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ARTICLE

Immune response in nasopharynx, lung, and blood elicited by experimental nasal pneumococcal vaccines containing live or heat-killed lactobacilli as mucosal adjuvants

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Abstract: This work analyzes the humoral and cellular immune responses induced by live (LcV) and heat-killed (LcM) *Lactobacillus casei* associated with the pneumococcal antigen (P-Ag) at the nasopharynx level, considering nasal-associated lymphoid tissue (NALT) as the primary inductive site of the mucosal immune system, and lung and blood as effector sites. Levels of P-Ag IgA and IgG antibodies, main types of B and T cells, and cytokines in mucosal and systemic compartments were evaluated. The results showed that both LcM+P-Ag and LcV+P-Ag vaccines effectively induced IgA and IgG anti-P-Ag Abs in the upper and lower respiratory tract and plasma. These results correlated with increased IgA+ cells in NALT and lung that was induced by the experimental vaccines. Moreover, numbers of IgG+ cells increased in the blood. Profiles of inflammatory and regulatory cytokines were evaluated and their possible implications for the defense against pneumococci was assessed. Considering the overall results, the potential mechanisms of immune stimulation induced by LcM and LcV used as adjuvants are discussed. LcV and LcM showed similar effects on the immune system. Strain viability is not crucial for the stimulation of the antigen-specific immune response, and LcM is a convenient and effective mucosal adjuvant.

Key words: NALT, heat-killed lactobacilli, nasal vaccines, mice, pneumococcal antigen, mucosal and systemic immune response.

Résumé : Ce travail analyse les réponses immunes humorales et cellulaires induites par *Lactobacillus casei* vivant (LcV) ou tué par la chaleur (LcM) associé à l'antigène du pneumocoque P-Ag dans le nasopharynx, considérant que le NALT est le site primaire d'induction du système immun muqueux, et les poumons et le sang, ses sites effecteurs. Les niveaux d'IgA et d'IgG dirigés contre P-Ag, les cellules B et T principales et les cytokines des compartiments muqueux et systémiques ont été évalués. Les résultats ont montré que les deux vaccins LcM+P-Ag et LcV+P-Ag induisent efficacement les IgA et IgG anti P-Ag dans les voies respiratoires supérieures et inférieures ainsi que dans le plasma. Ces résultats sont en corrélation avec l'augmentation du nombre de cellules IgA+ dans le NALT et les poumons, induite par les vaccins expérimentaux. De plus, le nombre de cellules IgG+ augmentait dans le sang. Les profils des cytokines inflammatoires et régulatrices ont été évalués et leur implication possible dans la défense contre le pneumocoque a été évaluée. Considérant l'ensemble des résultats, les mécanismes potentiels de la stimulation immunitaire induite par LcM et LcV utilisés comme adjuvants sont discutés. LcV et LcM exercent des effets similaires sur le système immunitaire. La viabilité de la souche n'est pas cruciale à la stimulation de la réponse immune spécifique à l'antigène, LcM constituant un adjuvant pratique et efficace. [Traduit par la Rédaction]

Mots-clés : NALT, lactobacillus tué par la chaleur, vaccin nasal, souris, antigène du pneumocoque, réponse immune muqueuse et systémique.

Introduction

Vaccines against *Streptococcus pneumoniae* have failed to eradicate pneumococcal infections; this pathogen is still one of the most important worldwide (Lynch and Zhanel 2009; Moffitt and Malley 2011). Mucosal vaccine delivery is a crucial target in research to ensure inexpensive, safe, and effective protection, especially for at-risk populations. Nasal immunization against respiratory pathogens has certain advantages over other antigen delivery pathways because it mimics the route of entry of pathogens and induces a protective immune response in both the local and systemic compartments (Holmgren and Czerkinsky 2005). In addition, the cost–benefit analysis as well as their easy and convenient application makes nasal vaccines the best available choice in the fight against respiratory pathogens. Adjuvants are often required to induce maximum Ab levels in mucosal tissues, since most antigens have little or no inherent immunostimulatory properties. At present, very few adjuvants have been approved for clinical use (Bertholet et al. 2009; Lambrecht et al. 2009). Cholera toxin and the related *Escherichia coli* enterotoxin are potent mucosal adjuvants for the enhancement of Ag-specific immune responses; however, their application to human health is not safe, since they can cause undesirable effects such as diarrhea or Bell's palsy (Couch 2004, Lewis et al. 2009); moreover, these toxins can migrate into and accumulate in the olfactory tissue when administered nasally (van Ginkel et al. 2000). Live and dead lactic acid bacteria (LAB) have been shown to elicit mucosal and systemic immune responses that afford protection against various infectious challenges (Li et al. 2009; Medina et al. 2010), and their use as mucosal adjuvants is promising. Most investigations have focused on the immunomodulatory effects of viable LAB, and only a few articles have studied the adjuvant effect of heat-killed LAB. Dead

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LAB are preferable to live ones for use in vaccines, since they ensure longer product shelf-life and allow easier storage and transportation. Nasal immunization with live and heat-killed *Lactobacillus casei* CRL 431 associated with peumoccocal antigen (Protective pneumococcal protein A: P-Ag) exerts protection against respiratory and systemic pneumococcal infections (Vintiñi and Medina 2011). In nasal vaccines, the primary inductive site of the mucosal immune system is the nasal-associated lymphoid tissue (NALT). This tissue is considered a potent target for mucosal vaccines (Wu et al. 1997; Brandtzaeg 2011) and contains all of the necessary lymphoid cells, including T cells, B cells, and APCs, for the induction and regulation of inhaled Ag-specific mucosal immune responses (Brandtzaeg 2011). NALT is rich in Th0-type CD4+ T cells, which can become either Th1- or Th2-type cells (Hiroi et al. 1998; Bienenstock and McDermott 2005). In addition, the generation of IgA-committed B cells and the induction of memory B cells (Shimoda et al. 2001; Shikina et al. 2004) occur in this tissue. The immune mechanism induced by LAB used as adjuvants on NALT is a little-studied topic, and there are no reports on heat-killed LAB at this level. In this work we analyzed the humoral and cellular immune responses induced by heat-killed and live *L. casei* as adjuvants co-administered with P-Ag at the nasopharynx level, considering NALT as the primary inductive site of the mucosal immune system, and lung and blood as effectors sites. Thus, the levels of P-Ag-specific antibodies and main types of B and T cells in the mucosal and systemic compartments were evaluated. Cytokine release in nasopharynx, lung, and blood was also measured.

Material and methods

Microorganisms and culture conditions

Lactobacillus casei CRL 431 (LcV) (Vintiñi et al. 2012), obtained from the CERELA culture collection, was cultured for 8 h at 37 °C (final log phase) in Man–Rogosa–Sharpe broth (Oxoid), harvested, and washed with sterile 0.01 mol·L^{-1} phosphate buffer saline (PBS), pH 7.2. The bacterial suspension was adjusted to the desired concentration (10^9 cell·day^{-1}·mouse^{-1}) for later administration by the nasal route. Heat-killed *L. casei* (LcM) was prepared by heating bacteria in a water bath at 80 °C for 30 min, and lack of bacterial growth was confirmed using MRS agar plates.

Pneumococcal antigen

Recombinant PppA (P-Ag) was purified from *E. coli*-PppA using a His-Bind purification kit (Novagen) as previously described (Medina et al. 2008). The reagent and P-Ag solution were tested by the E-toxate test for LPS (Sigma) and shown to be below the limit of detection (2 pg·mL^{-1}). P-Ag concentration was determined using Bradford's method, and 5 μg of this protein was used in immunization protocols.

Mice

Three-week-old male and female Swiss albino mice were obtained from the closed colony at CERELA, and each experimental group consisted of 5 or 6 mice for each period evaluated. During all of the experiments, animals were supplied with balanced rodent food and water ad libitum. Animals were cared for in accordance with standard guidelines (Canadian Council on Animal Care 1993). The experimental protocols were approved by the Animal Care and Ethics committee at CERELA, and all experiments complied with the current laws of Argentina.

Nasal immunization protocol and harvested sample collection

Young mice were nasally immunized with 50 uL of a mixture of 5 μg of P-Ag plus live (LcV) and heat-killed (LcM) *L. casei* (10^9 CFU·mouse^{-1}) as the mucosal adjuvant. LcV and LcM suspensions were prepared in PBS. The immunization was carried out using a protocol of 3 successive administrations that included 2 consecutive days each time, with a 14-day interval between them (days 0–1, 14–15, and 28–29). Groups that received LcV, LcM, P-Ag, and phosphate-buffered saline solution (PBS) were used as the controls. Fourteen days after the last immunization, samples of plasma, as well as nasal and bronchoalveolar fluids (BALF) were collected as previously described (Vintiñi and Medina 2011). Briefly, blood samples were obtained through cardiac puncture at the end of each treatment and collected in heparinized tubes. Then, blood was centrifuged at 4 °C for 5 min at 900*g*. The supernatant (plasma) was collected. Nasal lavages (NL) and BALF were pooled and after centrifugation for 10 min at 900*g* (at 4 °C) the supernatants were collected. All fluids were stored at 70 °C until analysis.

The immunization protocol used in this work was based on that from Green et al. (2005), where 5 μg of PppA were nasally administered together with different adjuvants. In this work, the dose of the adjuvants (LcV and LcM) was selected on the basis of our previous studies, in which we demonstrated that this strain induced a significant increase in the innate and acquired immune defense mechanisms of the host in a pneumococcal infection model in adult mice (Racedo et al. 2006; Vintiñi and Medina 2011).

Enzyme-linked immunosorbent assay (ELISA) for anti-PppA antibodies

To examine mucosal and systemic immune responses to pneumococcal Ag (rPppA = P-Ag), P-Ag-specific IgA and IgG antibody (Ab) levels in plasma, NL, and BALF were determined using ELISA on day 14 after the last immunization. Briefly, each well of microtiter plates was coated with P-Ag (100 μL of a 5 μg/ml stock in sodium carbonate–bicarbonate buffer (pH 9.6) per well). Nonspecific protein binding sites were blocked with PBS containing 5% non-fat milk. Samples were diluted (Serum 1:100; BAL 1:20 and NL 1:4) with PBS containing 0.05% (*v*/*v*) Tween 20 (PBS-T). Peroxidase-conjugated goat anti-mouse IgA and IgG (Fc specific; Sigma Chemical, St Louis, Missouri, USA) were diluted (1:500) in PBS-T. Antibodies were revealed with a substrate solution (*o*-phenylenediamine; Sigma Chemical) in citrate–phosphate buffer (pH 5, containing 0.05% H_2O_2) and the reaction was stopped by the addition of 1 mol·L^{-1} H_2SO_4. Readings were carried out at 493 nm (VERSAmax Tunable microplate reader; MDS Analytical Technologies, Sunnyvale, California, USA) and samples were considered negative for the presence of specific antibodies when OD493 < 0.1. Results are the mean of the optical density (OD) ± SD for each specific Ig (n = 6 mice per group).

Cytokine concentration determined by enzyme-linked immunosorbent assay (ELISA)

Cytokine concentrations in NL, BAL, and plasma were measured with a mouse Th1/Th2 ELISA Ready SET Go! Kit (BD Bioscience, San Diego, Calif.), including interleukin (IL)-2 and interferon (IFN)-γ as Th1-type, and IL-4 and IL-10 as Th2-type cytokines. IL-17A as a Th17-type cytokine was also measured using the ELISA kit from e-Bioscience (BD Biosciences). The sensitivity of assays to each cytokine was as follows: 4 pg·mL^{-1} for IL-2, IFN-γ, IL-17, and tumour necrosis factor (TNF)-α, and 2 pg·mL^{-1} for IL-4 and IL-10.

Flow cytometry studies

Mouse lungs were aseptically removed, finely minced, and incubated for 30 min with collagenase (SIGMA) in 15 mL of RPMI 1640 medium (Gibco). To prepare the single-cell suspension, collagenase-treated minced lungs were gently tapped into a plastic dish. After the removal of debris, red blood cells were depleted by hypotonic lysis. The cells were washed with RPMI medium supplemented with 100 U·mL^{-1} of penicillin and 100 mg·mL^{-1} of streptomycin, and then resuspended in RPMI supplemented with 10% heat-inactivated foetal calf serum (FCS). NALT suspension cells were obtained as follows. After removing the lower jaw and the tongue, the soft palate bearing the NALT was dissected from teeth and underlying tissue with a scalpel and carefully discon-

nected from the underlying bones. The paired NALT structure was detached from the soft palate with tweezers. The single-cell suspension was prepared immediately by gently mincing the tissue through a 45 mm nylon mesh into ice-cold RPMI medium containing 10% FCS. The cell suspension was washed and resuspended in RPMI supplemented with 10% heat-inactivated foetal calf serum (FCS). Live cells were counted using trypan blue exclusion and then resuspended at an appropriate concentration (lung: 5×10^6 cells·mL^{-1}; NALT: 0.3×10^6 cells·mL^{-1}). Blood was collected, as above, in heparin tubes. Red blood cells were lysed in FACS Lysis Solution buffer (Becton Dickinson), the remaining leukocytes were washed twice in PBS, and the concentration was adjusted to 1×10^6 leucocytes. Cell suspensions were pre-incubated with anti-mouse CD32/CD16 monoclonal antibody (Fc block) for 30 min at 4 °C. Then, aliquots of single-cell suspensions were stained with appropriate combinations of fluorescently labeled monoclonal antibodies (mAbs) that included the following: fluorescein isothiocyanate (FITC)-labeled anti-CD3, anti-B220; phycoerythrin (PE)-labeled anti-CD4, anti-IgA, anti-IgG, and peridinin chlorophyll-*a* protein (PercP) anti-CD8 (all mAbs from BD Pharmigen). Cell suspensions with double or triple labeling were used (B220/IgA; B220/IgG; CD3/CD4/CD8). Cells were stained with labeled mAbs in PBS + 2% fetal bovine serum (PBS–FBS) for 30 min at 4 °C, washed and re-suspended in ice-cold PBS–FBS. Then, cells were acquired on a Partec Past II flow cytometer (BD Bioscience) and data were analyzed with Flomax software.

Statistical analyses

Experiments were performed in triplicate and the results are the mean ± SD. After verification of a normal distribution of data, significant differences between means were determined by analysis of variance (ANOVA). 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

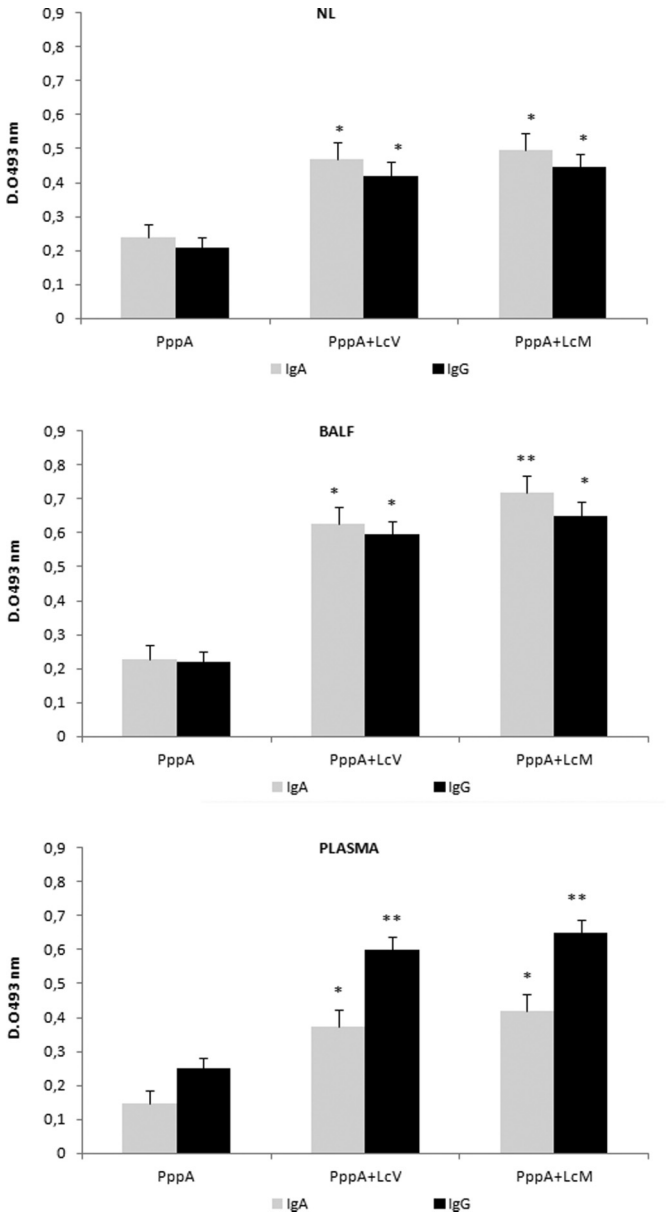
P-Ag-specific IgA and IgG responses in mucosal and systemic tissues of mice given P-Ag plus LcV and LcM

We evaluated whether nasal administration of LcV and LcM as mucosal adjuvants enhanced P-Ag-specific Ab responses (Fig. 1). No detectable values of these specific antibodies were observed in any of the samples collected from animals that received LcV, LcM, or PBS. Nasal administration of both P-Ag+LcM and P-Ag+LcV significantly increased the levels of Ag-specific IgA and IgG Ab responses in NL and BALF compared with the results from mice that received P-Ag alone. These results show that both LcV and LcM administered as nasal adjuvants effectively elicited Ag-specific Ab responses in mucosa-associated lymphoid tissue in the respiratory tract. Since nasal immunization induces systemic immunity, P-Ag-specific Ab responses in plasma were examined. Nasal immunization with P-Ag+LcV and P-Ag+LcM enhanced P-Ag-specific IgG and IgA Ab responses in plasma.

Flow cytometry evaluation of T and B cells in mucosal tissues

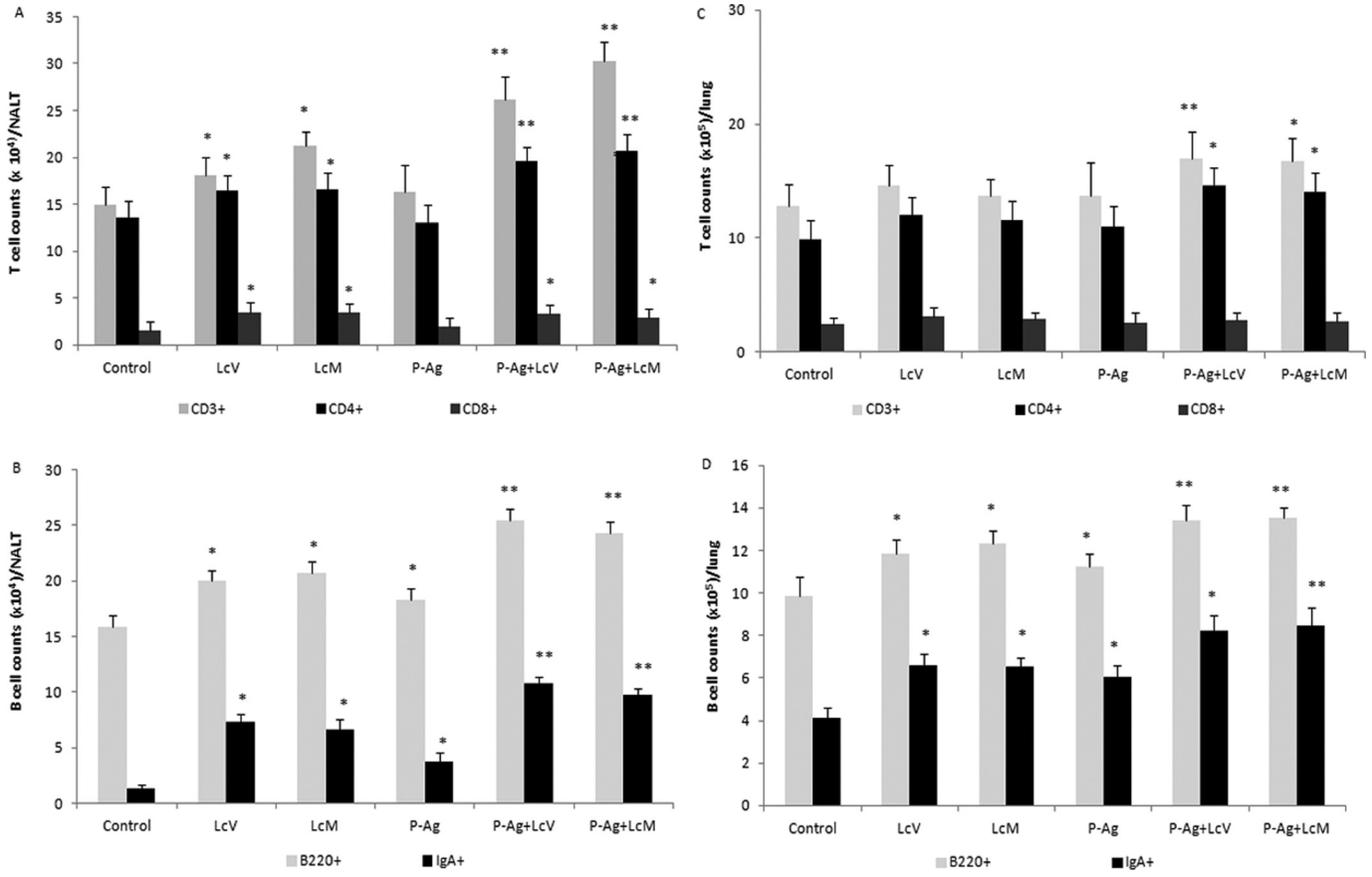
Flow cytometry assays were performed to evaluate the stimulation of the main types of B and T cells in mucosal tissue and blood after vaccination. For all phenotypic studies, a lymphocyte gate was set on the forward/side scatter plot. Knowing the values of total cell recovery from NALT and lung and the percentage of a particular cell subpopulation by flow cytometry, absolute numbers of lymphocyte subpopulations per organ (NALT and lung) were computed and absolute numbers of T and B cells are shown (Figs. 2A and 2B). The analysis of T cell subtypes revealed significant variations between different experimental groups. In the control mice, the percentage of T CD3+ cells in NALT was 36.5% ± 3.2%, out of which 93% ± 2.9% were T CD3+ cells and 7% CD8+ cells. At 14 days post-immunization, the number of CD3+, CD4+, and

Fig. 1. Mucosal and systemic IgA and IgG anti-pneumococcal antigen (P-Ag) antibodies response in nasal and bronchoalveolar lavages (NL and BALF) and plasma of mice nasally immunized with P-Ag and P-Ag associated with live (LcV) and heat-killed (LcM) *Lactobacillus casei*. Fourteen days after the last immunization, the levels of P-Ag-specific IgA and IgG Abs in NL, BALF, and plasma were determined by P-Ag-specific ELISA. Results are representative of 2 independent experiments and are the mean OD ± SD for each specific anti-P-Ag antibody; *, $p < 0.05$ and **, $p < 0.01$ compared with the PppA group.



CD8+ cells increased in the experimental groups nasally administered LcV, LcM, and their combinations, with P-Ag (P-Ag+LcV, P-Ag+LcM), while no significant increase in these T cells was found in the P-Ag group. In addition, the absolute number of B cells identified as B220+ and IgA+ cells increased with all the stimuli (Fig. 2B). IgA is the most important immunoglobulin (Ig) involved in mucosal tissue protection, and the increase in IgA+ cells is correlated with P-Ag-IgA Ab induced by the experimental vaccines. This fact shows that LcV and LcM exerted an adjuvant effect on the B cells in NALT. In the lung, nasal administration of LcV and LcM tended to increase the absolute number of CD3+ and CD4+

Fig. 2. Absolute number of T (CD3+, CD4+, CD8+) and B (B220+, IgA+) cells in the nasal-associated lymphoid tissue (NALT) and lung of mice stimulated with experimental vaccines (P-Ag+LcV, P-Ag+LcM) and respective controls (LcV, LcM, PppA, PBS) on day 14 after the last immunization. (A) Number of T cells. (B) Number of B220+ and IgA+ cells. Data are the mean ± SD values from 6 mice. Results are representative of 2 independent experiments; *, $p < 0.05$ and **, $p < 0.01$ compared with the control group (PBS).



cells (Fig. 2C). However, the values were not significantly different compared with the control without stimulation, while P-Ag alone did not increase CD3+, CD4+, or CD8+ cells. In contrast, experimental vaccines induced a significant increase in T CD3+ lymphocytes and CD4+ cells but not in CD8+ cells. With respect to B cells, a significant increase in B220+ and IgA+ cells was observed in all experimental groups compared with the control (Fig. 2D). However, the highest absolute number of IgA+ cells was induced by the experimental vaccines, without significant differences between both groups: P-Ag-LcV and P-Ag-LcM ($p < 0.18$). On the whole, the results correlated with the levels of anti-P-Ag-IgA Ab in NL and BALF.

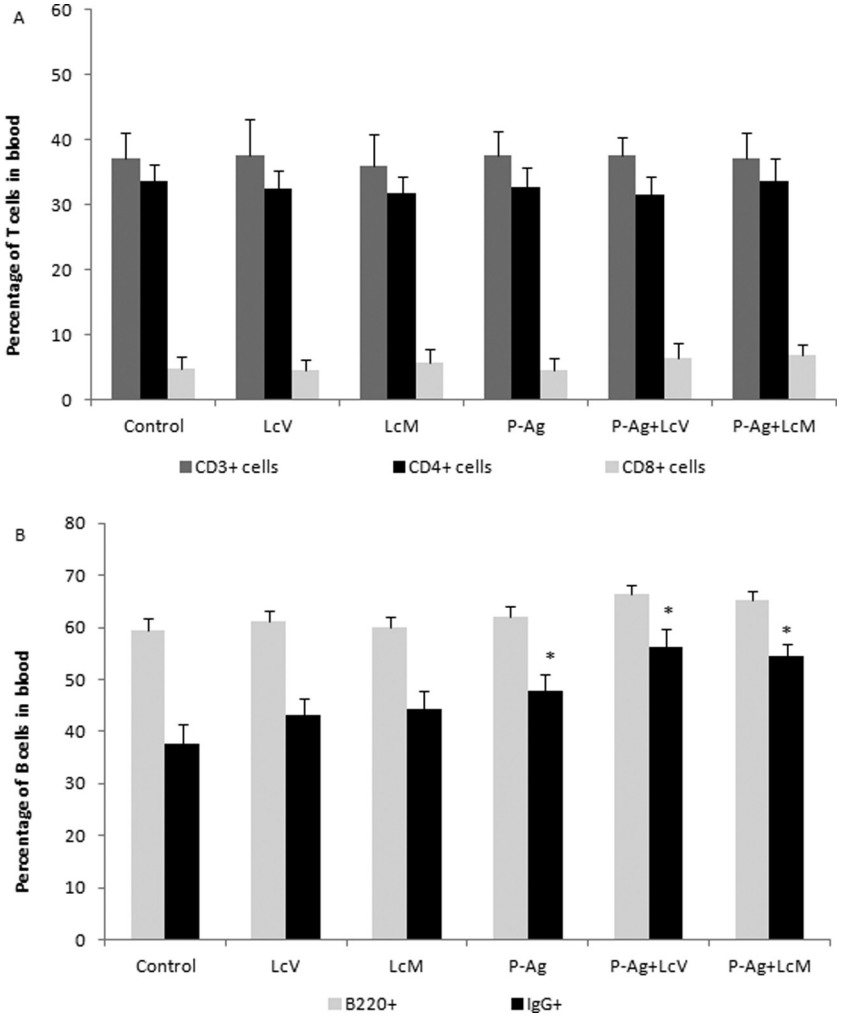
Flow cytometry evaluation of B and T cells in blood

Lymphocyte subpopulations were evaluated in the peripheral blood of immunized mice by flow cytometry analyses (Figs. 3A and 3B). Analyses of T CD3+, CD4+, and CD8+ cells in blood showed no significant differences between groups on day 14 after the last immunization. Since IgG is a crucial Ig in the systemic compartment involved in humoral immune responses, we studied the effect of nasal experimental vaccines on IgG+ cells. Although the percentage of B220+ cells showed no significant differences between experimental groups, IgG+ cells increased in the P-Ag and vaccinated groups. These results agree with the levels of anti-P-Ag IgG measured in the plasma. In addition, LcV and LcM tended to increase the numbers of IgG+ cells, indicating an adjuvant effect on the systemic humoral immune response; however, no significant differences were observed compared with control.

Cytokines profile in mucosal and systemic compartments

The profile of different cytokines induced 2 weeks after the last immunization were analyzed in mucosal and systemic compartments (Table 1) to evaluate the activation of the immune system induced by experimental vaccines. In mucosal fluids (NL and BALF), IL-2 and INF-γ levels were increased by experimental vaccines (P-Ag-LcV and P-Ag-LcM) compared with basal values. IL-2 levels showed no significant differences between P-Ag+LcV and P-Ag+LcM in BALF and NL. INF-γ levels increased in all of the experimental groups, but the highest values were observed in P-Ag+LcV (P-Ag+LcV compared with LcV = $p < 0.01$) and P-Ag+LcM (P-Ag+LcM compared with LcM = $p < 0.01$). IL-17 also increased in NL and BALF after immunization with LcV, LcM, P-Ag+LcV, and P-Ag+LcM, while P-Ag increased IL-17 only in NL. IL-4 was increased in NL and BALF in all of the experimental groups, indicating stimulation of the humoral immune response. On the other hand, ensuring a balanced immune response against a pneumococcal infection after immunization is an important factor that should be evaluated in experimental vaccines. Thus, IL-10 regulatory cytokine was assessed. In NL, all of the experimental groups showed increased IL-10 levels; in BALF the profile was similar, although P-Ag did not induce an increase in IL-10. A pro-inflammatory TNF-α cytokine was also measured in mucosal fluids (NL, BAL), with no significant differences being found in any of the experimental groups compared with the respective basal levels. The cytokine profile in plasma was also evaluated. INF-γ, IL-2, IL-4, and IL-17 levels in plasma increased in all of the experimental groups compared with controls. IL-10 increased significantly when mice were

Fig. 3. Percentages of T (CD3+, CD4+, CD8+) and B (B220+, IgG+) cells in the blood of mice stimulated with experimental vaccines (P-Ag+LcV, P-Ag+LcM) and the respective controls (LcV, LcM, PppA, PBS) on day 14 after the last immunization. (A) Percentage of T cells. (B) Percentage of B 220+ and IgG+ cells. Data are the mean ± SD values from 6 mice. Results are representative of 2 independent experiments; *, $p < 0.05$ and **, $p < 0.01$ compared with the control group (PBS).



immunized with experimental vaccines and P-Ag alone. LcM and LcV tended to increase IL-10, but no significant differences were observed compared with the controls. Finally, plasma TNF-α levels did not increase in any of the experimental groups (control value = 39 ± 5 pg·mL^{-1}). Results showed that nasal immunization with the experimental vaccines increased mucosal and systemic Th1, Th2, and Th17 cytokines, while the typical cytokine of the innate immune response TNF-α did not increase in either the systemic or the mucosal compartments.

Discussion

In this work we investigated whether nasal administration of LcM and LcV as mucosal adjuvants elicited bacterial Ag (P-Ag)-specific antibodies and cellular immune responses in respiratory mucosa and blood. Our results clearly showed that both LcM+P-Ag and LcV+P-Ag vaccines were effective in inducing IgA and IgG anti-P-Ag Abs in the upper and lower respiratory tract and plasma. Although immunization with P-Ag alone induced anti-PspA IgA and IgG in the respiratory tract and plasma of mice, levels were significant lower than those in the experimental vaccines. Thus, it is evident that LcV and LcM exerted an adjuvant effect on the humoral immune response that correlates with the capacity of *L. casei* to induce the Th2-cytokine IL-4 as well as IL-10 in NL, BAL, and plasma when nasally administered either alone (live and heat-killed) or combined with PppA. IL-4 and IL-10 contributed to expand specific B cell populations in mucosa and to increase the levels of mucosal specific-P-Ag Abs (Zhang et al. 2006). Mucosally induced S-IgA antibodies are a critical tool in vaccination to protect the host against pneumococcal infection. Th2 cells are involved in the development of humoral immunity and are characterized by the secretion of IL-4. The increase in IL-4 in mucosal compartments would indicate activation of the Th2 cell population. These cells also produce IL-5, which is involved in IgA class switching. Based on this fact, the activation of Th2 cells by an experimental vaccine would stimulate IgA production by IL-5 release. Studies are in progress to evaluate other cytokines such as IL-6, IL-5, and chemokines to clarify the mechanisms involved in IgA induction in nasopharynx and lung. The experimental vaccines increased IgA+ cells in NALT and lung. Nasal immunization has been shown to effectively induce Ag-specific IgA response via NALT for the upper respiratory tract (Hiroi et al. 1998; Yanagita et al. 1999) because NALT contains all the molecular and cellular environments necessary for the initiation of IgA B cell responses (Wu et al. 1997; Csencsits et al. 1999) and IgA-committed B cells as well as memory-type IgA-B cells are generated in NALT (Csencsits et al. 1999) and could migrate to the lung. IgA production at mucosal surfaces is regulated by the coordinated communication consisting of mucosal B cells, mucosal T cells, mucosal dendritic

Table 1. Profile of systemic and mucosal cytokines induced by experimental vaccines.

Cytokines (pg·mL^{-1})	IL-2	INF-γ	TNF-α	IL-4	IL-10	IL-17
NL						
Control	4.0±0.5^a	6.5±1.4^a	49.1±5.8^a	13.9±1.9^a	42.8±12.1^a	13.1±2.5^a
LcV	9.2±0.6^b	16.4±2.1^b	51.0±4.9^a	28.2±1.4^b	109.6±9.8^b	31.5±3.8^b
LcM	8.4±0.7^b	15.3±2.4^b	50.3±6.1^a	31.5±5.3^b	119.8±10.2^b	33.9±4.2^b
P-Ag	4.6±0.6^a	12.5±2.6^b	52.8±5.9^a	63.2±8.6^c	80.4±14.8^b	20.2±3.6^c
P-Ag+LcV	13.9±0.8^c	36.1±1.3^c	53.2±4.7^a	120.0±6.9^d	190.7±15.2^c	61.2±4.1^d
P-Ag+LcM	12.2±0.7^c	34.7±2.9^c	51.9±7.8^a	122.1±7.6^d	194.6±14.1^c	58.3±3.7^d
BALF						
Control	16.8±0.7^a	70.4±9.8^a	13.5±2.1^a	63.3±6.1^a	78.3±9.7^a	25.9±3.9^a
LcV	29.7±0.5^b	136.4±11.3^b	15.3±3.2^a	121±5.7^b	158.3±11.5^b	42.5±4.4^b
LcM	27.9±1.4^b	117.8±12.2^b	14.6±2.4^a	133.2±10.4^b	163.5±14.6^b	36.8±5.7^b
P-Ag	24.1±2.2^c	101.4±10.4^b	13.3±1.9^a	125.1±12.8^b	95.3±11.8^a	27.4±4.6^a
P-Ag+LcV	46.2±1.9^d	305.8±16.8^c	15.1±3.4^a	307±14.6^c	375.2±10.9^c	99.3±4.9^c
P-Ag+LcM	40.5±2.8^d	278.2±17.9^c	15.4±2.6^a	298.5±23.5^c	396.2±23.8^c	105.7±8.2^c
Plasma						
Control	9.4±1.6^a	5.1±0.9^a	34.5±4.8^a	10.7±2.3^a	96.6±10.3^a	17.5±2.6^a
LcV	18.8±1.9^b	14.9±0.7^b	35.4±5.2^a	26.1±3.1^b	114.2±12.1^a	22.8±1.7^b
LcM	19.6±2.3^b	14.6±1.3^b	36.4±4.1^a	25.6±2.6^b	115.2±10.4^a	23.8±2.1^b
P-Ag	19.5±2.5^b	13.6±2.1^b	37.9±3.8^a	28.9±3.2^b	127.3±11.2^a	29.9±3.2^b
P-Ag+LcV	29.1±2.4^c	20.8±1.8^c	38.7±5.3^a	41.3±3.6^c	203.2±10.7^b	39.5±3.3^c
P-Ag+LcM	27.2±2.2^c	21.7±1.6^c	39.1±5.7^a	39.7±2.9^c	208.3±14.2^b	43.1±2.3^c

Note: Cytokines induced in nasal lavages (NL), bronchoalveolar fluids (BALF), and plasma by nasal administration of LcV, LcM, P-Ag, and the experimental vaccines (P-Ag+LcV, P-Ag+LcM). Cytokine levels were measured on day 14 after the last immunization. Values are the mean ± SD of 6 mice per group. Different letters after a cytokine value indicate that the values differ at $p < 0.05$.

cells, and epithelial cells. Abs induced by protein Ags is T-cell-dependent because B–T cell cooperation is crucial to induce a strong immune response. Our results demonstrate that LcV, LcM, and the experimental vaccines tested increased total CD3+ T cells as well as CD4+ and CD8+ T cells in NALT, showing that the T lymphocyte population was expanded by these stimuli. In rodents, NALT is an important inductive tissue for the generation of mucosal immunity to inhaled antigens, capable of disseminating effector cells at distant mucosal sites (Kuper et al. 1992; Kiyono and Fukuyama 2004). In the lung, T CD4+ cells were significantly increased by the experimental vaccines, while LcV and LcM alone tended to increase these T helper cells, which are necessary for host defense against pneumococcal infection (Kadioglu et al. 2004). Activation of T cells implies the release of various cytokines and chemokines from different T-cell subsets, and recognition of antigenic epitopes within the context of MHC class 1 or 2 molecules. Both T-cell activation and release of specific cytokines are involved in the eventual process of B cell activation, isotype switch, and specific integrin expression on antigen-sensitized B cells that allow their migration. Th1, Th2, and Th17 cytokine profiles induced by adjuvants and experimental vaccines in NL and BALF were an indirect measure of Th cell activation and showed the adjuvant effect of LcV and LcM on cellular immunity. The increase in IL-2 supports T-cell proliferation, drives cytokine production, and is important for inducing immunological T memory (Foulds et al. 2006). The other Th1 cytokine, INF-γ, is significant in the neutrophil-mediated host protective responses against pneumococcal infection (Foulds et al. 2006), since it enhances chemokine expression and promotes pulmonary neutrophil recruitment into the infected lung (Sun et al. 2007). Moreover, a recent study has shown that an upregulation of IL-17- and IFN-γ-related gene expression together with the development of a Th17 response are relevant in protective immunity against *S. pneumoniae* infections (Marqués et al. 2012). IL-17 produced by Th17 cells plays an important role in protective immunity against *S. pneumoniae*, and in such cases it would be highly beneficial to induce preexisting Th17 memory by vaccination. It has been demonstrated that some pneumococcal proteins can induce T-cells, and that CD4-Th17 cells reduce pneumococcal colonization in mice in an Ab-independent way (Malley et al. 2005; Basset et al. 2007). With respect to CD8+ cells, Weber et al. (2011) suggested that CD8+ T cells modulate CD4+ T cell-mediated and (or) Th17 responses in pneumococcal infection in the lungs, resulting in reduced inflammation, damage, and pathogen dissemination. It seems probable that the increased number of CD8+ T cells in NALT would contribute to limiting the inflammatory responses to regulate inflammatory tissue injury. Further studies are necessary to evaluate this hypothesis. In addition, high levels of IL-10 were produced when mice were immunized with the experimental vaccines. IL-10, one of the most important cytokines with anti-inflammatory properties, is produced by a wide variety of cells such as monocytes/macrophages and T cell subsets including Tr1, Treg, and Th2 cells (Sabat et al. 2010). IL-10 regulates the functions of different immune cells and one of its relevant functions is to prevent and limit exacerbated specific and nonspecific immune responses that could injure the host's tissues. In our model, the induction of IL-10 by vaccination would be particularly important after infection with *S. pneumoniae*, where the adaptive response should be regulated to clear the pathogen without tissue damage. In this regard, a typical pro-inflammatory cytokine, TNF-α, was measured, and no increase in its levels was found in either mucosa or plasma. In a previous report it was demonstrated that mice that received LcV orally had increased TNF-α levels in BALF at 12 h after pneumococcal challenge (Racedo et al, 2006) while LcV did not induce an increase in this cytokine before infection. The capacity to rapidly secrete TNF-α to the airways would be related to innate host responses against pneumococci, which is improved by LcV administration, although levels decreased rapidly. In our model we only considered the effect of vaccination on the immune system, and on the basis of the above data it was expected that on day 14 after vaccination TNF-α levels would not be increased by comparison with the control mice. These results would indicate that the experimental vaccines do not induce a pro-inflammatory state in the host. In this sense, a recent report showed that nasal vaccination with pneumococcal proteins (PspA and PspC) induced low levels of TNF-α in the airways 13 h after pneumococcal challenge. This fact was correlated with increased protection against pneumococcal invasive challenge (Ferreira et al. 2009), and strong down-

regulation of this cytokine was observed in the vaccinated mice. In addition, low levels of IL-6 and other pro-inflammatory cytokines were found in protected mice after pneumococcal infection. Although the properties of pneumococcal proteins are dependent on each protein, in the present work similar results were obtained when TNF-alpha was evaluated. The correct balance between regulation/inflammation after vaccination is important to prevent a protective immune response that could damage the host when pneumococcal infection occurs. In this sense, other inflammation parameters are currently being evaluated after pneumococcal challenge in immunized mice, and preliminary results have shown that the protective response would be safe for the host. On the other hand, the experimental vaccines did not induce changes in T cell percentages in the systemic compartment; however, the cytokine profile induced by adjuvants and experimental vaccines was similar to those found in mucosa. Thus, activation of T cells in blood also occurred. In addition, high levels of IgA and IgG specific-P-Ag-Abs were induced in plasma compared with P-Ag alone, and the percentage of IgG+ cells was increased in the blood. A recent report concerning nasal vaccination against a respiratory virus showed that bone-marrow-resident antibody forming cells (AFC) had similar kinetics to specific serum antibodies (Sealy et al. 2010), with these cells being considered as a source of IgG antibodies. In our model, it seems likely that the antigen-specific IgG producing cells originated in bone marrow, and would be the effector cells responsible for plasma antibody levels. This fact reinforces the concept that nasal stimulation induces a strong specific immune response in the systemic compartment, indicating that LcV and LcM as nasal adjuvants could enhance the systemic immune response, a desirable property for a nasal vaccine. Overall, antibody levels were low in the nasal lavages compared with levels in the serum, possibly because antibodies are tethered to secretory components on cells lining the respiratory tract and are not easily released from the lumen wall (Murphy et al. 2008). It should be noted that LcV and LcM as nasal adjuvants showed similar effects on the immune system when they were nasally administered. Thus, the viability of the strain is not a crucial condition in the stimulation of the antigen-specific immune response and LcM emerge as a convenient and effective mucosal adjuvant.

Considering NALT as the primary inductive site of the common mucosal immune system (Wu et al. 1997), we hypothesized that the intranasal priming of NALT with live and heat-killed *L. casei* would activate the immune system and enhance the response against a specific pneumococcal antigen after vaccination. Direct stimulation at the nasopharynx level with experimental vaccines would induce specific P-Ag-IgA and T CD4+ cells in NALT that were able to migrate to the lung and populate this tissue with specific IgA-secreting cells and T memory cells. This fact would explain the increase in IgA and T CD4+ cells observed in the lung when mice were immunized with experimental vaccines. T CD8+ cells increased in NALT did not migrate into the lung, and their effect would limit tissue damage at the nasopharynx level (Weber et al. 2011). The induction and maintenance of antigen-specific T-cell responses is an essential feature in the design of effective mucosal vaccines, and nasal administration of LcM and LcV concomitantly with a protein antigen could enhance the antigen-specific antibody response. In addition, production of IgA AFC in plasma would be favored by the "IgA cycle". In previous studies, it was demonstrated that the oral administration of *L. casei* induced an increase in IgA cells in the bronchus-associated lymphoid tissue (BALT) in lung (Perdigón et al. 1999). It is evident that the effect of nasal stimulation with *L. casei* on the mucosal immune system is stronger than when the oral route is used because in our model IgA as well as CD4+ cells migrated to the lung. To the best of our knowledge, this is the first study dealing with the possible immune mechanisms involved in the stimulation of NALT by live and heat-killed lactic acid bacteria when they are used as adjuvants in nasal vaccination, taking into account their projection on the immune response in lung and systemic compartment. Further studies are necessary to confirm the hypothesis proposed, but this work represents a starting point to conduct further studies on the immune mechanisms induced by live and dead lactic bacteria administered as nasal vaccine adjuvants.

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