-Cy administration. The phagocityc activity of macrophages was performed according to Villena et al. [29]. The percentage of phagocytosis was expressed as the percentage of phagocyting macrophages in 200 cells; the counts were performed using an optical microscope.

The microbicidal activity of macrophages was assayed as follows. Polystyrene-adherent resident peritoneal macrophages were cultured with yeast cells at an effector-to-target ratio of 10:1 in a 96-well plate. As a control, yeast cells were cultured without macrophages under similar conditions. After 2 h of incubation, Triton X-100 was added to the wells to give a final concentration of 0.1% (v/v). The contents of the wells were removed by vigorous pipetting and washing with sterile double-distilled H_2O. Subsequently, each sample was serially

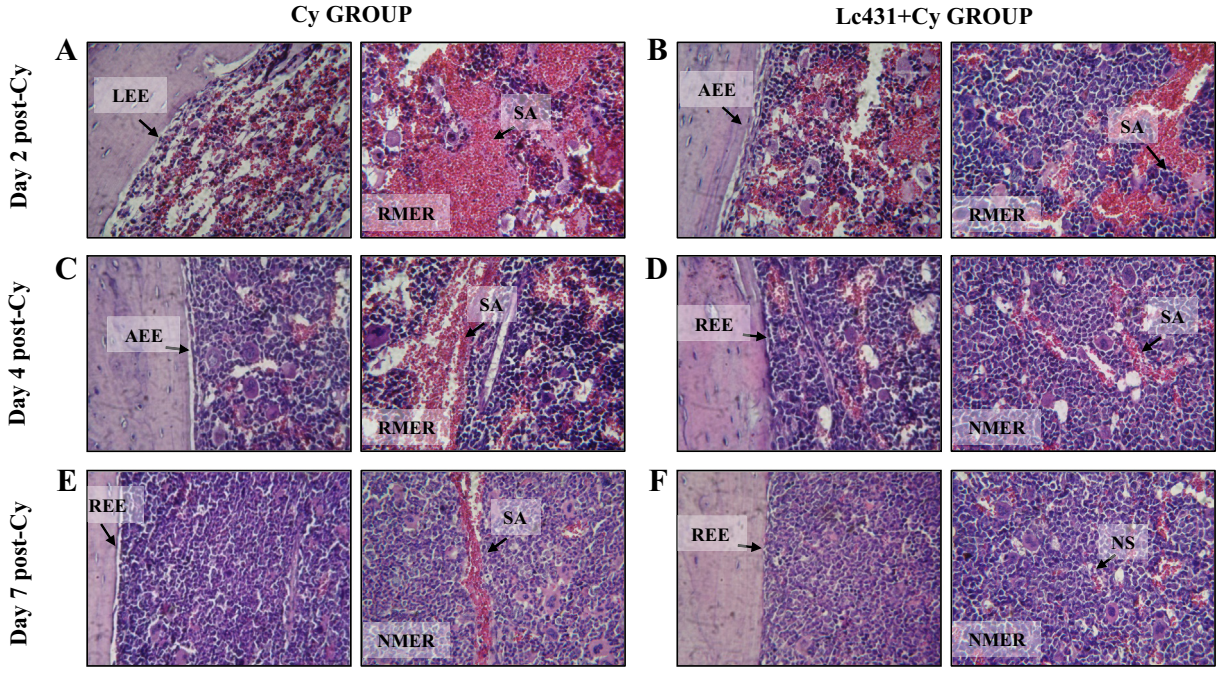

Fig. 2. Effect of lactobacilli on bone marrow tissue damage induced by cyclophosphamide. Male 6-week-old Swiss mice were fed with *Lactobacillus casei* CRL431 (Lc431 + Cy group) or *Lactobacillus rhamnosus* CRL1506 (Lr1506 + Cy group) for 2 consecutive days at a dose of 10^9 cells/mouse/day or 5 consecutive days at a dose of 10^8 cells/mouse/day respectively. The treated groups and the untreated control mice (Cy group) received one dose cyclophosphamide (Cy, 150 mg/kg) intraperitoneally. Histological examination of bone marrow architecture was performed on days 2 (A, B), 4 (C, D) and 7 (E, F) post-Cy administration. Femurs were removed and bone marrow was fixed in paraformaldehyde, decalcified in formic acid and sodium citrate, stained with hematoxylin and eosin, and examined with a light microscope (400× magnification). The results represent data from three independent experiments. Six animals per each time point per groups were used in the experiments. Loss of endostal epithelium (LEE), altered endostal epithelium (AEE), recovered endostal epithelium (REE), reduced myeloid/erythroid ratio (RMER), normal myeloid/erythroid ratio (NMER), sinusoidal alteration (SA), normal sinusoide (NS).

diluted and cultured on Sabouraud agar plates, and the number of viable yeasts able to form a colony was scored after overnight incubation at 37 °C. Microbicidal activity is presented as the percentage of viable yeasts [30].

2.7. Gr-1 and CD34 expression in blood and BM

The maturation of myeloid population was studied by Gr-1 and CD34 expression by flow cytometry as described previously [31]. The blood and BM cell suspensions were obtained as described above. Cells were washed twice in ice-cold PBS and pre-incubated with anti-mouse CD32/CD16 monoclonal antibody (Fc block) for 30 min at 4 °C. Cells were incubated in the antibody mixes for 30 min at 4 °C and washed with PBS. FITC-labeled anti-mouse CD34 and PE-labeled anti-mouse Gr-1 antibody were used. In all cases, cells were then acquired on a FacsCalibur flow cytometer (BD Biosciences) and data were analyzed with FlowJo software (TreeStar).

2.8. Resistance against Candida albicans infection

Candida albicans AV4 was first grown on Sabouraud agar for 18 h; freshly grown colonies were suspended in Sabouraud broth (Oxoid) and incubated at 37 °C overnight. The pathogen was harvested by centrifugation and washed three times with sterile PBS. Mice were infected with an intraperitoneal injection of 200 μl of the inoculum containing 10^7 log-phase cells of *C. albicans* in PBS. This dose was selected following preliminary studies evaluating the mortality rates in Cy-treated mice according to the number of *C. albicans* cells injected (data not shown). The size of the inoculum was confirmed by serial dilutions and quantitative subcultures on blood agar. Challenge with *C. albicans* was performed on the day 3 after Cy injection (Fig. 1B), which was considered day 0 post-infection (dpi).

The effects of Cy and lactobacilli treatments on the resistance against *C. albicans* infection were assessed by monitoring the survival rate. In addition, *C. albicans* colonies counts in liver, spleen and blood were studied. After challenge with *C. albicans*, mice were killed on day 0 (before infection) and on days 1, 3 and 5 post-infection, and their livers and spleens were excised, weighed and homogenized in 5 ml of sterile peptone water. The homogenates were diluted appropriately, plated in duplicate on Sabouraud agar and incubated for 18 h at 37 °C. Results were expressed as log of colony forming unit (CFU)/g of organ. Invasion of the bloodstream by fungi was monitored by obtaining blood samples by cardiac puncture with a heparinized syringe and plating on Sabouraud agar. Results were reported as CFU/ml of blood.

2.9. Immune cell populations during C. albicans infection

On days 1, 3 and 5 post-infection, total and differential number of blood and BM leukocytes and Gr-1 and CD34 expression in blood and BM were determined, using the methodology described previously.

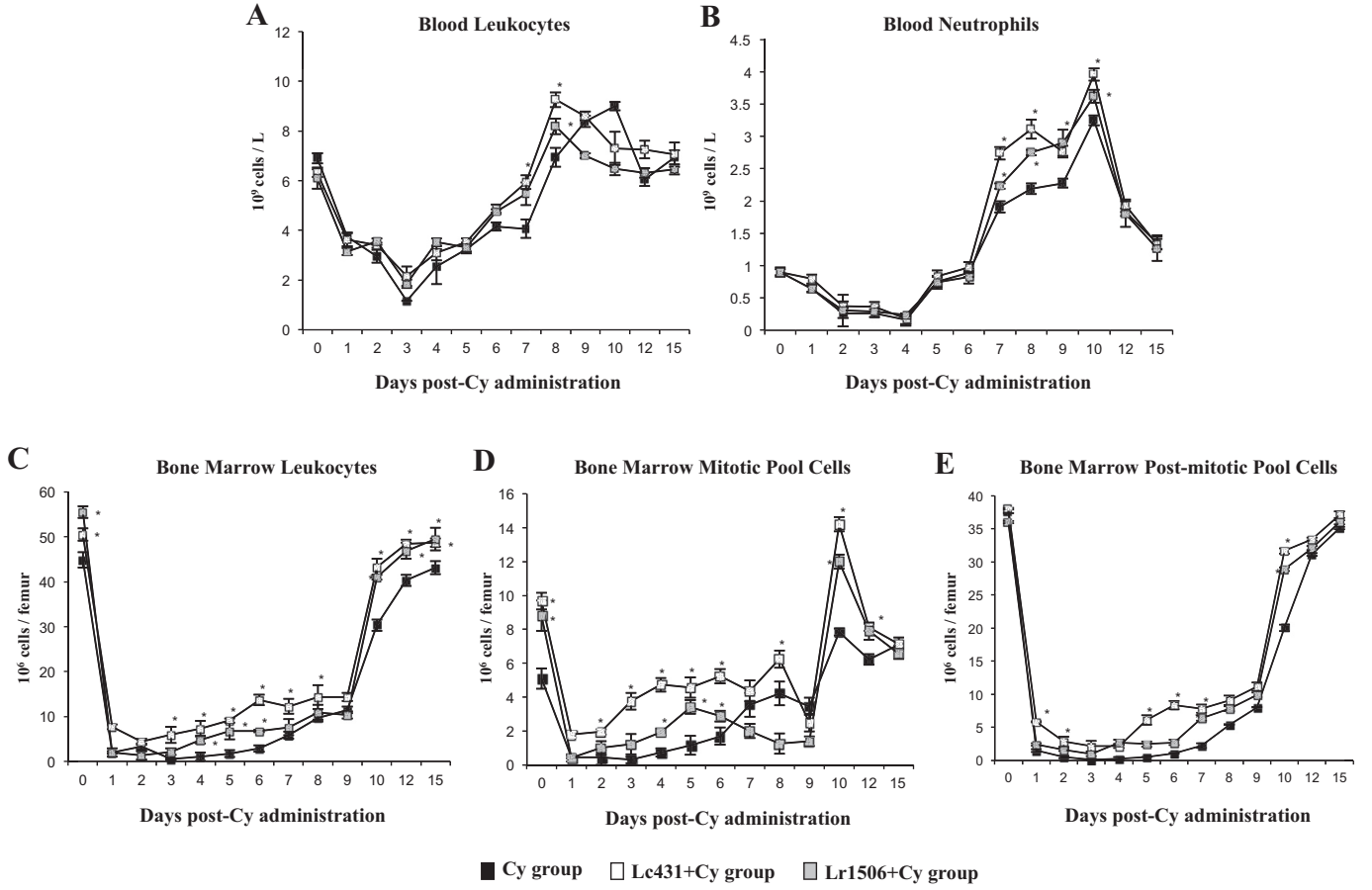

Fig. 3. Effect of lactobacilli on blood and bone marrow leukocytes changes induced by cyclophosphamide. Male 6-week-old Swiss mice were fed with *Lactobacillus casei* CRL431 (Lc431 + Cy group) or *Lactobacillus rhamnosus* CRL1506 (Lr1506 + Cy group) for 2 consecutive days at a dose of 10^9 cells/mouse/day or 5 consecutive days at a dose of 10^8 cells/mouse/day respectively. The treated groups and the untreated control mice (Cy group) received one dose cyclophosphamide (Cy, 150 mg/kg) intraperitoneally. Blood leukocyte (A) and neutrophils (B) counts, and bone marrow leukocytes (C), mitotic pool cells (D), and post-mitotic pool cells (E) counts were performed before Cy administration (day 0) and during 15 days post-Cy administration. The results represent data from three independent experiments. Six animals per each time point per groups were used in the experiments. Results are expressed as mean ± SD. *Significant difference from the control group ($P < 0.05$).

2.10. Statistical analysis

Experiments were performed in triplicate and results were expressed as mean values and standard deviations. A two-way ANOVA test was used. Tukey's test (for pair wise comparisons of the mean of the different groups) was used to test for differences between the groups. Differences were considered significant at $p < 0.05$.

3. Results

3.1. BM histological study

Administration of Cy induced important changes on BM tissue architecture. The most notable changes were observed early after chemotherapy. BM of Cy-treated mice showed hypocellularity with reduction of the myeloid/erythroid ratio. In addition, sinusoidal alteration, increased numbers of mature red blood cells and fat cells, and loss of endostal epithelium were observed on day 2 after Cy administration (Fig. 2A). BM architecture of Cy-treated mice showed similar alterations on day 4 (Fig. 2C) and showed signs of recovery from day 7, as observed by the normalization of cellularity and myeloid/erythroid ratio, as well as the reduction of fat cells and recovery of endostal epithelium (Fig. 2E). BM histology was normal on day 10 (data not shown). In mice treated with lactobacilli, BM alterations during the early days after Cy treatment were significantly lower than controls. In Lc431 + Cy mice the myeloid/erythroid ratio was not severely reduced after Cy administration (Fig. 2B). In addition, BM showed the presence of myeloid and lymphoid cells together with mature red blood cell. Moreover, BM of Lc431 + Cy showed signs of recovery from day 4 (Fig. 2D) and showed a normal histology on day 7 (Fig. 2F). Lr1506 + Cy mice showed BM characteristics similar to those observed in Lc431 + Cy mice during all the studied period (data not shown).

3.2. Blood and BM leukocytes

Cy administration induced a significantly decrease in the number of blood leukocytes from day 1 after treatment, reaching a minimum on day 3. From day 3 there was an increase of leukocytes, which reached normal values on day 8 (Fig. 3A). On days 9 to 11 post-Cy administration, blood leukocytes were higher than normal. These cells returned to basal levels on day 12 (Fig. 3A). Blood neutrophils decreased significantly between days 2 and 6 post-Cy administration (Fig. 3B). From day 7 there was an increase of neutrophils, and these cells reached levels that were significantly higher than normal (Fig. 3B). Neutrophils' number returned to normal values on day 15 post-chemotherapy. Mice treated with lactobacilli strains showed changes in blood leukocytes and neutrophils that were similar to those of the Cy group, showing a marked leukopenia and neutropenia after Cy administration. However, Lc431 + Cy and Lr1506 + Cy groups showed numbers of blood leukocytes that were higher than controls on day 3 (Fig. 3A). In addition, lactobacilli-treated mice showed an earlier increase of blood leukocytes, which reached normal values one day earlier than the Cy control group.

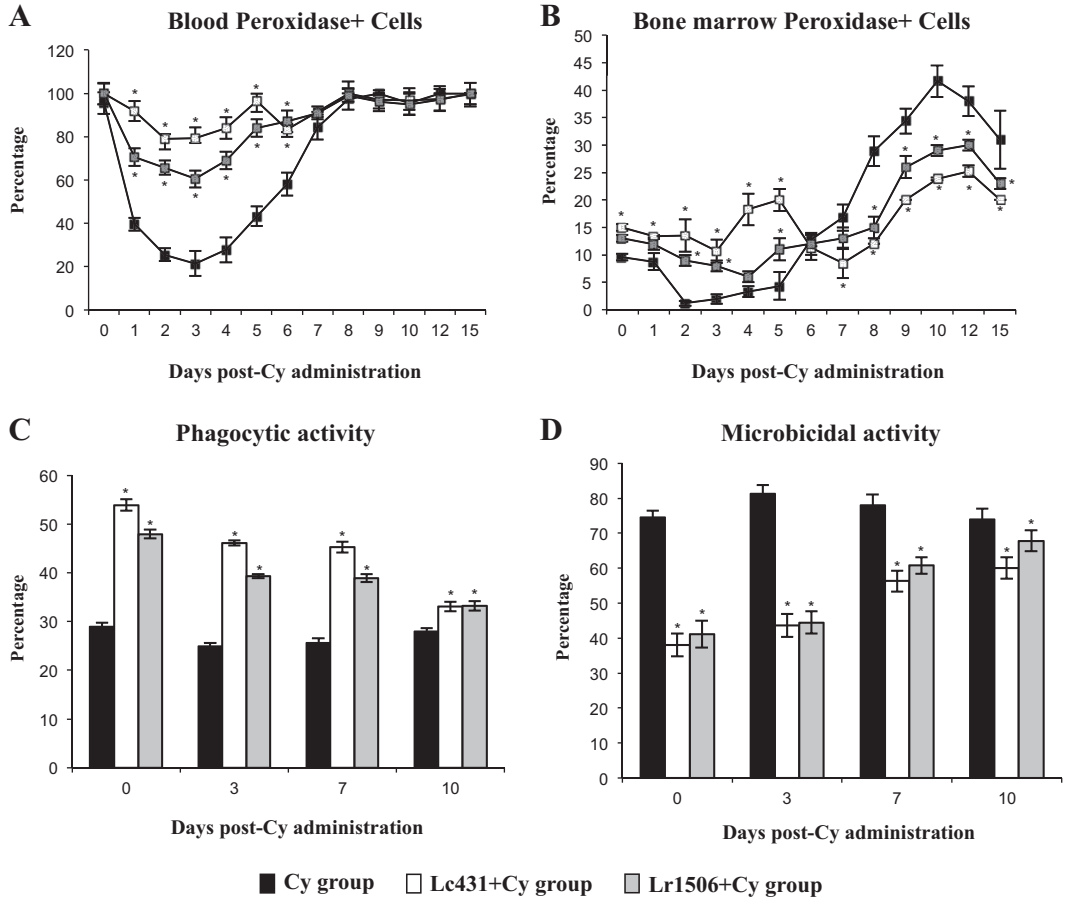

Fig. 4. Effect of lactobacilli and cyclophosphamide on blood and bone marrow myeloperoxidase positive cells and peritoneal macrophages activities. Male 6-week-old Swiss mice were fed with *Lactobacillus casei* CRL431 (Lc431 + Cy group) or *Lactobacillus rhamnosus* CRL1506 (Lr1506 + Cy group) for 2 consecutive days at a dose of 10^9 cells/mouse/day or 5 consecutive days at a dose of 10^8 cells/mouse/day respectively. The treated groups and the untreated control mice (Cy group) received one dose cyclophosphamide (Cy, 150 mg/kg) intraperitoneally. Peroxidase positive cells in blood (A) and bone marrow (B), and the percentage of phagocytosis (C) and the microbicidal activity (E) of peritoneal macrophages were performed before (day 0) and after Cy administration. The results represent data from three independent experiments. Six animals per each time point per groups were used in the experiments. Results are expressed as mean ± SD. *Significant difference from the control group ($P < 0.05$).

Then, on day 8, blood leukocytes increased reaching values that were higher than normal. In Lr1506 + Cy mice blood leukocytes returned to the normal values on day 9, while this effect was observed on day 10 for the Lc431 + Cy group (Fig. 3A). The kinetics of blood neutrophils in lactobacilli-treated mice was similar to the Cy group. The only difference was observed between days 7 and 10 post-Cy administration, when blood neutrophils levels in Lc431 + Cy and Lr1506 + Cy mice were significantly higher than those observed in the Cy controls (Fig. 3B).

In the BM, Cy treatment induced a significant decrease in the total cell counts, which was accompanied by decrease in both mitotic pool and post-mitotic pool cells (Fig. 3C–E). Total numbers of BM leukocytes as well as BM post-mitotic pool cells were significantly decreased from day 1 to 9 post-Cy administration (Fig. 3C, E). From day 9, the numbers of these cells increased and reached normal values on day 15 post-Cy administration. Similarly, the number of BM mitotic pool cells was decreased from day 1 after Cy treatment. Mitotic pool cells showed values under 2.10^6 cells/femur until day 5 (Fig. 3D). From day 6, these cells increased reaching values that were significantly higher than basal between days 10 and 15. Notably, lactobacilli-treated mice showed significantly increased levels of BM total leukocytes and mitotic pool cells before Cy administration (day 0) (Fig. 3C, D). After Cy treatment, BM leukocytes numbers and post-mitotic pool cells in Lc431 + Cy and Lr1506 + Cy mice were similar to Cy controls during all the studied period (Fig. 3D, E). However, values of both cell populations were significantly higher in lactobacilli-treated mice when compared to Cy controls. In the Lc431 + Cy group, BM mitotic pool cells were decreased between days 1 and 3 post-Cy administration, however values were higher than those observed in Cy control mice (Fig. 3D). These cells reached normal values between days 4 and 9 and showed significantly higher levels from day 10 to 12 when compared to normal values. BM mitotic pool cells in Lr1506 + Cy mice showed a different kinetic when compared to the Lc431 + Cy group. Values of these cells in Lr1506 + Cy mice were higher than Cy controls between days 4 and 6, while they were lower than controls between days 7 and 9 after Cy treatment (Fig. 3D). From day 10, BM mitotic pool cells were higher than controls.

3.3. Phagocytic cells activation

Cy administration induced a significant decrease in the percentage of blood Px + cells between days 1 and 6 after the treatment. The minimum value was observed on day 3 that was 4.5 folds lower than the basal value. This parameter reached normal values from day 7 (Fig. 4A). Blood Px + cells were also decreased in Lc431 + Cy and Lr1506 + Cy groups between days 1 and 4 however, these mice showed values that were significantly higher than those observed in the Cy group. Moreover, the percentage of blood Px + cells was normalized from day 5 in lactobacilli-treated mice, two days earlier than Cy controls (Fig. 4A). In the BM, Cy decreased the percentage of Px + cells between days 2 and 5. From day 7 post-Cy administration, this parameter was increased reaching a peak on day 10, and returning to normal values on day 20 (Fig. 4B). Before Cy injection (day 0), mice treated with lactobacilli

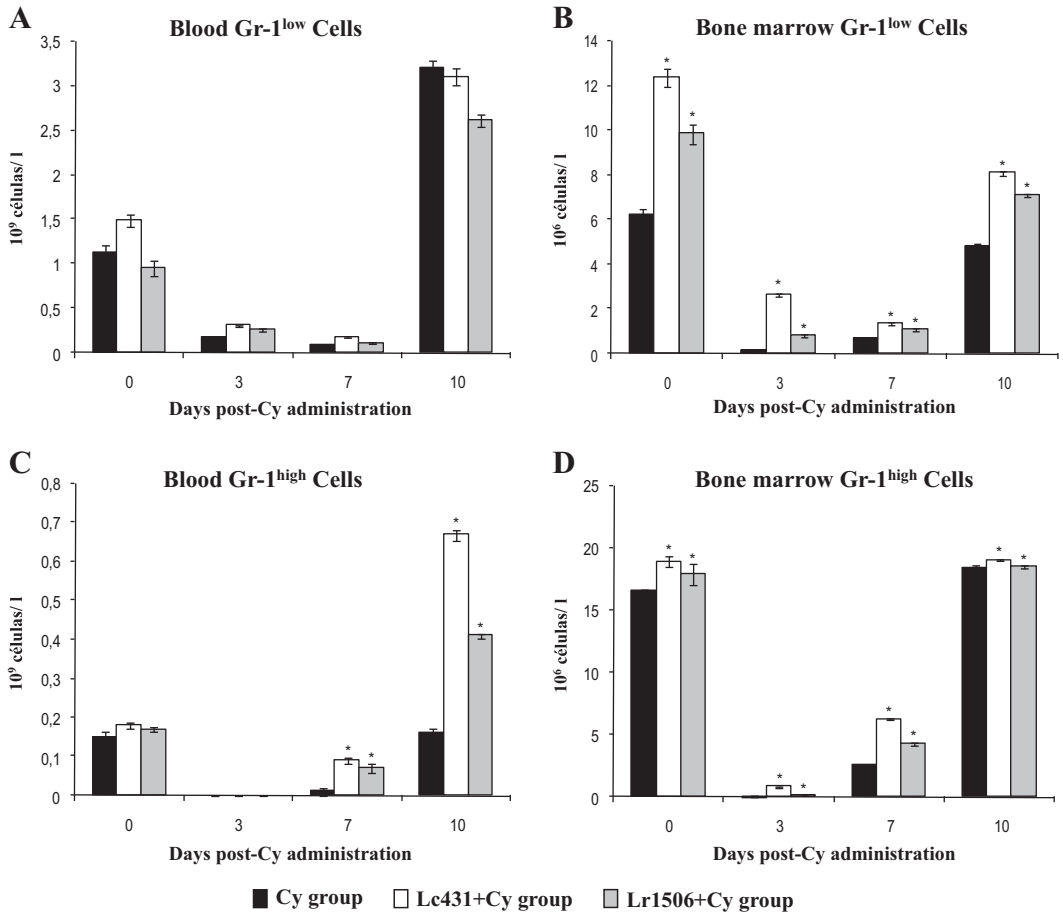

Fig. 5. Effect of lactobacilli on blood and bone marrow Gr-1 cells changes induced by cyclophosphamide. Male 6-week-old Swiss mice were fed with *Lactobacillus casei* CRL431 (Lc431 + Cy group) or *Lactobacillus rhamnosus* CRL1506 (Lr1506 + Cy group) for 2 consecutive days at a dose of 10^9 cells/mouse/day or 5 consecutive days at a dose of 10^8 cells/mouse/day respectively. The treated groups and the untreated control mice (Cy group) received one dose cyclophosphamide (Cy, 150 mg/kg) intraperitoneally. Gr-1^{low} cells in blood (A), and bone marrow (B), and Gr-1^{high} cells in blood (C), and bone marrow (D) were evaluated before (day 0) and after Cy administration. The results represent data from three independent experiments. Six animals per each time point per groups were used in the experiments. Results are expressed as mean ± SD. *Significant difference from the control group ($P < 0.05$).

presented percentages of BM Px + cells that were higher than controls. In Lc431 + Cy mice, Cy administration did not reduce BM Px + cells between days 1 and 4 as observed in Cy controls (Fig. 4B). Moreover, this parameter was significantly increased on days 4 and 5. Between days 6 and 8, Lc431 + Cy mice showed normal values of BM Px + cells while this parameter was again higher than normal between days 9 and 15 post-Cy administration (Fig. 4B). Lr1506 + Cy mice showed changes in BM Px + cells that were similar to those in Lc431 + Cy mice with the exception of days 4 and 5, when these cell population in Lr1506 + Cy group was lower than in Lc431 + Cy mice.

Cy induced no changes in peritoneal macrophages' microbicidal and phagocitic activities (Fig. 4C, D). Lactobacilli treatments significantly increased macrophages activity before Cy administration. In addition, in lactobacilli-treated mice macrophages microbicidal and phagocytic activities remained higher than controls during 10 days after Cy administration (Fig. 4C, D).

3.4. Blood and BM CD34$^+$ and Gr-1$^+$ cells

Studies on day 0 (before Cy injection) showed that lactobacilli treatments did not induce significant changes in blood Gr-1^{high} (mature neutrophils) or Gr-1^{low} cells (Fig. 5A, C). However, both Gr-1^{high} and Gr-1^{low} (blasts and myelocytes) cells in BM were significantly increased in mice treated with Lc431 or Lr1506 with respect to the Cy group (Fig. 5B, D). In all experimental groups, the administration of Cy induced a decrease of blood and BM Gr-1^{high} and Gr-1^{low} cells, which reached its minimum value on day 3 post-Cy administration (Fig. 5). However, groups treated with lactobacilli showed numbers of BM Gr-1^{high} and Gr-1^{low} cells that were higher than those observed in the Cy group during all the studied period (Fig. 5A, B). In addition, blood Gr-1^{high} cells were higher than controls on days 7 to 10 post-Cy administration (Fig. 5C).

We found that approximately 6% (3.6 10^6 cells/femur) of nucleated BM cells from untreated mice were committed early and intermediate progenitors (CD34$^+$ cells) (Fig. 6A). Moreover, the majority of CD34$^+$ cells were also Gr-1^{low} positive, which represent immature myeloid progenitors. Both CD34$^+$ and double-positive CD34$^+$/Gr-1$^+$ cells in BM were significantly increased by lactobacilli treatments before Cy administration (day 0) (Fig. 6). Cy reduced the number of BM CD34$^+$ and CD34$^+$/Gr-1$^+$ cells in all groups; however Lc431 + Cy and Lr1506 + Cy groups showed higher numbers of these cells with respect to the Cy group on day 3 post-Cy administration (Fig. 6).

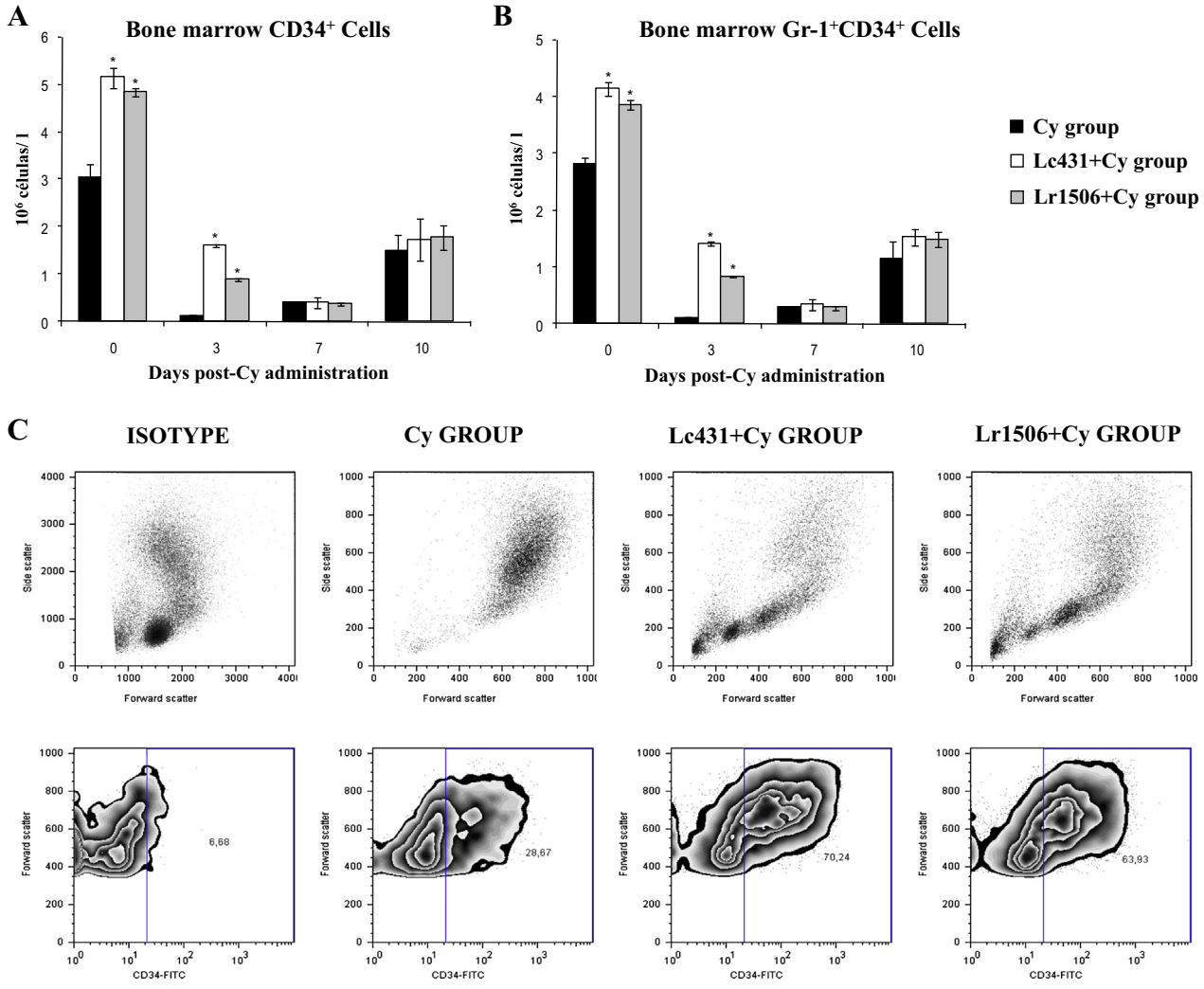

Fig. 6. Effect of lactobacilli on bone marrow CD34 and Gr-1 cells changes induced by cyclophosphamide. Male 6-week-old Swiss mice were fed with *Lactobacillus casei* CRL431 (Lc431 + Cy group) or *Lactobacillus rhamnosus* CRL1506 (Lr1506 + Cy group) for 2 consecutive days at a dose of 10^9 cells/mouse/day or 5 consecutive days at a dose of 10^8 cells/mouse/day respectively. The treated groups and the untreated control mice (Cy group) received one dose cyclophosphamide (Cy, 150 mg/kg) intraperitoneally. Bone marrow CD34$^+$ (A) and CD34$^+$Gr-1$^+$ (B) cells were evaluated before (day 0) and after Cy administration. (C) Representative dot plot for bone marrow CD34$^+$ cells. The results represent data from three independent experiments. Six animals per each time point per groups were used in the experiments. Results are expressed as mean ± SD. *Significant difference from the control group ($P < 0.05$).

3.5. Resistance against C. albicans infection

The effect of Cy and lactobacilli treatments on the resistance against *C. albicans* infection was assessed by monitoring the survival curves of the different groups of mice intraperitoneally challenged with 10^7 yeast cells. This infective dose was selected following preliminary studies evaluating the mortality rates in Cy treated mice according to the number of *C. albicans* cells injected (data not shown). Under this condition, the percentage of mice alive was 40% in the Cy control group after 21 days post-infection, while the percentages of survival in Lc431 + Cy and Lr1506 + Cy groups were 70% and 60% respectively (Fig. 7A). This statistically significant difference indicated that Cy mice are more susceptible to invasive candidiasis than mice treated with lactobacilli. In addition, *C. albicans* cells numbers in liver, spleen, and blood were determined on days 1, 3 and 5 post-infection. The Cy group showed a higher colonization level in liver and spleen compared to the lactobacilli-treated mice (Fig. 7B, C). Moreover, Cy control mice showed positive hemocultures until the last day of the study (day 5 post-infection), while the Lc431 + Cy and Lr1506 + Cy groups were able to eliminate *C. albicans* from blood on day 3 post-infection (Fig. 7D).

3.6. Blood and BM leukocytes, CD34$^+$ and Gr-1$^+$ cells during C. albicans infection

Challenge with *C. albicans* induced slightly increases in leukocyte and neutrophil counts in blood of Cy control mice (Fig. 8A, B). Notably, the groups treated with the lactobacilli showed a significant increase in blood leukocyte and neutrophil numbers after challenge with the opportunistic pathogen (Fig. 8A, B). In BM of Cy control mice, the challenge with *C. albicans* increased total leukocytes numbers as well as mitotic and post-mitotic pool cells from day 3 post-infection, and remained in the same values until day 5 post-infection (Fig. 8C–E). Lactobacilli-treated mice showed a similar kinetic in BM cell changes when compared to Cy controls, however, total leukocytes counts and BM mitotic pool cells were higher than controls on day 5 post-infection (Fig. 8C, D). In addition, Lr1506 + Cy mice showed higher levels of BM post-mitotic pool cells than controls (Fig. 8E).

Blood Gr-1^{low} and Gr-1^{high} cells were significantly increased after the challenge with *C. albicans* in all the experimental groups (Fig. 9A, C). However, Lc431 + Cy showed values that were higher than Cy controls on days 3 to 5 post-infection. The Lr1506 + Cy group showed increased values of Gr-1^{low} and Gr-1^{high} cells on days 1 and 5 post-infection respectively (Fig. 9A, C). *C. albicans* infection induced a marked increase in the number of BM Gr-1^{low} cells while a slight increase of BM Gr-1^{high} cells was observed (Fig. 9D). In Lc431 + Cy mice, BM Gr-1^{low} cells were significantly higher than Cy control group on day 1 post-infection while these cells were lower than Cy controls on day 5 post-infection (Fig. 9B, D). In addition, Lr1506 + Cy mice showed higher values of BM Gr-1^{low} cells on day 3 post-infection (Fig. 9B). Both lactobacilli-treated mice showed significantly higher levels of BM Gr-1^{high} cells on day 5 post-infection (Fig. 9D).

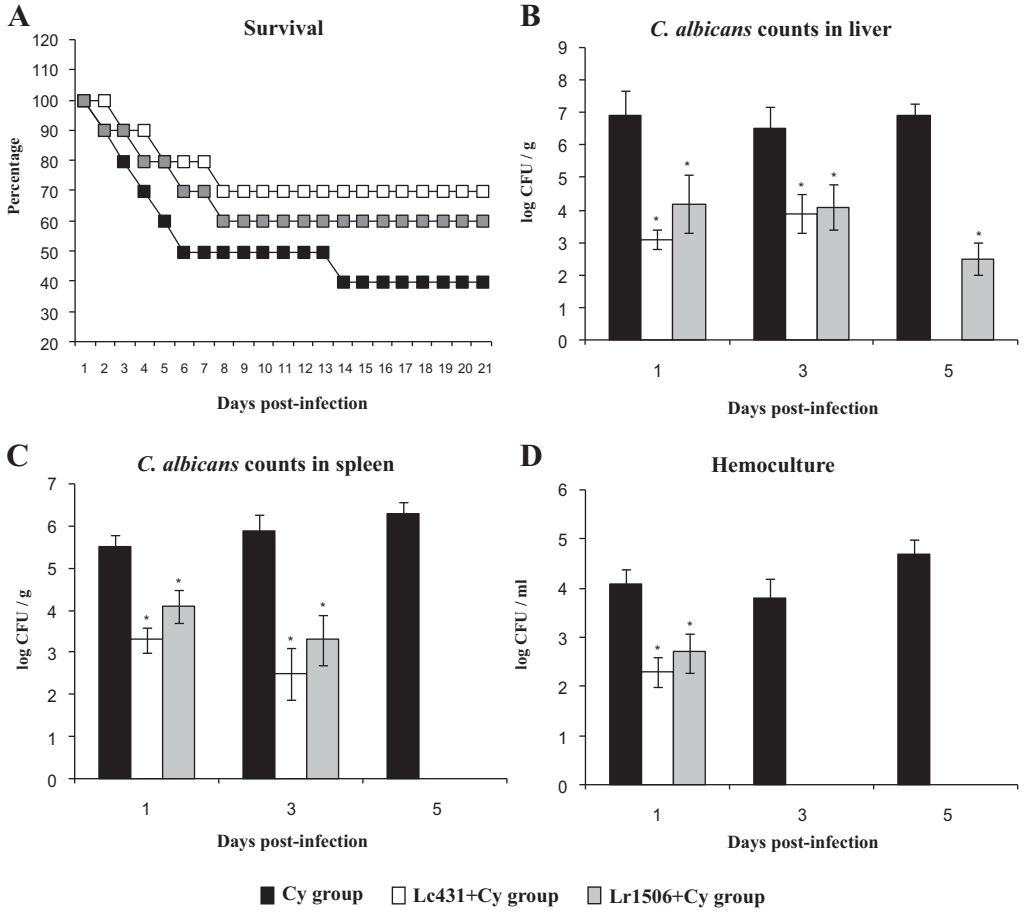

Fig. 7. Effect of lactobacilli on resistance against *Candida albicans* infection in cyclophosphamide-treated mice. Male 6-week-old Swiss mice were fed with *Lactobacillus casei* CRL431 (Lc431 + Cy group) or *Lactobacillus rhamnosus* CRL1506 (Lr1506 + Cy group) for 2 consecutive days at a dose of 10^9 cells/mouse/day or 5 consecutive days at a dose of 10^8 cells/mouse/day respectively. The treated groups and the untreated control mice (Cy group) received one dose cyclophosphamide (Cy, 150 mg/kg) intraperitoneally. Challenge with *Candida albicans* (intraperitoneal injection of 10^7 cells) was performed on the day 3 after Cy administration, which was considered day 0. Survival of infected mice (A). *C. albicans* cell counts in liver (B), spleen (C), and blood (D). The results represent data from three independent experiments. Six animals per each time point per groups were used in the experiments. Results are expressed as mean ± SD. *Significant difference from the control group ($P < 0.05$).

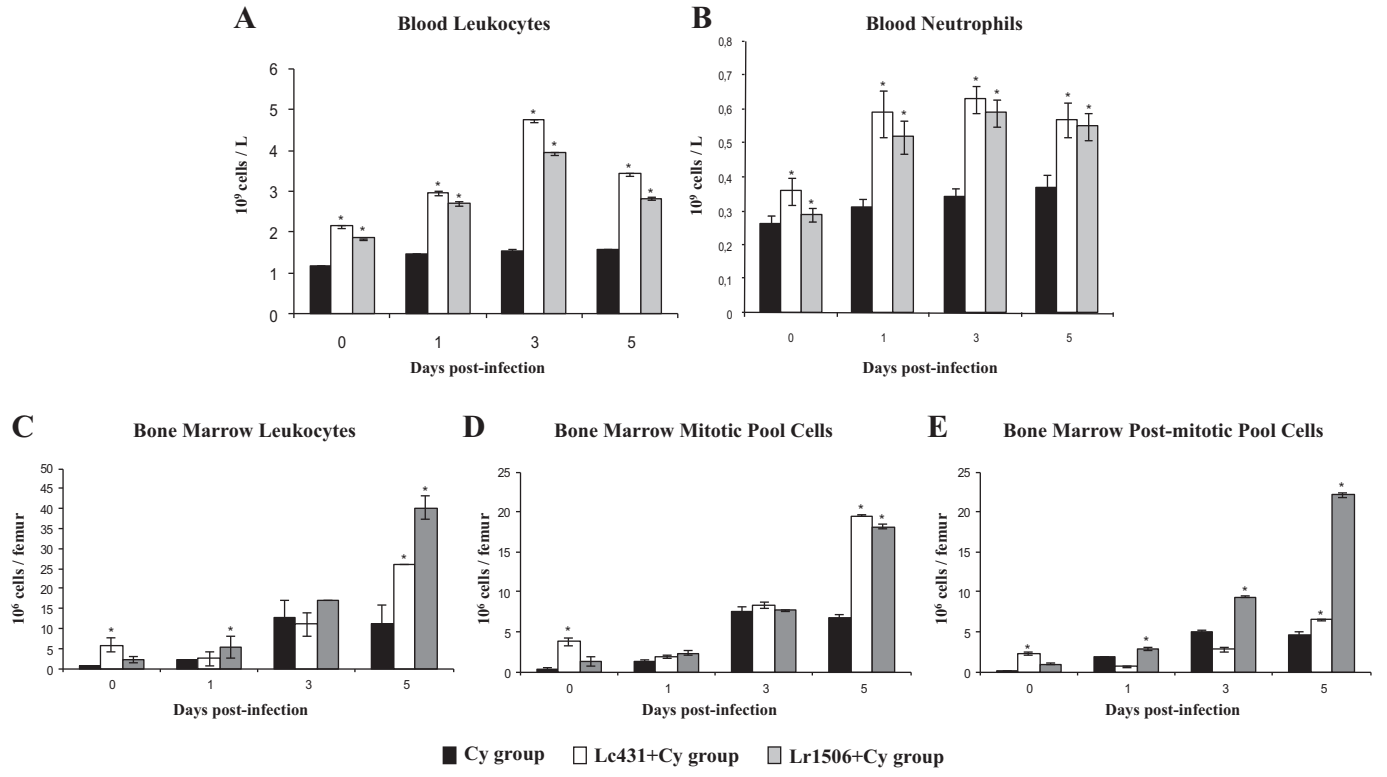

Fig. 8. Effect of lactobacilli on blood and bone marrow leukocytes changes after *Candida albicans* challenge in cyclophosphamide-treated mice. Male 6-week-old Swiss mice were fed with *Lactobacillus casei* CRL431 (Lc431 + Cy group) or *Lactobacillus rhamnosus* CRL1506 (Lr1506 + Cy group) for 2 consecutive days at a dose of 10^9 cells/mouse/day or 5 consecutive days at a dose of 10^8 cells/mouse/day respectively. The treated groups and the untreated control mice (Cy group) received one dose cyclophosphamide (Cy, 150 mg/kg) intraperitoneally. Challenge with *Candida albicans* (intraperitoneal injection of 10^7 cells) was performed on the day 3 after Cy administration, which was considered day 0. Determinations were performed on day 0 (before infection) and on days 1, 3 and 5 post-infection. Blood leukocyte (A) and neutrophil (B) counts, and bone marrow leukocytes (C), mitotic pool cells (D), and post-mitotic pool cells (E) counts. The results represent data from three independent experiments. Six animals per each time point per groups were used in the experiments. Results are expressed as mean ± SD. *Significant difference from the control group ($P < 0.05$).

Finally we studied blood and BM $CD34^+$ and $CD34^+/Gr\text{-}1^+$ cell populations after the challenge with *C. albicans*. As shown in Fig. 10, infection induced a significant increase of BM $CD34^+$ cells and slightly augmented blood $CD34^+$ and $CD34^+/Gr\text{-}1^+$ cell populations in Cy mice. Notably, blood $CD34^+$ and $CD34^+/Gr\text{-}1^+$ cells in the Lr1506 + Cy group reached values that were significantly higher than Cy controls on day 3 post-infection (Fig. 10A, B). In addition, BM $CD34^+$ and $CD34^+/Gr\text{-}1^+$ cells in Lr1506 + Cy mice were higher than Cy controls on day 5 post-infection (Fig. 10C, D). Lc431 + Cy mice showed levels of blood and BM $CD34^+/Gr\text{-}1^+$ cells that were higher than controls on days 3 (Fig. 10B) and 5 (Fig. 10D) post-infection respectively. Moreover, BM $CD34^+$ cells increased on day 5 post-infection in this group when compared to Cy controls (Fig. 10C).

4. Discussion

The development of blood and immune cells occurs in the complex microenvironment of the BM. This process is maintained by the immunoregulatory activity of stromal cells and immune cells through the production of cytokines and growth factors [32]. Thus, the normal function of the immune system is closely related to hematopoiesis. Myelosuppression and immunosuppression induced by chemotherapy are major problems restricting the therapeutic effects during cancer treatment. Therefore, new strategies to prevent or reduce the toxicity of chemotherapy at the level of hematopoiesis would be essential to improve therapeutic effects and prognosis for these patients.

In the present study, a single dose of Cy (150 mg/kg) to adult immunocompetent mice was able to induce a state of immunosuppression with significant alterations of hematopoiesis. In line with the results presented in this study, it was first described in histological studies that the BM of animals treated with Cy had a drastic deterioration of medullary tissue architecture, evidenced by marked hypocellularity and impairment of myeloid and erythroid compartments [33]. These changes correlated with significant reduction of the total counts of BM cells as reported by several works [14,33–37]. In addition, in our mice model, Cy administration induced a significant reduction of myeloid cell counts and Px + cells in both blood and BM. We observed that chemotherapy affected both myeloid cells capable of replication (mitotic pool cells), and those with ability to differentiate and mature (post-mitotic pool cells). Moreover, flow cytometry studies demonstrated that administration of a single dose of Cy was able to induce a reduction of BM $CD34^+$ and $CD34^+Gr\text{-}1^+$ cells, which are committed precursors, accompanied by a significant decrease of blood and BM $Gr\text{-}1^{high}$ and $Gr\text{-}1^{low}$ cells. These findings are consistent with previous results from Yankelevich et al. [12] that described alterations of myeloid cells and their precursors after treatment with Cy (200 mg/kg). Furthermore, Zhu et al. [38] demonstrated that daily administration of 100 mg/kg of Cy for 3 days causes deterioration of the clonogenic activity of BM hematopoietic precursors. Moreover, similarly to our present work, several other studies showed alterations in myelopoiesis produced by the administration of Cy together with a marked leucopenia and neutropenia after Cy treatment [14,39–42], accompanied by a reduction in the percentage of Px + cells in blood. It is also known that treatment with Cy affects functionality of cells of innate immune responses. In contrast to other studies [14,37,40,42], no modifications in peritoneal macrophages' phagocytic activity were observed in Cy-treated mice. These discrepancies are probably related to differences in the doses of Cy used to induce immunosuppression. Therefore, in the model of

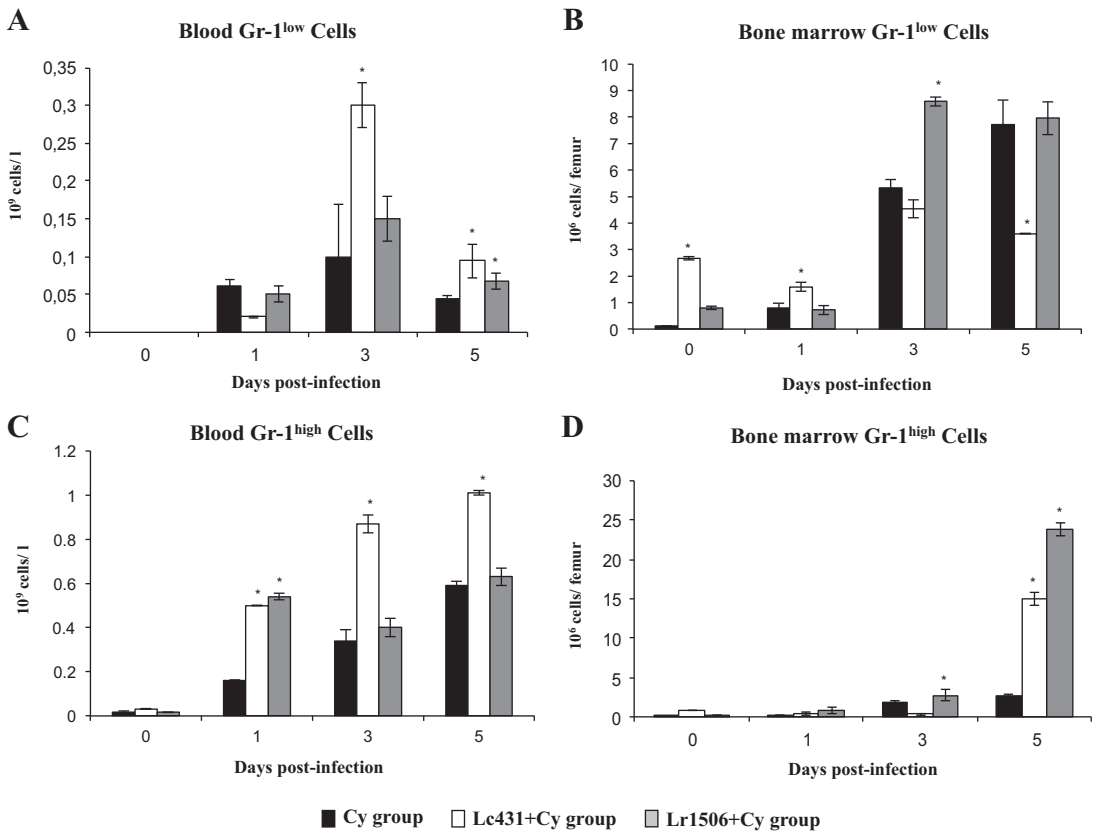

Fig. 9. Effect of lactobacilli on blood and bone marrow Gr-1 cells after *Candida albicans* challenge in cyclophosphamide-treated mice. Male 6-week-old Swiss mice were fed with *Lactobacillus casei* CRL431 (Lc431 + Cy group) or *Lactobacillus rhamnosus* CRL1506 (Lr1506 + Cy group) for 2 consecutive days at a dose of 10^9 cells/mouse/day or 5 consecutive days at a dose of 10^8 cells/mouse/day respectively. The treated groups and the untreated control mice (Cy group) received one dose cyclophosphamide (Cy, 150 mg/kg) intraperitoneally. Challenge with *Candida albicans* (intraperitoneal injection of 10^7 cells) was performed on the day 3 after Cy administration, which was considered day 0. Determinations were performed on day 0 (before infection) and on days 1, 3 and 5 post-infection. Gr-1^{low} cells in blood (A), and bone marrow (B), and Gr-1^{high} cells in blood (C), and bone marrow (D). The results represent data from three independent experiments. Six animals per each time point per groups were used in the experiments. Results are expressed as mean ± SD. *Significant difference from the control group ($P < 0.05$).

immunosuppression by Cy used in this work, the most important side effect was the interference with the proliferation and differentiation of BM cells causing myelosuppression, while no effect was observed in the functional capacity of already mature myeloid cells.

Safe immunomodulatory agents able to reduce myelosuppression and improve immune response in Cy-treated hosts are being investigated. For example, some plant extracts with specific biological properties, administered to Cy-immunosuppressed animals, are known to increase immune responses [41,43,44]. In addition, it was described that some polysaccharides from diverse origins are able to protect against myelosuppression and/or immunosuppression caused by Cy [36,38,45–47]. However, the potential beneficial effect of probiotic LAB in the recovery of the impaired immune response after treatment with immunosuppressive drugs has been less studied. In this regard, it has been shown that a probiotic strain of *L. plantarum* is able to stimulate the proliferation of splenocytes in Cy-immunocompromised hosts in response to LPS [39]. Recently, other researchers showed that both *L. plantarum* HY7712 and *L. casei* HY7213 accelerate the recovery of the immunosuppression induced by Cy. Both HY7712 and HY7213 strains improved activity of NK cells and cytotoxic T lymphocytes derived from BM and spleen; and restored the phagocytic activity of peritoneal macrophages [14,37]. To our knowledge, no other study described the beneficial effect of probiotic bacteria during Cy treatment. In the present work, we demonstrated that the preventive administration of *L. casei* CRL431 or *L. rhamnosus* CRL1506, prior to injection of Cy, was effective in reducing the alterations of BM myelopoiesis caused by chemotherapy.

Lactobacilli treatments had a significant influence in BM immature myeloid progenitors. Before Cy administration, orally administered *L. casei* CRL431 or *L. rhamnosus* CRL1506 was able to significantly increase the numbers of BM $CD34^+$ hematopoietic progenitors and immature Gr-1^{low} myeloid cells. Then, the BM of lactobacilli-treated mice had higher numbers of immature myeloid progenitors when the animals received Cy, which would explain the higher capacity of these groups to return to normality. In fact, after Cy treatment a remarkable reduction of the BM architecture alteration was observed in lactobacilli-treated mice. Again, the most significant change induced by lactobacilli was observed in myeloid cells of the mitotic pool and BM myeloid precursors ($CD34^+$ and Gr-1^+ cells), since the preventive administration of *L. casei* or *L. rhamnosus* induced an early recovery of these cells when compared to Cy controls. These findings are consistent with the effects induced by *L. casei* CRL431 and other probiotic strains on BM mitotic pool cells of mice immunosuppressed by malnutrition [23,26,31]. In addition, the changes induced in the BM by probiotic lactobacilli administration were reflected in blood, since a quick recovery in the numbers of leukocytes, Px^+ cells, and neutrophils was observed. Therefore, the capacity of some orally administered probiotic lactobacilli to influence cell division in immature myeloid BM cells would be useful to diminish the cytotoxic effects of Cy and accelerate the recovery of these cell populations after chemotherapy.

We also verified whether the capacity of *L. casei* CRL431 or *L. rhamnosus* CRL1506 to accelerate the recovery of myeloid immune cells conferred increased protection of Cy-treated mice against infections. For this purpose Cy-immunosuppressed mice were challenged with *C. albicans* as typical opportunistic pathogen in this kind of hosts [48]. Results showed that Cy administration significantly increased the overall susceptibility of mice to *C. albicans* infection. We found

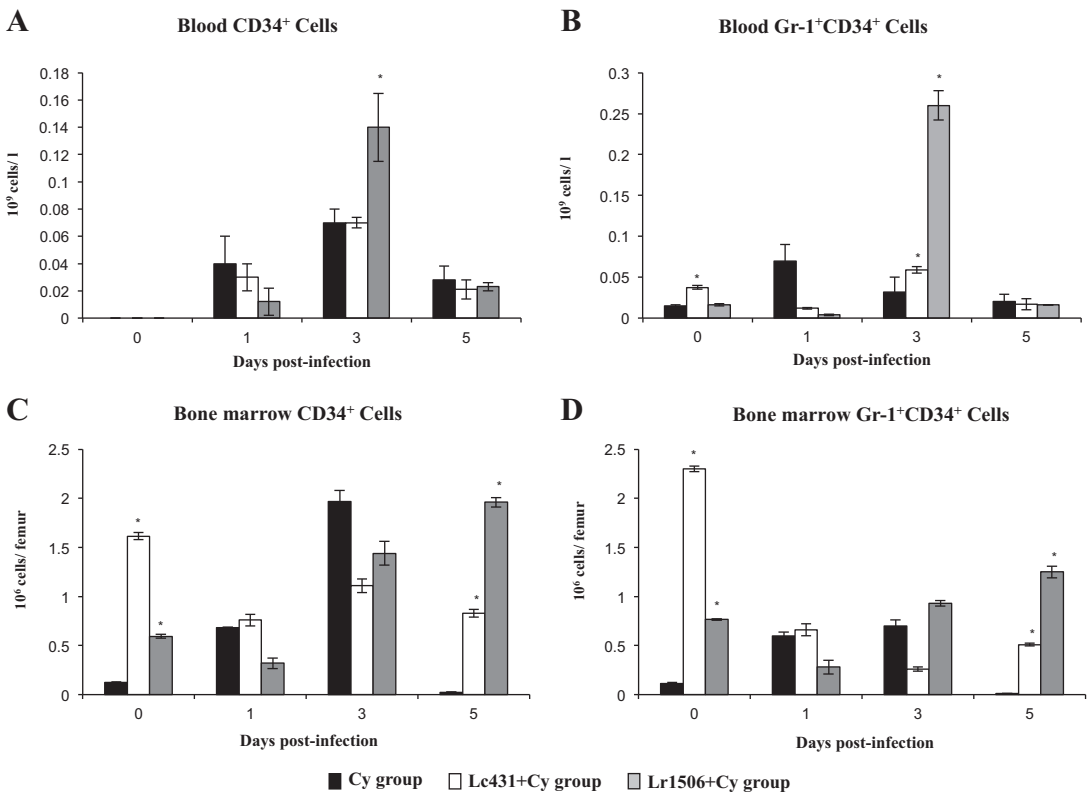

Fig. 10. Effect of lactobacilli on blood and bone marrow CD34 and Gr-1 cells after *Candida albicans* challenge in cyclophosphamide-treated mice. Male 6-week-old Swiss mice were fed with *Lactobacillus casei* CRL431 (Lc431 + Cy group) or *Lactobacillus rhamnosus* CRL1506 (Lr1506 + Cy group) for 2 consecutive days at a dose of 10^9 cells/mouse/day or 5 consecutive days at a dose of 10^8 cells/mouse/day respectively. The treated groups and the untreated control mice (Cy group) received one dose cyclophosphamide (Cy, 150 mg/kg) intraperitoneally. Challenge with *Candida albicans* (intraperitoneal injection of 10^7 cells) was performed on the day 3 after Cy administration, which was considered day 0. Determinations were performed on day 0 (before infection) and on days 1, 3 and 5 post-infection. Blood $CD34^+$ (A), and $CD34^+Gr\text{-}1^+$ (B) cells and bone marrow $CD34^+$ (C), and $CD34^+Gr\text{-}1^+$ (D) cells. The results represent data from three independent experiments. Six animals per each time point per groups were used in the experiments. Results are expressed as mean ± SD. *Significant difference from the control group ($P < 0.05$).

decreased survival rates in Cy control group mice as well as higher fungal loads in liver, spleen and blood. On the contrary, when mice received the preventive administration of *L. casei* CRL431 or *L. rhamnosus* CRL1506, their resistance to *C. albicans* challenge was significantly improved. Experimental models evaluating host defense against *C. albicans* have shown that both innate resistance and acquired cell-mediated immunity are involved in the anti-*Candida* response. However, phagocytic cells (polymorphonuclear neutrophils and mononuclear phagocytes) are necessary for preventing *C. albicans* dissemination [49]. In this sense, we showed that treatment with probiotic lactobacilli improved the myelopoietic response necessary to limit and eradicate *C. albicans* infection. These results are consistent with previous studies in which immunosuppressed malnourished treated with probiotic LAB showed a more efficient immune and myelopoietic response against challenge with *S. pneumoniae* [22,24,31] or *C. albicans* [29].

How orally administered lactobacilli influence myelopoiesis in Cy-treated mice is an interesting topic for future research. In this regard, we have made some advances in our mice model of immunosuppression by malnutrition. The release of neutrophils from the BM is strictly regulated by chemokines and chemokine receptors. The chemokine CXCL12 (stromal derived factor-1, SDF-1), through interaction with its major receptor CXCR4, plays a key role in controlling neutrophil homeostasis in the steady-state [50,51]. Treatment with G-CSF results in a decrease in CXCL12 expression in the BM [52]. These observations suggest the hypothesis that disruption of CXCR4 signaling is a key step mediating neutrophil release by G-CSF. We showed that the respiratory challenge with *S. pneumonie* increased levels of G-CSF mRNA and reduced CXCL12 expression in the BM of immunocompetent mice while peripheral circulation through the CXCR4/CXCL12-associated mechanism was significantly impaired in malnourished mice [53]. Moreover, we have recently demonstrated that the probiotic strain *L. rhamnosus* CRL1505 improves emergency granulopoiesis and that CXCR4/CXCR12 signaling would be involved in this effect [53].

Other keys to understand the mechanism of probiotic actions on the BM could be inferred from some recent studies evaluating the effect of microbiota in normal hematopoiesis. There is evidence that, during colonization of gut mucosa by commensal bacteria, peptidoglycan is constantly turned over and either excreted or translocated across the intestinal mucosa into the circulation. Translocated peptidoglycan can accumulate in the BM since it was reported that this molecule could be detected in the neutrophil fraction [54]. Moreover, depletion of the microbiota markedly lowered systemic peptidoglycan concentrations, which correlated with less killing of *S. pneumoniae* and *S. aureus* by BM-derived neutrophils. Then, these data showed a mechanism for systemic immunomodulation by the microbiota and provided a direct example of a probable mechanism for a probiotic activity of the microbiota, demonstrating that translocated microbial products benefit the host by modulating BM myelopoiesis and enhancing systemic innate immune function. In addition, it was recently found that fermentable fibers in the diet promote the outgrowth of gut bacteria from the Bacteroidetes phylum, leading to increased local and systemic levels of short-chain fatty acids, which in turn influence dendritic cell (DC) hematopoiesis and functionality [55]. Authors showed that short-chain fatty acids, such as propionate, enhance the hematopoiesis of DCs precursors from BM, and these DCs exhibit an impaired ability to activate Th2 effector cells in the lung. As a consequence, allergic airway inflammation cannot be sustained and rapidly resolves. The work highlighted the importance of dietary fermentable fibers and microbiota, and

provided a cellular mechanism for an intestinal–BM–lung axis in controlling allergic airway inflammation, similarly to our previous works with probiotic bacteria [23,26,31,53].

In conclusion, we demonstrate for the first time that the preventive treatment with immunomodulatory lactobacilli such as *L. casei* CRL431 or *L. rhamnosus* CRL1506 [22,28] is able to increase immature myeloid progenitors in the BM, allowing an early recovery of myeloid cells after Cy administration. Probiotic LAB are also capable to induce an early recovery of neutrophils in blood, improve phagocytic cells recruitment to infectious sites and increase the resistance against opportunistic pathogens. Although deeper studies regarding the cellular and molecular mechanisms of probiotic actions are needed, these findings support the idea that strains like CRL431 and CRL1506 may accelerate the recovery of cyclophosphamide-caused immunosuppression by immunopotentiating myeloid cells, without evident side effects. Then, probiotic lactobacilli could be used as alternatives for lessening chemotherapy-induced immunosuppression in cancer patients.

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