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ORIGINAL ARTICLE

Nasal administration of *Lactococcus lactis* improves local and systemic immune responses against *Streptococcus pneumoniae*

Marcela Medina[1,2], Julio Villena[1,2], Susana Salva[1], Elisa Vintiñi[2], Philippe Langella[3] and Susana Alvarez[1,2]

[1]Faculty of Biochemistry, Chemistry and Pharmacy, National University of Tucuman, [2]Reference Center for Lactobacilli (CERELA-CONICET), Tucuman, Argentina; and [3]Unit of Ecology and Physiology of the Digestive System, INRA, Jouy en Josas Cedex, France

ABSTRACT

Lactococcus lactis* NZ9000 is a non-pathogenic non-invasive bacterium extensively used for the delivery of antigens and cytokines at the mucosal level. However, there are no reports concerning the per se immunomodulatory capacity of this strain. The aim of the present study was to investigate the intrinsic immunostimulating properties of the nasal administration of *L. lactis* NZ9000 in a pneumococcal infection model. Mice were preventively treated with *L. lactis* (2, 5 or 7 days with 10^8 cells/day per mouse) and then challenged with *Streptococcus pneumoniae*. The local and the systemic immune responses were evaluated. Our results showed that nasal administration of *L. lactis* for 5 days (LLN5d) increased the clearance rate of *S. pneumoniae* from lung and prevented the dissemination of pneumococci into blood. This effect coincided with an upregulation of the innate and specific immune responses in both local and systemic compartments. LLN5d increased phagocyte activation in lung, blood and bone marrow, determined by NBT and peroxidase tests. Anti-pneumococcal immunoglobulin (Ig)A in bronchoalveolar lavages (BAL) and IgG in BAL and serum were increased in the LLN5d group. Lung tissue injury was reduced by LLN5d treatment as revealed by histopathological examination and albumin concentration and lactate dehydrogenase activity in BAL. The adjuvant effect of *L. lactis* in our infection model would be an important advantage for its use as a delivery vehicle of pneumococcal proteins and nasal immunization with recombinant *L. lactis* emerges as an effective route of vaccination for both systemic and mucosal immunity against pneumococcal infection.

Key words adjuvant, *Lactococcus lactis*, nasal administration, pneumococcal infection.

Bacterial respiratory diseases remain a major cause of morbidity and mortality in both developed and developing communities. In Argentina, *Streptococcus pneumoniae* is one of the most important respiratory pathogens (1). This bacterium can migrate to mucosal areas that are normally sterile and lead to a variety of mucosal infections such as otitis media or pneumonia. In addition, pneumococci can induce invasive cases of septicemia and meningitis. The high morbidity associated with this microorganism remains a leading cause of mortality in the young and the elderly (2). The deaths caused by *S. pneumoniae* are concentrated in developing countries, where the rates are

Correspondence
Susana Alvarez, Centro de Referencia para Lactobacilos (CERELA-CONICET), Chacabuco 145, 4000 San Miguel de Tucumán, Tucumán, Argentina. Fax: +54 381 400 5600; email: salvarez@cerela.org.ar

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List of Abbreviations: BAL, bronchoalveolar lavage; LAB, lactic acid bacteria; LLN, nasal administration of *Lactococcus lactis*; NBT, nitro blue tetrazolium.

four- to 100-fold higher than in developed countries. This is due to multiple causes that are risk factors for pneumonia: low weight of newborns, severe malnutrition, environmental pollution, lack of specific vaccines and poor health systems (1).

The recent dramatic rise in the prevalence of clinical isolates that are multi-drug resistant raises the possibility that antibiotic therapy may become less effective in the treatment of pneumococcal diseases. The current vaccine for adults, which includes 23 capsular polysaccharides, is far from ideal. The older age groups, which are the most highly exposed to the risk of infection, show the least efficacious responses (3). Newer vaccines, in which a smaller number of polysaccharides are chemically conjugated to non-pneumococcal proteins, elicit improved immune responses in such high-risk groups. However, conjugate vaccines are rather expensive to produce, which may ultimately limit their use (3). In addition, as the distribution of serotypes responsible for pneumococcal diseases differs in various regions of the world, the spectrum of coverage provided by conjugate vaccines may not be the same. This is particularly relevant in developing countries, where pneumococcal serotypes responsible for both invasive and mucosal diseases differ from those in industrialized countries (4). Moreover, conjugate vaccines are expected to have a limited period of maximum impact because of niche replacement by *S. pneumoniae* of serotypes not included in the vaccine.

Improvements in engineering technology afford new possibilities for vaccine development, extending the potential use of LAB as biotherapeutic agents from a few natural strains to recombinant strains (5). The lactic acid bacterium model *Lactococcus lactis* NZ9000, extensively engineered for the production of heterologous proteins (6, 7) that include bacterial and viral antigens (8), has proved to elicit an immune response after inoculation by different mucosal routes (9). The development of natural antibodies to different strains of *L. lactis* has been investigated and the relative immunogenicity of individual bacterial proteins varies according to mouse strain and route of inoculation (10).

The route of administration of a vaccine is decisive in the presentation of the antigen and in the localization of responsiveness. Classic routes of application (i.m. or s.c.) mainly lead to systemic immunization. However, as most pathogens enter the body via the mucosa of the gastrointestinal, respiratory or urogenital tracts, protection of individuals against invasion of infectious agents via the mucosa should preferably include activation of the mucosal immune system (11).

Our group has experience in using LAB to improve the immune response against respiratory pathogens (*Pseudomonas aeuruginosa* and *S. pneumoniae*) in normal and immunocompromised mice (12–14). Our aim is to construct recombinant strains of *L. lactis* that express pneumococcal immunogenic proteins in order to provide protective immunity for the host. For the present study, we chose *L. lactis* NZ9000, which has been extensively engineered for the production of heterologous proteins using the nisin controlled gene expression (NICE) system, and assessed its per se adjuvant capacity. Numerous studies have demonstrated that the nasal administration of an antigen is the most efficient route to elicit optimal protective immunity in both the mucosal and the systemic immune compartments, so that nasal immunization is commonly used to induce immunity along the respiratory tract (15). Thus, the aim of the present research was to evaluate the effect of nasal *L. lactis* administration on the local and the systemic immune response using a pneumococcal infection model.

MATERIALS AND METHODS

Animals

Six-week-old male Swiss albino mice (22–28 g) were obtained from the closed colony at CERELA. Animals were housed in plastic cages and environmental conditions were kept constant, in agreement with the standards for animal housing. Each experimental group consisted of 25–30 mice (5–6 mice per group at each time point), which were housed individually during the experiments. The Ethical Committee of Animal Care at CERELA approved experimental protocols.

Microorganisms

Lactococcus lactis NZ9000 was obtained from the NIZO collection. *L. lactis* was grown for 8 hr at 37 °C (final log phase) in M17 broth (Difco Laboratories, Lawrence, MA, USA); the bacteria were harvested by centrifugation at 3000 × *g* for 10 min and then washed three times with sterile 0.01 M PBS, pH 7.2. Capsulated *Streptococcus pneumoniae* was isolated from the respiratory tract of a patient from the Department of Clinical Bacteriology of the Niño Jesús Children's Hospital in San Miguel de Tucumán, Argentina. The *S. pneumoniae* strain belongs to the 14 serotype, one of the 10 most frequent serotypes of *S. pneumoniae* isolated in pneumococcal infections in Argentina (1).

L. lactis administration and experimental infection

Different doses of *L. lactis*: 10^6, 10^7, 10^8, 10^9 and 10^{10} cells/day/mice were assayed and the dose 10^8 cells/day per mouse was selected, as this was the dose that stimulated

the immune system without causing translocation or inducing an exaggerated inflammatory response (J. Villena *et al*., unpubl. data, 2006). *L. lactis* was given to different groups of mice by nasal routes for 2, 5 or 7 consecutive days at a dose 10^8 cells/mouse per day. *L. lactis* was suspended in sterile PBS and 25 μL of an inoculum containing the appropriate dose were given by dripping into each nostril. The control group received sterile PBS in the same conditions. All mice were fed a conventional balanced diet ad libitum during experiments.

S. pneumoniae was first grown on blood agar for 18 hr. Freshly grown colonies were suspended in Todd Hewitt broth (Oxoid, Cambridge, UK) and incubated overnight at 37 °C. The pathogens were harvested by centrifugation at $3600 \times g$ for 10 min at 4 °C and then washed three times with sterile PBS. Cell density was adjusted to 4×10^7 cells/mL. The size of the inoculum was confirmed by serial dilutions and quantitative subcultures on blood agar. The choice of the infecting dose was based on the bacterial cell counts recovered from the blood of animals suffering from severe pneumonia (14). Challenge with *S. pneumoniae* was carried out on the day after the end of each *L. lactis* treatment (on the 3rd, 6th or 8th day). Mice were challenged nasally with the pathogen by dripping 25 μL of an inoculum containing 10^6 cells of *S. pneumoniae* (log phase) in PBS into each nostril, which was then involuntarily inhaled. To facilitate migration of the inoculum to the alveoli, mice were held in a head-up vertical position for 2 min. Untreated mice (control group) were infected in the same way. Blood, bronchoalveolar fluid, lung tissue and bone marrow were sampled for pathogenesis infection studies.

Bacterial cell counts in lung and blood

Mice were killed before challenge (day 0) and on days 1, 5, 10, and 15 post-infection. Their lungs were excised, weighed and homogenized in 5 mL sterile peptone water. Homogenates were diluted appropriately, plated in duplicate on blood agar and incubated for 18 hr at 37 °C. *S. pneumoniae* was identified by standard techniques (16) and the results were expressed as log (base 10) c.f.u./g of organ. Progression of bacterial growth to the bloodstream was monitored by blood samples obtained by cardiac puncture with a heparinized syringe. Samples were plated on blood agar and bacteremia was reported as negative or positive hemocultures after incubation for 18 hr at 37 °C.

Leukocyte counts in blood and bronchoalveolar lavages

Blood samples were obtained as described above and BAL samples were obtained according to the technique previously described (13). Briefly, the trachea was exposed and intubated with a catheter and two sequential bronchoalveolar lavages were performed in each mouse by injecting 0.5 mL sterile PBS; the recovered fluid was centrifuged for 10 min at $900 \times g$; the pellet was used to determine total and differential counts of BAL leukocytes and the fluid was frozen at −70 °C for subsequent antibody analyses. The total number of leukocytes in blood and BAL were determined with a hemocytometer. Differential cell counts were carried out by counting 200 cells in blood or BAL smears stained with May Grünwald-Giemsa.

Bone marrow differential cell counts

Bone marrow samples were obtained from the femoral shaft with physiological solution. Differential cell counts (percentage) were carried out by counting 400 cells in bone marrow smears stained with May Grünwald-Giemsa. We performed a morphological differentiation between the myeloid and lymphoid lineages on the bases of size, nuclear shape and nucleus-to-cytoplasm size ratio. Myeloid cells were grouped into mitotic pool, which includes the cells capable of replication (myeloblasts, promyelocytes and myelocytes), and the post-mitotic pool, whose cells usually do not replicate but are able to evolve toward more mature and differentiated cells (metamyelocytes, band cells and neutrophils). The cells of the lymphoid lineage were also counted and were expressed as percentage of total bone marrow leukocytes. Bone marrow lymphocyte subsets (CD4+, CD8+ and CD19+ cells) were also analyzed. Cell suspensions were prepared by flushing the femoral shaft with PBS containing heparin and albumin (1%) and the pellet was used to make smears. Labeling of CD4+, CD8+ or CD19+ cells was performed by the direct immunofluorescence technique. Cell smears were fixed onto slides with methanol (4%) for 30 min. The fixed cells were subsequently incubated with anti-mouse CD4+, CD8+ or CD19+ conjugated with fluorescein isothiocyanate (Sigma, Buenos Aires, Argentina). Incubations were carried out in a humidified chamber at 37 °C for 30 min. Samples were analyzed under an ultraviolet light with a fluorescence microscope (×100). Four hundred cells were counted and the results were expressed as percentages.

Local and systemic phagocyte activation

Measurement of myeloperoxidase activity of blood neutrophils was carried out using a cytochemical method (Washburn test) with benzidine as a myeloperoxidase chromogen (13). Cells were graded as negative or weakly, moderately or strongly positive and were used to calculate the score. The bactericidal activity (oxidative burst) of macrophages and neutrophils was measured in the pellet

of BAL and in the pellet of peritoneal lavages using the NBT reduction test (Sigma-Aldrich, St Louis, MO, USA). NBT was added to each sample with (positive control) or without addition of the bacterial extract; then, samples were incubated at 37 °C for 20 min. In the presence of oxidative metabolites, NBT (yellow) is reduced to formazan, which forms a blue precipitate (17). Smears were prepared and, after staining, samples were examined under a light microscope for blue precipitates. One hundred cells were counted and the percentage of NBT-positive (+) cells was determined.

Anti-pneumococcal antibodies

Specific antibodies (IgA and IgG) in serum and BAL were measured prior to infection (day 0) and on days 1, 5, 10, 15 and 21 after challenge. A previously developed enzyme-linked immunosorbent assay (ELISA) technique was used (13). Briefly, plates were coated with a heat-killed *S. pneumoniae*-sodium carbonate-bicarbonate buffer (1:100) suspension, pH 9.6. Non-specific protein binding sites were blocked with PBS containing 5% non-fat milk. Samples were diluted (serum 1:20; BAL 1:2) with PBS containing 0.05% (v/v) Tween 20 (PBS-T). Peroxidase-conjugated goat anti-mouse IgA or IgG (Fc specific, Sigma Chemical, St Louis, MO, USA) were diluted (1:500) in PBS-T. Antibodies were revealed with a substrate solution [3-3′, 5-5′-tetramethylbenzidine (Sigma Chemical)] in citrate-phosphate buffer (pH 5, containing 0.05% H_2O_2) and the reaction was stopped by the addition of 1 M H_2SO_4. Readings were carried out at 493 nm (VERSAmax Tunable microplate reader; MDS Analytical Technologies, Sunnyvale, CA, USA). Antibody concentration of each unknown sample was expressed as mg/L determined from a standard curve made with commercial mouse IgA or IgG (Sigma-Aldrich).

Biochemical assay of BAL fluid and histopathological examination

Albumin content, a measure to quantitate increased permeability of the bronchoalveolar–capillarity barrier, and lactate dehydrogenase (LDH) activity, an indicator of general cytotoxicity, were determined in the acellular BAL fluid. Albumin content was determined colorimetrically based on albumin binding to bromcresol green using an albumin diagnostic kit (Wiener Lab, Buenos Aires, Argentina). LDH activity, expressed as units per liter of BAL fluid, was determined by measuring the formation of the reduced form of nicotinamide adenine dinucleotide (NAD) using the Wiener reagents and procedures (Wiener Lab).

Tissue damage was evaluated on day 0 and on days 1, 10 and 15 post-infection. Lungs were aseptically removed, fixed in 4% formalin and embedded in histowax (Leica, Buenos Aires, Argentina). Histopathological assessment was performed on 5 μm tissue sections stained with hematoxylin-eosin for light microscopy. At least four tissue sections from various areas of the lung of each mouse in all experimental groups were examined.

Statistical analysis

Experiments were carried out in triplicate and results were expressed as mean ± standard deviation (SD). After verification of a normal distribution of data, two-way ANOVA was used. Tukey's test (for pairwise comparisons of the means) was used to test for differences between the groups. Differences were considered significant at $P < 0.05$.

RESULTS

Nasal administration of *L. lactis* reduces lung colonization and prevents pathogen passage to blood

Nasal administration of *L. lactis* (10^8 cells/mouse per day) for 5 and 7 days (LLN5d and LLN7d) were able to reduce pneumococcal colonization in lung (Fig. 1) and prevent pathogen dissemination in blood, showing negative hemocultures throughout the assayed period (data not shown). In contrast, LLN2d was unable to reduce the

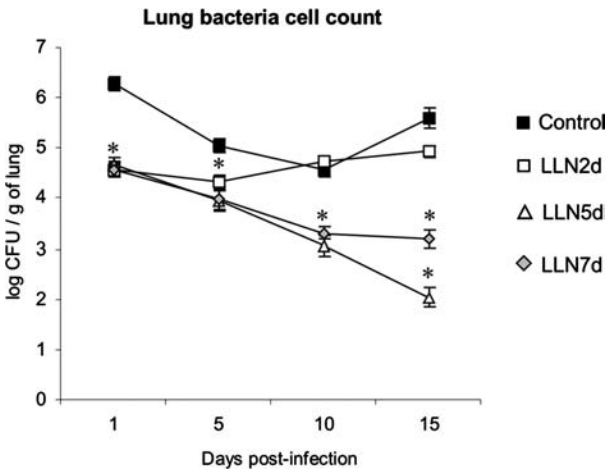

Fig. 1. Kinetics of *Streptococcus pneumoniae* clearance from lungs. Mice were challenged nasally with *S. pneumoniae* (10^6 cells/mouse) after treatment with *Lactococcus lactis* (10^8 cells/mouse per day) by the nasal route for 2, 5 or 7 days (LLN2d, LLN5d and LLN7d groups, respectively). Control mice were challenged with the pathogen after treatment with sterile PBS. Lung homogenates were examined at various time points for bacterial counts. Results are expressed as mean ± SD ($n = 6$ mice/group at each time point). *Significant differences compared to control mice $P < 0.05$.

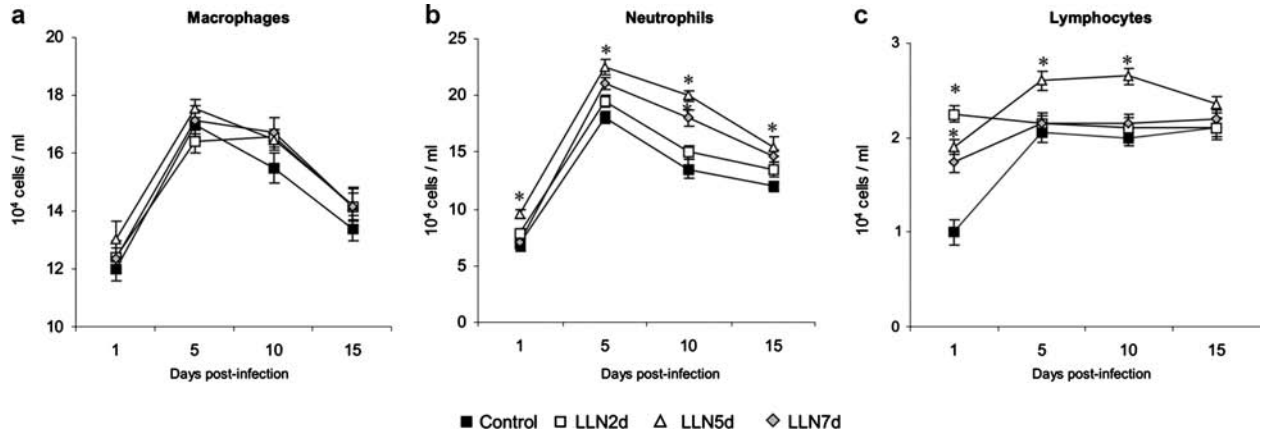

Fig. 2. Bronchoalveolar (a) macrophage, (b) neutrophil and (c) lymphocyte counts. Mice were challenged nasally with *Streptococcus pneumoniae* (10^6 cells/mouse) after treatment with *Lactococcus lactis* (10^8 cells/mouse per day) by the nasal route for 2, 5 or 7 days (LLN2d, LLN5d and LLN7d groups, respectively). Control mice were challenged with the pathogen after treatment with sterile PBS. Results are expressed as mean ± SD ($n = 6$ mice/group at each time point). *Significant differences compared to control mice $P < 0.05$.

number of pathogens in lung or prevent their passage to blood.

L. lactis administration increases innate and specific immune response in the respiratory tract

Alveolar macrophages were the predominant resident leukocytes in the alveolar spaces of non-infected mice; lymphocytes were a minority population and neutrophils were not observed (18). There was no difference between treated and control mice in the number of BAL leukocytes before challenge (data not shown). The pneumococcal infection induced an increase in BAL leukocytes in all groups (Fig. 2). Values of BAL neutrophils and lymphocytes significantly higher than in the control group were observed with LLN5d and LLN7d treatments (Fig. 2b,c). Even though the pneumococcal infection increased the numbers of macrophages (Fig. 2a), there were no significant differences between treated and control mice. Before the challenge, all treatments significantly increased the percentage of BAL NBT+ cells compared to the control group (Table 1). The pneumococcal infection increased the bactericidal activity of BAL phagocytes in all groups. However, the percentage of NBT+ cells was significantly higher in mice treated with *L. lactis* than in the control mice (Table 1).

Local antibody response was examined in all experimental groups. Anti-pneumococcal IgG and IgA in BAL showed a peak on the 15th day post-infection in all groups; consequently, only the data for this day is shown in Table 2. All treatments induced a significant increase in specific pneumococcal IgA and IgG antibodies in BAL compared to controls (Table 2).

L. lactis administration enhances systemic innate and specific immune responses

There were no differences between treated and control mice in the number of blood leukocytes before the challenge (Fig. 3a). Pneumococcal infection increased the number of leukocytes in all experimental groups; however, LLN5d treatment induced higher values of blood leukocytes than in the control group (Fig. 3a). These changes were mediated principally by neutrophils, which were significantly increased in the LLN5d group compared to control mice (Fig. 3b).

To evaluate whether *L. lactis* administration was able to activate phagocytes in systemic compartments, blood neutrophil peroxidase activity and the percentage of NBT+ cells in peritoneal lavages were assessed. There were no differences between treated and control groups in blood peroxidase scores before the challenge with the pathogen (Table 3). However, after infection, this parameter increased significantly in all groups compared to control mice (Table 3). The microbicidal activity of phagocytes from peritoneal lavages was significantly higher in mice that received nasal treatments compared to the control group (Table 1).

Specific IgG antibodies in serum were significantly increased in mice that received nasal treatments compared to the control group (Table 2). There were no differences in the levels of specific IgA between treated and control mice (Table 2).

On the basis of the results presented above, LLN5d was selected as the optimal treatment to improve resistance against pneumococcal infection; consequently, the following studies were focused on this experimental group.

Table 1 Microbicidal activity of BAL and peritoneal phagocytes before and after challenge (day 1) with 10^6 cells of *Streptococcus pneumoniae*

	% NBT+ cells in BAL		% NBT+ cells in peritoneal cavity	
Treatment	Before challenge	After challenge	Before challenge	After challenge
LLN2d	37.87 ± 1.21*	73.53 ± 1.33*	31.52 ± 0.93*	45.24 ± 1.21*
LLN5d	43.51 ± 1.17**	79.28 ± 0.98*	37.89 ± 0.64**	54.91 ± 1.13**
LLN7d	39.61 ± 0.98*	77.79 ± 0.87*	33.24 ± 1.31*	48.87 ± 0.89*
Control	25.77 ± 0.85	63.87 ± 1.13	23.75 ± 1.03	38.88 ± 1.31

Mice were treated preventively with *Lactococcus lactis* (10^8 cells/mouse) by nasal route for 2, 5 or 7 days (LLN2d, LLN5d and LLN7d groups, respectively). Activity of phagocytes was measured by the NBT test and is expressed as percentage of NBT-positive cells (%NBT+).
Values are mean ± SD ($n = 5$ mice/group at each time point).
Significant differences compared to control mice *$P < 0.05$ and **$P < 0.01$

Table 2 Serum and BAL anti-pneumococcal antibodies after challenge (day 15) with 10^6 cells of *Streptococcus pneumoniae*

	BAL		Serum	
Treatment	IgG	IgA	IgG	IgA
LLN2d	3.66 ± 0.73*	3.19 ± 1.02*	55.22 ± 2.34*	10.73 ± 0.14
LLN5d	4.77 ± 0.76**	5.04 ± 0.95**	66.49 ± 1.43**	13.25 ± 0.23
LLN7d	3.87 ± 0.89*	3.16 ± 1.01*	60.67 ± 1.02**	11.67 ± 0.52
Control	2.52 ± 0.91	2.61 ± 0.89	28.51 ± 1.83	9.71 ± 0.63

Mice were treated preventively with *Lactococcus lactis* (10^8 cells/mouse) by nasal route for 2, 5 or 7 days (LLN2d, LLN5d and LLN7d groups, respectively). Specific immunoglobulin (Ig)G and IgA antibodies are expressed in mg/L.
Values are mean ± SD ($n = 6$ mice/group at each time point).
Significant differences compared to control mice *$P < 0.05$ and **$P < 0.01$.
IG, immunoglobulin.

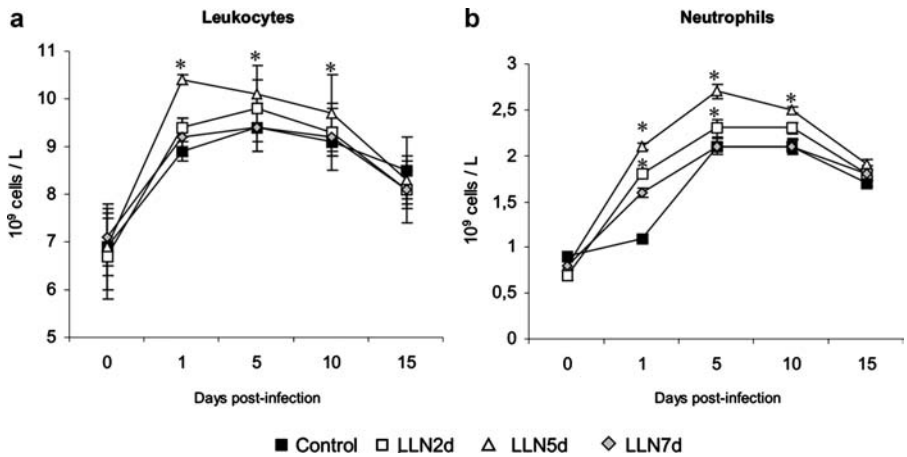

Fig. 3. Blood (a) leukocyte and (b) neutrophil counts. Mice were challenged nasally with *Streptococcus pneumoniae* (10^6 cells/mouse) after treatment with *Lactococcus lactis* (10^8 cells/mouse per day) by the nasal route for 2, 5 or 7 days (LLN2d, LLN5d and LLN7d groups, respectively). Control mice were challenged with the pathogen after treatment with sterile PBS. Results are expressed as mean ± SD ($n = 5$ mice/group at each time point). *Significant differences compared to control mice $P < 0.05$.

L. lactis induced a beneficial effect on bone marrow myeloid and lymphoid populations

LLN5d treatment induced no changes in the bone marrow myeloid population before challenge (Fig. 4a,b). The pneumococcal infection reduced the percentage of cells in the post-mitotic pool (metamyelocytes, band cells and neutrophils) in LLN5d and control up to day 1 (Fig. 4b); from then onwards mice treated with *L. lactis* showed higher values than the control group. In contrast, the in-

Table 3 Blood neutrophils peroxidase activity before and after challenge (day 1) with 10^6 cells of *Streptococcus pneumoniae*

Treatment	Before challenge	After challenge
LLN2d	125.3 ± 1.4	178.6 ± 2.6*
LLN5d	131.2 ± 1.2	181.0 ± 0.9*
LLN7d	124.9 ± 0.8	176.6 ± 3.1*
Control	124.0 ± 1.2	162.5 ± 3.5

Mice were treated preventively with *Lactococcus lactis* (10^8 cells/mouse) by nasal route for 2, 5 or 7 days (LLN2d, LLN5d and LLN7d groups, respectively). Peroxidase activity of blood neutrophils is expressed as score numbers.
Values are mean ± SD ($n = 6$ mice/group at each time point).
*Significant differences compared to control mice $P < 0.05$.

Table 4 Bone marrow cells peroxidase activity before and after challenge (day 1) with 10^6 cells of *Streptococcus pneumoniae*

Treatment	Before challenge	After challenge
LLN5d	32.7 ± 1.6*	36.3 ± 1.6*
Control	25.7 ± 3.3	25.8 ± 1.6

Mice were treated preventively with *Lactococcus lactis* (10^8 cells/mouse) by the nasal route for 5 days (LLN5d). Peroxidase activity of bone marrow cells is expressed as percentage.
Values are mean ± SD ($n = 6$ mice/group at each time point).
*Significant differences compared to control mice $P < 0.05$.

fection increased the percentage of cells in the mitotic pool (blasts, promyelocytes and myelocytes), but values were significantly lower in the LLN5d group compared to the control mice (Fig. 4a). Peroxidase activity of bone marrow myeloid cells is presented in Table 4. LLN5d treatment significantly increased the percentage of peroxidase-positive cells compared to controls.

In contrast, pneumococcal infection induced a significant increase in bone marrow lymphocytes on day 5 post-infection in both groups, but percentages were significantly higher in mice that received the LLN5d treatment (Fig. 4c). Bone marrow CD19+, CD4+ and CD8+ populations were also studied. In the LLN5d group, CD19+ cells were significantly increased on days 5 and 10 post-infection (Fig. 4d) and CD4+ cells were decreased on day 1 post-infection and significantly increased on day 10 post-infection (Fig. 4e). There were no significant differences between treated and control groups in the percentage of bone marrow CD8+ cells (Fig. 4f).

L. lactis treatment reduced lung tissue damage caused by pneumococcal infection

The challenge with *S. pneumoniae* increased significantly BAL albumin concentration and LDH activity in both experimental groups, which indicates that infection increased bronchoalveolar capillarity barrier permeability and cell damage in lungs (Fig. 5). LLN5d treatment was able to reduce significantly both LDH and albumin compared to the control group. These findings would indicate lower lung tissue damage in mice treated with *L. lactis* by the nasal route. In order to confirm these results, histological analyses were carried out. Lung histopathological examination of control mice revealed a gradual and intense inflammatory response with progressive parenchymal involvement, including widespread cellular infiltration, increased fibrosis in bronchial walls and vessels, hemorrhage and reduction of the alveolar airspaces (Fig. 6a). Lung examination of mice that received LLN5d treatment revealed signs of moderate inflammation with focal cellular infiltration, without hemorrhage and with conserved alveolar airspaces (Fig. 6b). We also observed an increase in immune cells, probably lymphocytes, in the lamina propria of the bronchus-associated lymphoid tissue (BALT) in relation to control mice, especially on day 10 post-infection.

DISCUSSION

The present study demonstrated that nasal administration of *L. lactis* was able to increase the clearance rate of *S. pneumoniae* from lung and to prevent the dissemination of pneumococci into blood. These effects would be related to an upregulation of the innate and specific immune responses in both local and systemic compartments. The nasopharyngeal-associated lymphoid tissue (NALT) contains all the lymphoid cells required for the induction and regulation of the mucosal immune response to antigens delivered into the nasal cavity (15). Hussell and Humphreys (19) suggested that the NALT could fulfill an important role by reducing the pathogen burden to a level that only induces minimal inflammation in the lower lung. In consequence, the intranasal priming of NALT with *L. lactis* before challenge with *S. pneumoniae* probably was able to reduce pathogen numbers in the nasal cavity and the number of pneumococci that reach the lung. The effect induced by the nasal inoculation of *L. lactis* could be explained by a decreased adherence of *S. pneumoniae* to the respiratory epithelium, as it has been reported that nasally given LAB could exclude competitively pneumococcal cells (20). In addition, the increased activation of macrophages observed in mice treated with *L. lactis* would enhance the protective effect. This activation would induce a more effective neutrophil recruitment (21, 22) as was observed in the LLN5d group after pneumococcal infection. These findings agree with our previous results that showed an increased stimulation of the phagocyte activity of alveolar macrophages in mice that received orally given *Lactobacillus casei* (12).

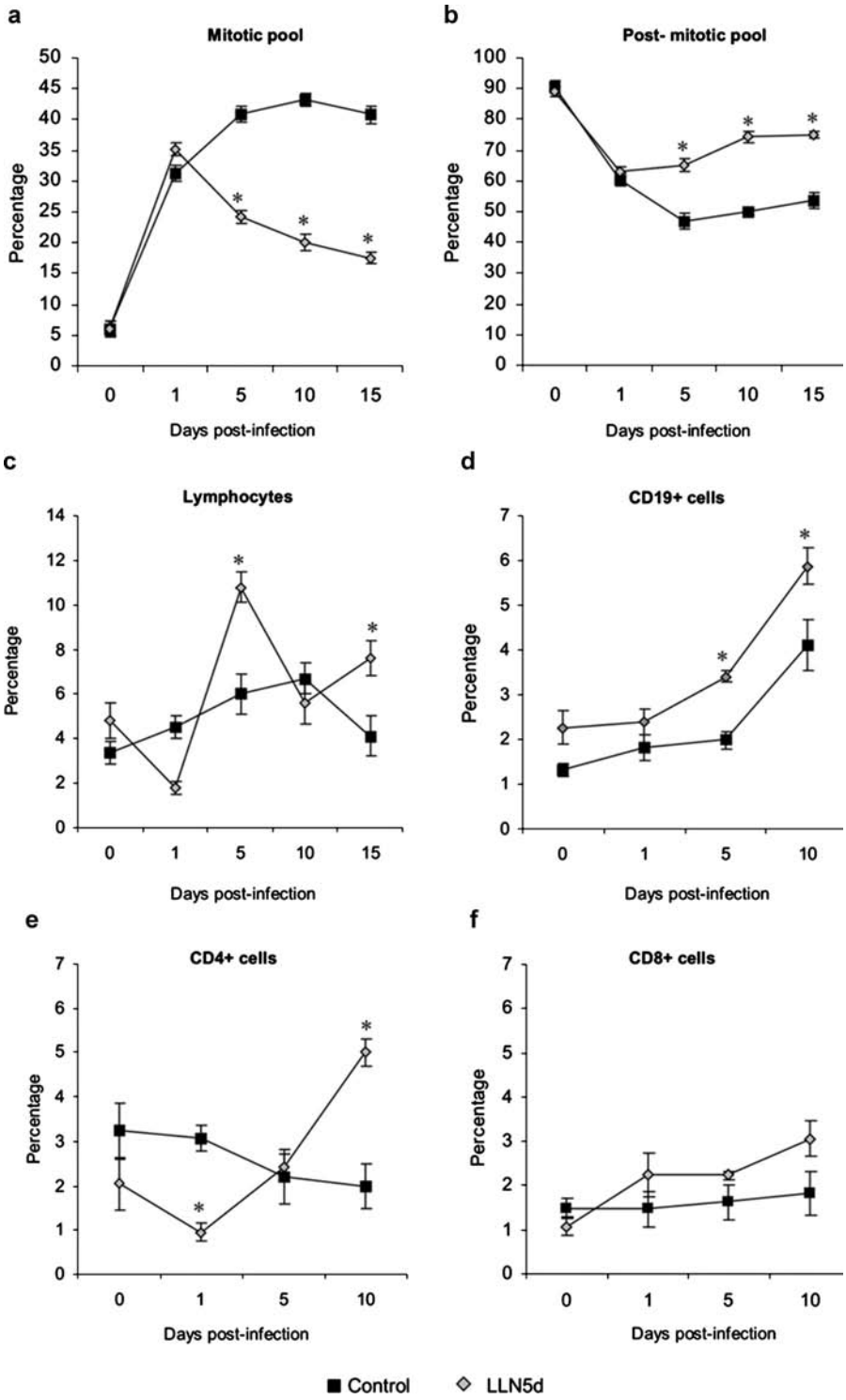

Fig. 4. Bone marrow myeloid cells were grouped into (a) mitotic pool (blasts, promyelocytes and myelocytes), (b) post-mitotic pool (metamyelocytes, band cells and neutrophils), (c) bone marrow lymphocytes, (d) CD19+, (e) CD4+ and (f) CD8+ cells. Mice were challenged nasally with *Streptococcus pneumoniae* (10^6 cells/mouse) after nasal treatment with *Lactococcus lactis* (10^8 cells/mouse) for 5 days (LLN5d). Control mice were challenged with the pathogen after treatment with sterile PBS. Results are expressed as mean ± SD ($n = 6$ mice/group at each time point). *Significant differences compared to control mice $P < 0.05$.

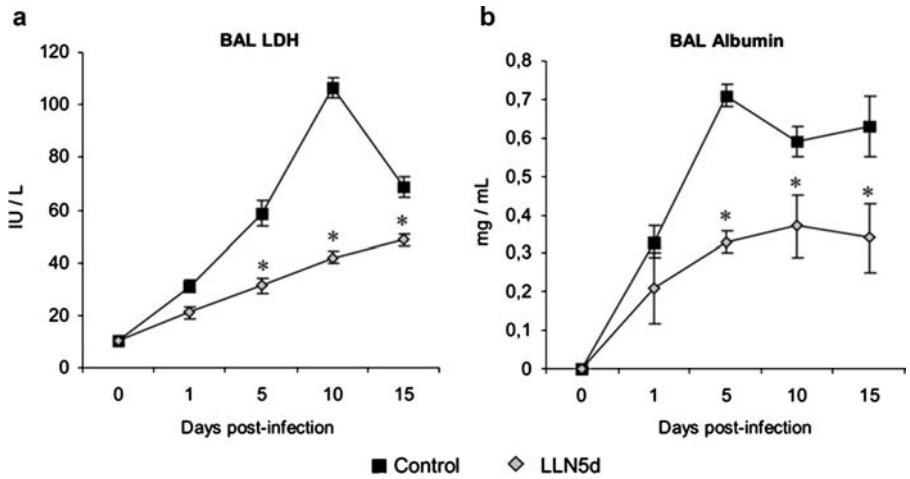

Fig. 5. (a) BAL lactate dehydrogenase (LDH) activity and (b) BAL albumin concentration. Mice were challenged nasally with *Streptococcus pneumoniae* (10^6 cells/mouse) after nasal treatment with *Lactococcus lactis* (10^8 cells/mouse) for 5 days (LLN5d). Control mice were challenged with the pathogen after treatment with sterile PBS. Results are expressed as mean ± SD ($n = 6$ mice/group at each time point). *Significant differences compared to control mice $P < 0.05$.

Pulmonary lymphocytes play an important role in lung defense against infection because they influence important events of inflammation and tissue repair, such as the recruitment of phagocytes from blood and fibroblast activation (23). The lymphocyte population in BAL was significantly increased in the LLN5d group compared to the controls. These lymphocytes are probably IgA+ cells that have migrated from mucosal inductive sites. In previous work, we have demonstrated the ability of different strains of orally given LAB to induce the migration of B lymphocytes to BALT (24). This fact would be related with the increase of BAL anti-pneumococcal IgA and IgG observed in the nasal treatment with *L. lactis* for 5 days. Secretory IgA constitutes the main mediator of specific humoral immunity at mucosal surfaces by neutralizing bacterial toxins and viral particles (25) and inhibiting adherence of bacteria to epithelial cells (26). Moreover, IgA may participate actively in protective phagocytosis and killing of *S. pneumoniae* once the inflammatory process is initiated (27). IgG would be involved in the protection against respiratory pathogens because of its ability to opsonize bacteria and, to a lesser extent, to mediate lysis by the complement (28). Studies of lung tissue injury showed that nasal treatment reduced significantly the damage caused by the infection compared to the control.

In contrast, nasal administration of *L. lactis* for 5 days was able to induce activation of a systemic innate immune response which was evidenced by the increase in the microbicidal function of blood neutrophils and peritoneal cavity phagocytes. The capacity of LAB to induce cytokine production is well documented (29, 30), these molecules probably being responsible for this effect on blood and peritoneal cavity. In addition, the LLN5d group showed a decrease of the percentage of the post-mitotic pool (metamyelocytes, band cells and neutrophils) in bone marrow, probably related with the release of mature neutrophils and the production of granulocytes in response to inflammatory signals (18). Other evidence of systemic activation is the increase of serum anti-pneumococcal IgG in LLN5d mice, which is the most important factor contributing positively to opsonophagocytic activity against *S. pneumoniae* (31). IgG antibodies present in serum protect the host against pulmonary infection and presumably cross the alveolar–capillary barrier into the alveoli, where they provide resistance to infection (32). The levels of IgG antibodies in the LLN5d group could be related with the increase of bone marrow CD19+ cells observed in this experimental group.

With respect to CD4+ cells, a decrease compared to the controls was observed on day 1 post-infection, probably caused by the mobilization of cells towards the site of infection. Recent studies have shown that bone marrow CD4+ T cells would be involved in the maintenance of hematopoiesis under normal conditions (33); then the increase of the bone marrow CD4+ cells observed on day 10 post-infection in the LLN5d group would be an interesting subject for future investigations.

Some protein antigens of *S. pneumoniae* have been evaluated for protective efficacy in animal models of pneumococcal infection (34). Infection with *S. pneumoniae* frequently results in bacteremia and sepsis because of its capacity to invade the bloodstream (35, 36). Consequently, vaccination strategies against this pathogen, which enters the body through the respiratory mucosal layer, should

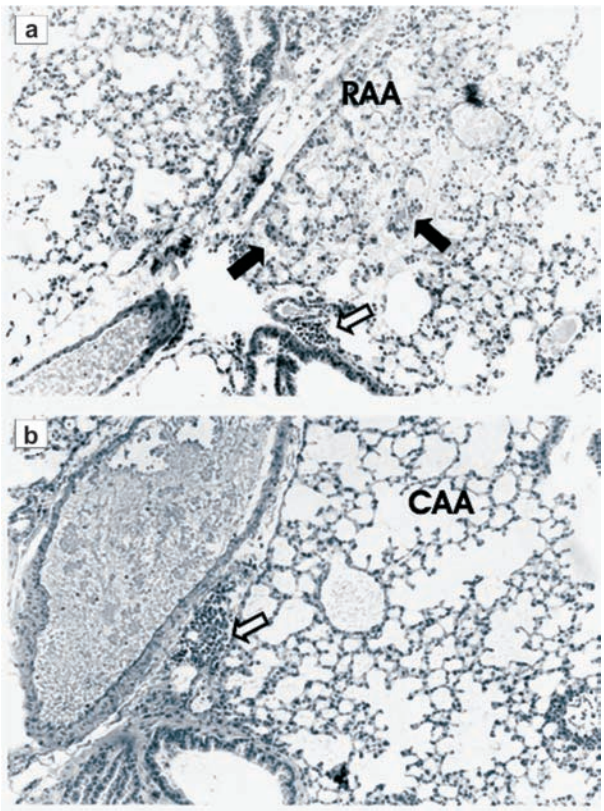

Fig. 6. Histological examination of lungs. (a) Lung histology of control mice on day 10 after challenge with *Streptococcus pneumoniae* (10^6 cells/mouse). Hemorrhage (black arrow) and reduction of alveolar airspaces (RAA) are evident in control mice. (b) Lung damage was less pronounced in mice treated preventively with *Lactococcus lactis* (10^8 cells/mouse) for 5 days by the nasal route, which showed signs of a moderate inflammatory response without hemorrhage, with conserved alveolar airspaces (CAA) and increased cellularity in the lamina propria of the bronchus-associated lymphoid tissue (white arrows). Original magnification ×200.

involve both the systemic and the mucosal immune responses. According to our results, the preventive nasal administration of *L. lactis* in the appropriate dose reduced pathogen counts in lung, preventing the passage of pneumococci to blood and inducing a local and systemic specific immune response. The adjuvant effect of *L. lactis* in our infection model would represent an important advantage for its use as a delivery vehicle of pneumococcal proteins. Then, nasal immunization with recombinant *L. lactis* emerges as an effective route of vaccination for both systemic and mucosal immunity against pneumococcal infection. Taking into account the fact that the immunoenhancing properties of LAB are dependent on strain, dose and administration route, the precise determination of these characteristics is required for the use of a certain strain as an antigen delivery vehicle for mucosal vaccination.

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