

Lactococcus lactis as an adjuvant and delivery vehicle of antigens against pneumococcal respiratory infections

Marcela Medina,^{1,2,*} Elisa Vintiñi,¹ Julio Villena,¹ Raul Raya¹ and Susana Alvarez^{1,2,*}

¹Laboratorio de Bioquímica y Clínica Experimental; Centro de Referencia para Lactobacilos (CERELA-CONICET); Tucumán, Argentina; ²Facultad de Bioquímica, Química y Farmacia; Universidad Nacional de Tucumán; San Miguel de Tucumán; Tucumán, Argentina

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Most studies of *Lactococcus lactis* as delivery vehicles of pneumococcal antigens are focused on the effectiveness of mucosal recombinant vaccines against *Streptococcus pneumoniae* in animal models. At present, there are three types of pneumococcal vaccines: capsular polysaccharide pneumococcal vaccines (PPV), protein-polysaccharide conjugate pneumococcal vaccines (PCV) and protein-based pneumococcal vaccines (PBPV). Only PPV and PCV have been licensed. These vaccines, however, do not represent a definitive solution. Novel, safe and inexpensive vaccines are necessary, especially in developing countries. Probiotic microorganisms such as lactic acid bacteria (LAB) are an interesting alternative for their use as vehicles in pneumococcal vaccines due to their GRAS (Generally Recognized As Safe) status. Thus, the adjuvanticity of *Lactococcus lactis* by itself represents added value over the use of other bacteria, a question dealt with in this review. In addition, the expression of different pneumococcal antigens as well as the use of oral and nasal mucosal routes of administration of lactococcal vaccines is considered. The advantages of nasal live vaccines are evident; nonetheless, oral vaccines can be a good alternative when the adequate dose is used. Another point addressed here is the use of live versus inactivated vaccines. In this sense, few researchers have focused on inactivated strains to be used as vaccines against pneumococcus. The immunogenicity of live vaccines is better than the one afforded by inactivated ones; however, the probiotic-inactivated vaccine combination has improved this matter considerably. The progress made so far in the protective immune response induced by recombinant vaccines, the successful trials in animal models and the safety considerations of their application in humans suggest that the use of recombinant vaccines represents a good short-term option in the control of pneumococcal diseases.

Introduction

Respiratory infections represent a serious worldwide health problem that affects mainly young children, the elderly people and immunocompromised individuals. Respiratory Syncytial Virus (RSV) is the most common viral cause of lower respiratory tract infections (LRTIs), especially in young children, and *Streptococcus pneumoniae* (pneumococcus) and *Haemophilus influenzae* are the most common bacterial causes.¹

At present, *S. pneumoniae* (pneumococcus) is a leading cause of morbidity and mortality among children all over the world and a major cause of diseases such as pneumonia, meningitis and sepsis. An estimated 700,000 to one million children, most of whom live in developing countries, die of pneumococcal diseases every year.²⁻⁴ In these countries, pneumococcal disease is common in children under two years of age, including newborn infants, while in the elderly population incidence rates are unknown. The most common form of the disease is bacteremic pneumococcal pneumonia, whose highest incidence is associated with both extremes of age (children <2 years of age and adults >65 years of age). The next common disease is pneumococcal meningitis, especially in infants and young children, followed by blood stream infection or sepsis and otitis media. Pneumococci also colonize the nasopharynx of up to 50% of the children in the world while up to 5% of the adults are pneumococcal carriers, which increase the risk of spreading the infection.

The world trend in the control of pneumococcal diseases is towards the design of mucosal vaccines because they are practical, non invasive and effective for the induction of an adequate response at both the mucosal and the systemic levels. In this sense, lactic acid bacteria (LAB), microorganisms with GRAS (Generally Recognized As Safe) status, some of them with important immunostimulant properties and positive effects on human and animal health,⁵⁻⁷ represent an interesting alternative for the development of a mucosal vaccine. The most common lactic acid bacterium used as a vehicle of antigen presentation is *Lactococcus lactis*, a bacterium extensively engineered for the production of heterologous bacterial and viral proteins.⁸⁻¹⁰

The aim of this review is to update the most relevant findings concerning the use of the recombinant *L. lactis* as an adjuvant and delivery vehicle of antigens against pneumococcal infections

*Correspondence to: Marcela Medina and Susana Alvarez;
Email: mmedina@cerela.org.ar and salvarez@cerela.org.ar
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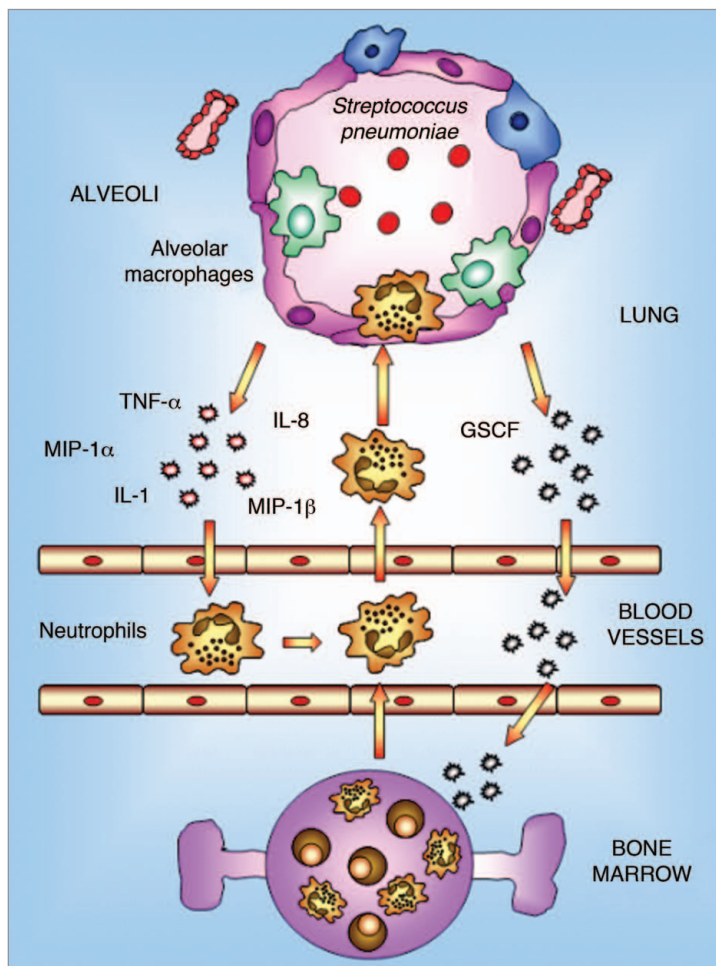


Figure 1. Innate immune response in lung. Alveolar macrophages constitute the first line of phagocytic defense against infectious agents and play a prominent role in lung immunity by initiating inflammation and immune responses. In the event that invading pathogens are too virulent or represent too large a load to be contained by macrophages alone, alveolar macrophages are capable of generating mediators that orchestrate the recruitment of large numbers of neutrophils from the pulmonary vasculature into the alveolar space. These neutrophils provide auxiliary phagocytic capacities that play a critical role in host defense against pathogens.

and, from a prophylactic point of view, to gain insight into the protective immune effect induced by the oral and nasal administration of a recombinant vaccine.

Pneumococcal Vaccines

For many years pneumococcal infections were controlled by antimicrobial treatments which have contributed to the increase in the rate of antimicrobial resistance among *S. pneumoniae* strains. Increasing pneumococcal resistance to antibiotics indicates the urgent need for effective vaccines to prevent and control pneumococcal diseases. Thus, eliciting an adequate protective immunity through vaccination represents a major challenge.

At present, there are three established approaches in vaccine development against pneumococcus: capsular polysaccharide

pneumococcal vaccines (PPV), protein-polysaccharide conjugate pneumococcal vaccines (PCV) and protein-based pneumococcal vaccines (PBPV). Only PPV and PCV have been licensed and are in current clinical use while PBPV are still under investigation.

Pneumococcal epidemiology is based on 91 serotypes,¹¹ each corresponding to the different structures of the capsular polysaccharide (CP), one of the major virulence determinants of *S. pneumoniae*. These polysaccharides differ in immunological properties, chemical composition and virulence potential and relatively few of *S. pneumoniae* serotypes are responsible for most serious pneumococcal diseases. Antibodies to the capsular polysaccharide confer protection against disease and vaccines in use are based on the induction of these antibodies. However, this protection is serotype specific. Thus, the currently available 23-valent PPV, licensed in 1983, has the potential to prevent pneumococcal disease and contains CP antigens against the 23 most prevalent serotypes in clinical isolates.¹² These antigens induce type-specific antibodies by a T-cell independent way that improves opsonization and promotes phagocytosis and killing of pathogens by cells of innate immunity.¹³ The degree of protection against different serotypes and within different populations is uncertain. After PPV vaccination, the protective immune response is good in children >2 years but in younger infants, elderly people and immunocompromised individuals the specific humoral response may be poor. In addition, these individuals may respond only against some of the serotypes contained in the vaccine.^{12,13} Different meta-analyses in adults have provided inconclusive results concerning the effectiveness of PPV in preventing pneumonia in high-risk groups, especially in the elderly.^{12,14}

Based on the low effectiveness of PPV in young children, a new generation of vaccines with good immunogenicity in infants was developed. These conjugate vaccines (PCV) are based on a combination of CPs with a highly immunogenic protein (i.e., a non-toxic diphtheria toxoid). These vaccines induce good B- and T-cell specific immune responses and protect against vaccine serotypes that induce invasive pneumococcal disease. The currently available PCV was licensed in 2000 and contains antigens against the seven most common pneumococcal serotypes (PCV-7) that cause disease in children. PCV-7 has already been included in the national infant immunization programme of more than 10 developed countries (<http://www.who.int/nuvi/pneumococcus>). In developing countries, other serotypes should be included in the formulation of PCV-7 to be effective. Thus, new conjugate vaccines including additional serotypes (9 and 11) are expected to be available in the next few years. On the other hand, although these vaccines can be a good alternative for developed countries, they are too expensive for developing countries so that support from governments and international agencies would be required. It is important to keep in mind that PCVs do not represent a definitive solution because recent researches have shown that serotypes eradicated by the vaccine are being replaced by non-vaccine pneumococcal serotypes and the impact of a pneumococcal vaccination programme would be reduced if serotype replacement were significant.¹⁵⁻¹⁷

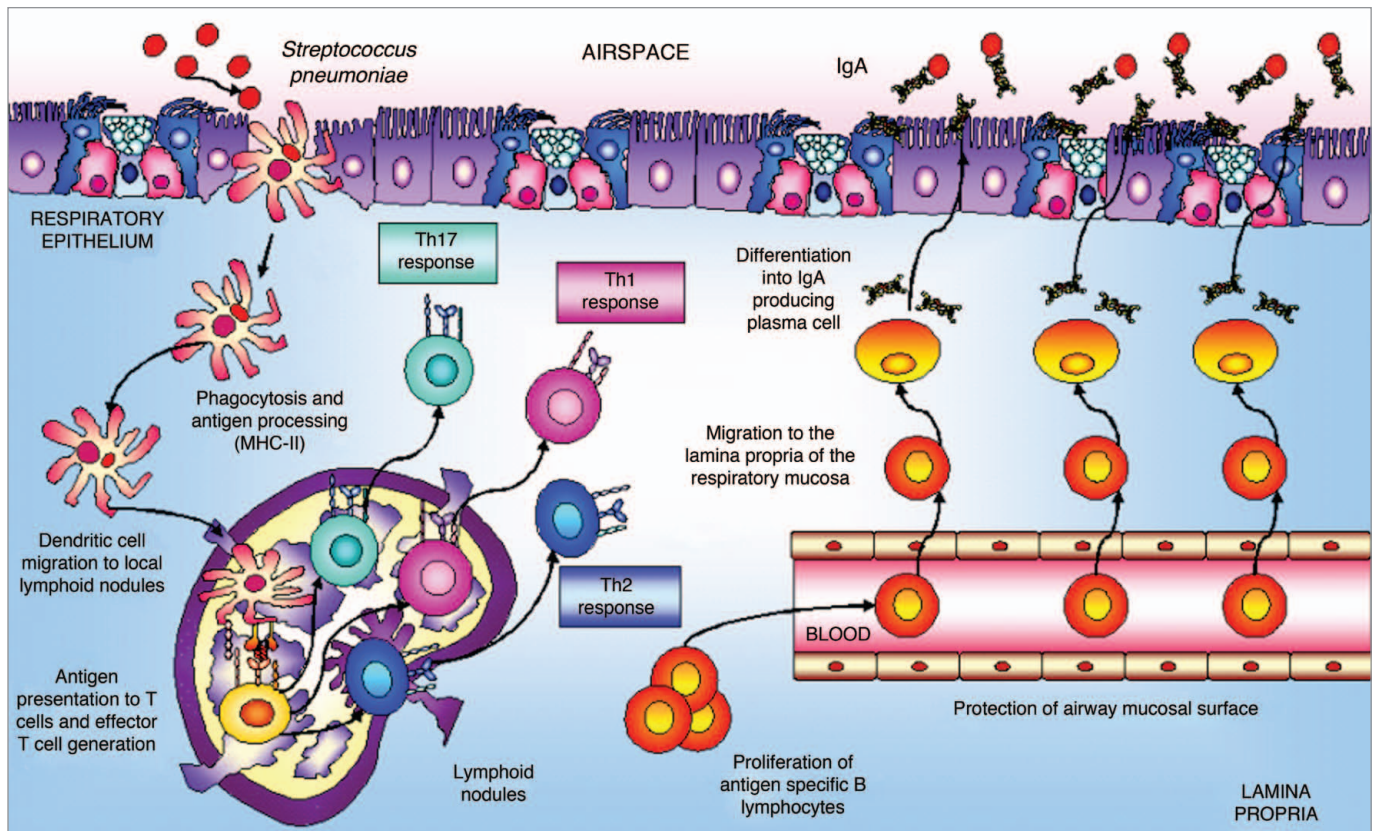


Figure 2. Antigen uptake and migratory pattern induction in the airways. The production of specific IgA in the respiratory tract during an infectious process is very important because it prevents colonization of mucosal sites and subsequent spreading into the systemic circulation.

Besides the polysaccharide capsule of *S. pneumoniae*, numerous pneumococcal proteins are important virulence factors and are also associated with its pathogenicity. In particular, new strategies in the fight against this pathogen involve the identification of pneumococcal surface proteins that are serotype independent. These proteins represent a valuable option for the design of a vaccine through the induction of specific antibodies that neutralize or reduce the virulence of the pathogen. Several pneumococcal proteins alone or in combination with others have been evaluated in animal models. These studies have shown that some of these PBPV formulations were able to protect against invasive infection and nasopharyngeal carriage.¹⁸⁻²¹ Compared with PPV and PCV, the major potential advantages of PBPV are that it could induce a serotype-independent protection and that it can be administered through the oral or nasal mucosal routes. Adjuvants are necessary for protein administration through the mucosal route and cholera toxin or heat-labile enterotoxin has been used. However, these adjuvants may not be clinically safe.²² At present, none of the formulations containing proteins have been licensed for use in human health.

Thus, new, safe and inexpensive vaccines for global application to populations at risk are necessary to control pneumococcal diseases, especially in developing countries. In this sense, probiotic microorganisms such as LAB represent an interesting alternative for their use as vehicles in vaccine formulations since they have important immunomodulatory properties^{6,7} that include

the improvement of the immune response against respiratory pathogens.^{23,24}

Pneumococcal Infection: Immunity in the Respiratory Tract

The pulmonary immune response against respiratory diseases involves both the innate and the adaptive immune responses. The former is mediated by the phagocytic defense against the pathogen, principally carried out by alveolar macrophages and neutrophils (Fig. 1). Alveolar macrophages have important phagocytic, microbicidal and secretory functions and play a prominent role in lung immunity by initiating inflammatory and immune responses. In the steady state, the main function of alveolar macrophages is the phagocytosis and sequestration of antigens from the immune system to shield local tissues from the development of specific immune responses.²⁵ Alveolar macrophages take up most of the particulate material delivered intranasally, but they do not migrate to regional lymph nodes, nor are they considered to have a significant role in antigen presentation.²⁶ Although resting alveolar macrophages are normally maintained in a quiescent state and produce small amounts of pro-inflammatory cytokines, in the presence of a pathogenic bacteria they are capable of producing proinflammatory chemokines and cytokines such as TNF α and IL-1, which coordinate the innate immune response.²⁷ These

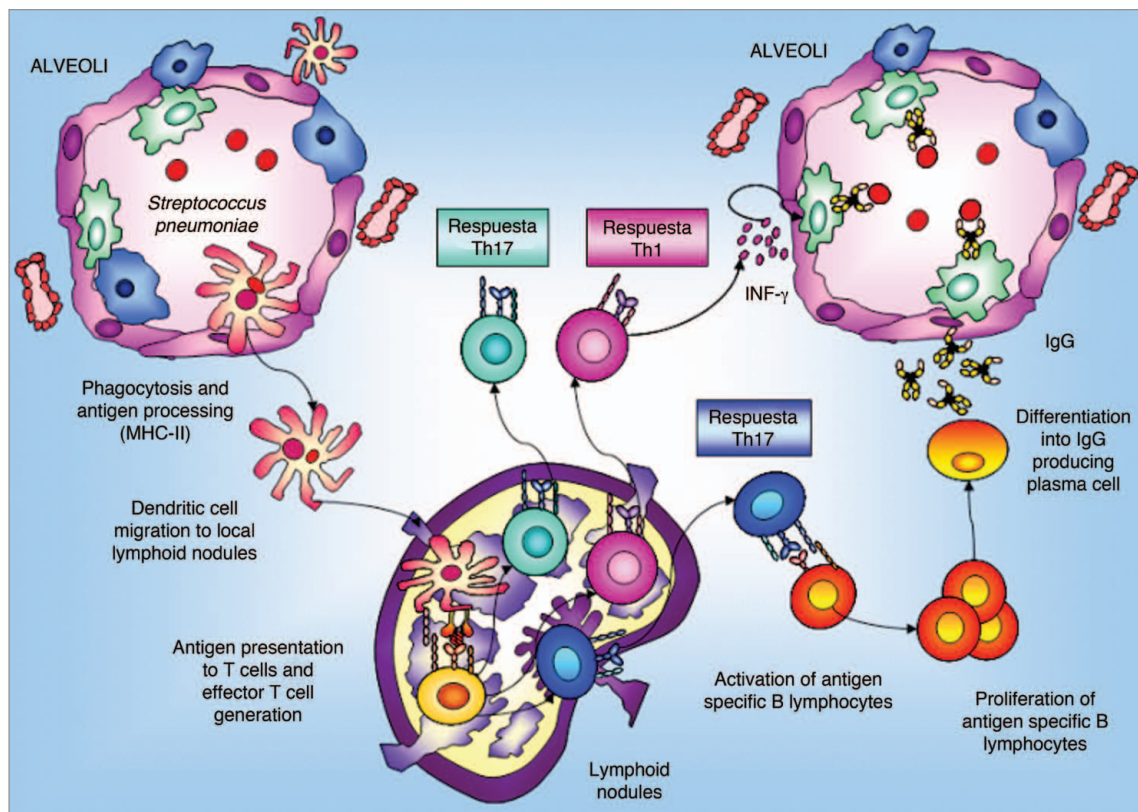


Figure 3. Antigen uptake and migratory pattern for immune induction in deep lung. IgG production plays an important role in the protection against pathogens that reach the alveolar space. Opsonizing IgG antibodies are important for complement fixation and for improvement in the efficiency of macrophages killing. At the systemic level, immune activation also induces production of antibodies responsible for preventing the passage of pathogens to the blood.

biological mediators induce the recruitment of neutrophils and monocytes from the lung capillaries. The recruited neutrophils provide auxiliary phagocytic capacities that are critical for the effective eradication of offending pathogens. On the other hand, it is worth noting that the functional phenotype of recently recruited monocytes shows a marked contrast with that of resident alveolar macrophages; monocytes, in particular, can function as effective antigen-presenting cells (APCs) before their maturation into immunosuppressive alveolar macrophages, which occurs over a period of days.²⁵

One of the most important APCs in lungs are dendritic cells,²⁸ which play a central role in initiating protective T-cell immune responses in the respiratory tract. Dendritic cells, with long, intertwined cytoplasmic processes at the base of the airway epithelium and distributed throughout the lung, form a network ideally positioned to sample inhaled antigens for subsequent presentation to naive T cells. In the steady state, resident lung dendritic cells are specialized for antigen uptake and processing but lack the capacity for efficient antigen presentation. However, when a dangerous antigen reaches the respiratory tissues, dendritic cells are able to take up and process these antigens and to migrate to mediastinal lymph nodes via afferent lymphatics (Fig. 2). The migration of respiratory dendritic cells to draining lymph nodes is accompanied by their maturation and by the increased co-stimulatory expression of CD40,

CD80, CD86 and MHC-II molecules. In regional lymphoid organs dendritic cells present antigenic peptides to naive T cells and stimulate the clonal development of antigen-specific T cells.²⁹ The activated T cells proliferate and migrate through the efferent lymphatics into the bloodstream. Effector T cells exit into the bronchial mucosa through postcapillary venules in the lamina propria or disseminate from the bloodstream throughout the peripheral immune system. In the absence of bacterial stimuli, the airways environment is characterized by a weak Th2 response to inhaled harmless antigens.²⁸ Under inflammatory conditions, cytokines in the respiratory tract change dramatically. CD4⁺ T cells play an important role in the initiation of immune responses by providing help to other cells. Upon antigenic stimulation, naive CD4⁺ T cells proliferate and differentiate into different effector subsets characterized by the production of specific cytokines and effector functions. T helper cells could be divided into distinct subsets, T helper type 1 (Th1), Th2,³⁰ and Th17, characterized by distinct cytokine profiles and effector functions. Th1 cells produce large quantities of IFN γ , activate macrophages and are highly effective in clearing intracellular pathogens. In mice, Th1 cells induce B cells to produce IgG2a.³¹ Th2 cells, on the other hand, produce IL-4, IL-5, IL-13 and IL-25. Th2 cells are especially important for humoral immunity and, in mice, they direct B cell secretion of IgE and IgG1.³¹ The discovery of the IL-17 family of

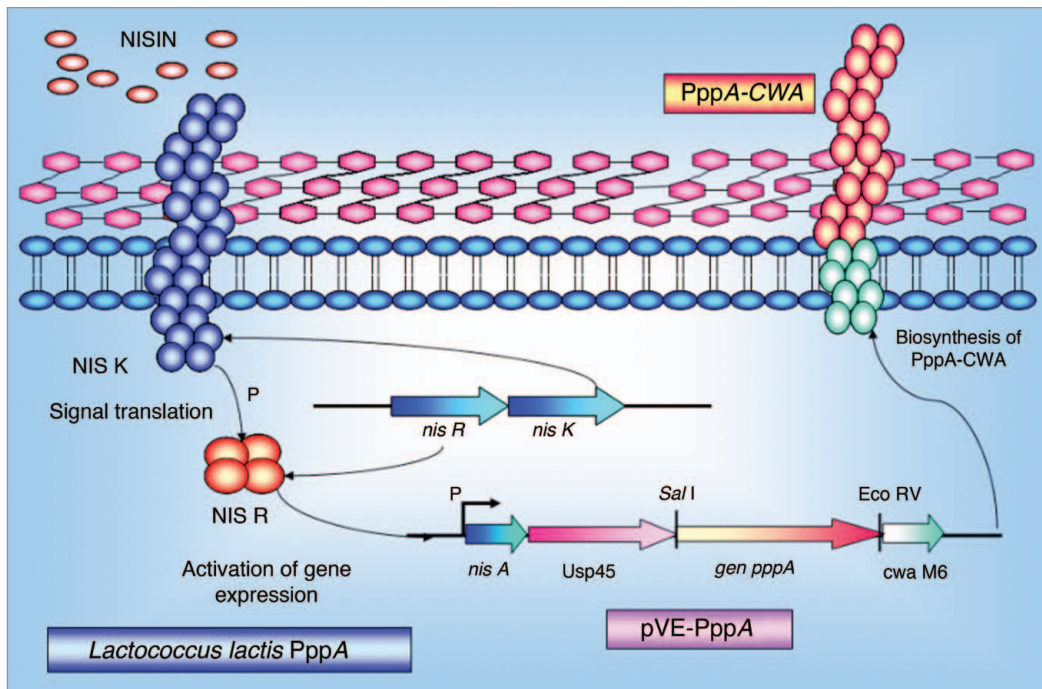


Figure 4. Schematic representation of the nisin-controlled gene expression (NICE) system (40) used to express the recombinant PppA pneumococcal protein in *L. lactis*. *L. lactis* NZ9000 cells containing the signal transduction genes *nisK* and *nisR* in their chromosome were transformed with a plasmid carrying the *pppA* gene under the control of the inducible promoter P_{nisA} . Expression of the PppA recombinant protein was induced by the addition of sub-inhibitory amounts of the nisin and its insertion in the cell wall was mediated by the secretion (sec) and cell wall (CW) anchoring signals.

cytokines and the analysis of IL-23-mediated effector functions on T cells have suggested the existence of an additional subset of CD4⁺ T cells that produce IL-17, designated Th17. CD4⁺ Th17 cells seem to play a critical role in the enhancement of host protection against extracellular bacteria and fungi, which are not efficiently cleared by Th1 and Th2 responses.³² After infection, the activated CD4⁺ Th2-cells contribute to stimulate B cells to proliferate and mature into polymeric IgA-producing cells and to develop specific antibodies.^{33,34} The IgA-committed B cells then migrate to mucosal effector sites where the antigen-sensitized IgM⁺IgA⁺ B cells undergo terminal differentiation to IgA-producing plasma cells. The production of specific IgA in the respiratory tract during an infectious process prevents colonization of mucosal tissues and subsequent spreading into the systemic circulation.³⁵ Additionally, specific IgA antibodies can bind antigens and minimize their entry with a consequent reduction in inflammatory reactions, which prevents potentially harmful effects on the tissue.

In the specific case of *S. pneumoniae*, when this pathogen reaches the alveolar space in the deep lung, resident cells secrete cytokines and chemokines to attract APCs:²⁸ macrophages³⁵ and dendritic cells (Fig. 3). The induction of the specific immune response starts when immature APCs recognize and take up the antigen. Then, these APCs are induced to migrate via the afferent lymphatics into the draining lymph nodes of the lung where the primary immune response occurs. During this migration APCs mature and become efficient, losing their capacity to take up antigens and to activate the expression of

the costimulatory molecules CD80 and CD86 that are necessary to stimulate naive T cells. Mature APCs present the processed antigen in association with MHC-II, which is recognized by the T-cell receptor (TCR) of naive CD4⁺ cells. The recognition of the antigen-MHC by the TCR and the stimulation of the costimulatory signal induce T-cell proliferation, differentiation and migration to peripheral tissues. In addition, activated T cells interact with B cells and induce stimulation of B cell clonal expansion, isotype switching and affinity maturation. Once IgM⁺IgG⁻ have become IgM⁺IgG⁺ antigen-specific B cells, they traffic back to the lung where they terminally differentiate and expand into either antibody-secreting plasma cells or memory B cells.³⁵ These plasma cells are committed to the production of IgG, which has an important role in the protection against pathogens that reach the alveolar space. Opsonizing IgG antibodies are important for complement fixation and for enhanced efficiency in macrophage killing. This immune activation also induces the production at the systemic level of antibodies responsible for preventing the passage of pathogens to the blood and their subsequent dissemination. Besides, systemic antibody transudation increases during inflammatory reactions when vascular permeability is increased, which enhances pulmonary clearance of pathogenic microorganisms.³⁶

Knowledge of the mechanisms involved in the lung immune response against respiratory pathogens is necessary because it is in this context that recombinant vaccines will have to exert their preventive effect.

Table 1. Lactic acid bacteria as vaccine vehicles against *S. pneumoniae*

Antigen	Amplified from	LAB strain (recombinant)	Expresión system	Localization	Route Immunization	Type immune response	Protection	Ref.
Proteins								
PspA	TIGR4	<i>L. lactis</i> F17847	pTnis- inducible with nisin	Citoplasm	Nasal Live and Inactivated vaccine	Live vaccine: Th1-type (IgG1:IgG2a = 3,49) Inactivated vaccine:Th2-type response (higher IgG1:IgG2a ratio) Nasal/IgG (mainly IgG1) but not IgA detected. Anti-PspA induces complement deposition	Significant protection in sepsis and respiratory infection models Increase survival of PspA-immunized mice against intraperitoneal challenge	Hanniffy et al, 2007 Campos et al, 2008 ⁹⁵
	Strain 245/00 Serotype 14 clade 1 PspA	<i>Lb. casei</i> CECT5275	pTINX-constitutive expression from lactococcal promoter P1	Citoplasm				
PsaA	Strain 472/96 serotype 6B	<i>Lb. casei</i> CECT5275 <i>Lb. plantarum</i> NCD01193 <i>Lb. helveticus</i> ATCC15009 <i>L. lactis</i> MG1363	pTINX-constitutive expression from lactococcal promoter P1	Citoplasm and cell wall	Nasal	IgG (serum) and IgA in saliva, nasal and bronchial washes (except <i>Lb casei</i>)	Reduced recovery of <i>S. pneumoniae</i> from nasal mucose in immunized animals after <i>S. pneumoniae</i> challenge.	Oliveira et al, 2006
PppA	T14, serotype 14	<i>L. lactis</i> NZ9000	pVE5547, inducible with nisin	Cell wall	Nasal and oral/Live (L) vaccine Nasal/ Inactivated (D) vaccine	Local (IgA, IgG) and systemic responses (IgG)	L vaccine: Improved resistance to systemic and respiratory pneumococcal challenge, both in active and passive immunized mice. Cross protection against serotypes 3, 6b and 23F D vaccine: Improved resistance to respiratory challenge.	Medina et al, 2008; Villena et al, 2008; Vintiñi et al, 2009
PpmA	D39	GEM (based on hot TCA-treated non-living <i>L. lactis</i> NZ9000)	<i>L. lactis</i> PA1001 ^a (pPA32)- Nisin inducible; expressed as PpmA-PA	Cell wall	Nasal	Systemic and mucosal	NO (only in combination with IgA1p-PA and SlrA-PA)	Audouy et al, 2006; Audouy et al, 2007
SlrA	D39	GEM (based on hot TCA-treated non-living <i>L. lactis</i> NZ9000)	<i>L. lactis</i> PA1001 (pPA162)- Nisin inducible; expressed as SlrA-PA	Cell wall	Nasal	Systemic and mucosal	NO (only with IgA1p-PA and PpmA-PA or in combination with IgA1p-PA)	Audouy et al, 2006; Audouy et al, 2007
IgA1p	TIGR4	GEM (based on hot TCA-treated non-living <i>L. lactis</i> NZ9000)	<i>L. lactis</i> PA1001 (pPA152)- Nisin inducible; expressed as IgA1p-PA	Cell wall	Nasal	Systemic and mucosal (lower levels with regard to PpmA-Pa and SlrA-PA)	NO (only with PpmA-PA and SlrA-PA or in combination with SlrA-PA)	Audouy et al, 2006; Audouy et al, 2007
Polysaccharides								
Serotype 3	<i>S. pneumoniae</i> WU2	<i>L. lactis</i> MG1363	Constitutive expression from original promoter	Mainly associated with cells	Intraperitoneal	T-cell independent response (IgM, Ig1 and Ig3)	n.d.	Gilbert et al, 2000
Serotype 14	"serotype 14" strain	<i>L. lactis</i> NZ9000	Two-plasmids system. Plasmid with regulatory genes inducible with nisin	Mainly supernatant	n.d.	n.d	n.d.	Nierop Groot et al, 2008

^a MG1363 (*acmA*; *htrA*; *nisRK*²) strain, lacking the AcmA cell-wall hydrolase activity and the housekeeping protease HtrA (which has been shown to degrade the AcmA cell-wall binding domain). The NisRK genes are required for nisin induced expression. n.d., not done.

Expression System of Pneumococcal Antigens in Lactic Acid Bacteria

Due to their non-pathogenic status and the availability for recombinant gene expression, lactic acid bacteria are attractive candidates for use as vaccine carriers. The possibility of using LAB as vaccine vehicles has been thoroughly reviewed by Wells and Mercenier.³⁷ Most studies that evaluated LAB as vaccine vectors to elicit *S. pneumoniae*-antigen specific immune responses used

protein antigens instead of polysaccharides (Table 1), *Lactococcus lactis* NZ9000 being the most common lactic acid bacterium employed as a vehicle.

Gilbert et al.³⁸ evaluated *L. lactis* as an attractive producer host for polysaccharides of pneumococcal origin and showed that recombinant *L. lactis* strains produced serotype 3 capsule polysaccharides (CPS) with a composition and structure identical to that of the native CPS produced by *S. pneumoniae*, while Nierop Groot et al.³⁹ did the same for serotype 14. Besides, the

polysaccharide produced by recombinant *L. lactis* was not contaminated with C polysaccharide, a highly reactive immunogen produced by the pneumococcus. Immunization of mice with recombinant *L. lactis* producing type 3 CPS elicited a T-cell independent response characteristic of those elicited by unconjugated bacterial polysaccharides, showing that *L. lactis* is a potential host for capsule vaccine antigens.³⁸

On the other hand, formulations of LAB expressing the recombinant proteins of PspA, PsaA, PppA, PpmA, SlrA and IgA1p have been shown to be efficacious as vaccine carriers against pneumococci in animal models. Two main strategies have been applied in these studies: the use of recombinant lactic acid bacteria vectors and the use of a non-genetically modified gram-positive enhancer matrix (GEM) particle. In the first strategy, *S. pneumoniae* proteins were expressed in the cytoplasm or bacterial cell wall of the recombinant lactic acid bacteria using the nisin-controlled expression (NICE) system (Fig. 4).⁴⁰ The genes of interest were placed on a plasmid under the control of the inducible promoter P_{nisA} and their expression was induced by the addition of sub-inhibitory amounts of the lantibiotic nisin, in a strain containing the genes for the signal transduction system *nisK* and *nisR*. Depending on the presence or absence of secretion and cell wall (CW) anchoring signals, the proteins were expressed in the cytoplasm (absence of both secretion and CW signals) or in the cell wall (presence of both signals). Three LAB vaccines expressing the *S. pneumoniae* proteins, PsaA (pneumococcal surface antigen), PspA (pneumococcal surface protein A) and PppA (pneumococcal protective protein A) (Table 1) were capable of eliciting significant protection against systemic and respiratory *S. pneumoniae* lethal challenges. The immune effects of each vaccine are discussed below.

In the second strategy, a non-living GEM particle was developed as an antigen delivery device.^{41,42} GEM particles were made from non-living *L. lactis* cells (cells were treated with a 1M hot acid solution for 30 min and washed thoroughly with PBS) free of DNA and cytoplasmic material that retained their bacterial size and surface display of antigens of the original bacterial cells. Heterologous antigens were anchored to the GEM particles by means of a lactococcal peptidoglycan binding domain called the protein anchor (PA). This binding domain is derived from the *L. lactis* AcmA cell-wall hydrolase and contains three repeats of a LysM-type cell-wall binding motif. Antigens, produced as antigen-PA fusions after nisin induction, were secreted into the culture growth medium and, after removal of the recombinant producer cells, were purified from the complex medium in a one-step procedure with the GEM particles. Antigen-PA molecules bind non-covalently and with affinity to GEM particles (10^6 PA/GEM particle) 15 times more strongly than they bind to living cells and multiple antigen-PA fusions can be bound to the same GEM particle. GEM-antigen vaccines are stable at room temperature, lack recombinant DNA, and can be used as antigen delivery particles and adjuvants capable of stimulating the mucosal and systemic responses of the immune system, a subject that will be dealt with in the next section.

***Lactococcus lactis* Nasally Administered as an Adjuvant and Delivery Vehicle of Pneumococcal Antigens**

***Lactococcus lactis* as a nasal adjuvant.** The route of administration of a vaccine is crucial to the improvement in the defenses against respiratory pathogens. Classic vaccination pathways (intramuscular or subcutaneous) mainly lead to systemic immunization. However, the natural route of entry of most pathogens is the mucosa of the gastrointestinal, respiratory or urogenital tracts. Hence, in order to efficiently prevent respiratory infections, the activation of the mucosal immune system is essential.⁴³ Since 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, nasal immunization is commonly used to induce immunity along the respiratory tract.⁴⁴ 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.⁴⁴ Hussell and Humphreys⁴⁵ suggested that NALT could fulfill an important role by reducing the pathogen burden to a level that would induce only minimal inflammation in the lower lung. Using an experimental model of *S. pneumoniae* infection, Cangemi de Gutierrez et al.⁴⁶ showed that the preventive intranasal inoculation of *Lactobacillus fermentum* caused a decrease in the number of pathogens throughout the respiratory tract together with an increase both in the number of activated macrophages in lung and in the lymphocyte population in the tracheal lamina propria. On the other hand, recombinant *L. lactis* NZ 9000 has been proven to elicit an immune response after inoculation by different mucosal routes.⁴⁷ In this respect, it has been reported that mice nasally immunized with a strain of *L. lactis* that expresses an M protein antigen were protected against pharyngeal infection following a challenge with *Streptococcus pyogenes*.⁴⁸ However, the immunological intrinsic properties of this lactococcus were not evaluated. Furthermore, the effect of nasal *L. lactis* administration on the local and the systemic immune response using a pneumococcal infection model⁴⁹ was evaluated. Nasal administration of *L. lactis* cells increased the clearance rate of *S. pneumoniae* from lung and prevented the dissemination of pneumococci into blood. It seems probable that the *L. lactis* cells decreased both the adherence of *S. pneumoniae* to the respiratory epithelium and the number of pneumococci that reached the lung, since it has been reported that nasally administered LAB could competitively exclude pneumococcal cells.⁵⁰ In addition, the increased activation of macrophages observed in mice treated with *L. lactis* would induce a more effective neutrophil recruitment, as reported by Standford et al.⁵¹ and Nelson and Summer.⁵² The pulmonary lymphocyte population, which plays an important role in lung defense against infection,⁵³ was also significantly increased in the bronchoalveolar fluid (BAL) of mice after nasal administration of *L. lactis*. This fact would be related to the increase observed in BAL anti-pneumococcal IgA and IgG. Secretory IgA constitutes the main mediator of specific humoral immunity at mucosal surfaces by neutralizing bacterial

toxins and viral particles⁵⁴ and inhibiting adherence of bacteria to epithelial cells.⁵⁵ IgA may participate actively in protective phagocytosis and killing of *S. pneumoniae* once the inflammatory process is initiated.⁵⁶ Moreover, this immunoglobulin possesses intrinsic modulatory properties that stimulate the mucosal and systemic immune responses.⁵⁷ IgG would be involved in the protection against respiratory pathogens because of its ability to opsonise bacteria and, to a lesser extent, to mediate lysis by the complement.⁵⁸ Studies of lung tissue injury showed that nasal treatment significantly reduced the damage caused by the infection compared to the control. Nasal administration of *L. lactis* was also able to induce the activation of the systemic innate immune response, which was revealed by the increase in the microbicidal function of blood neutrophils and peritoneal cavity phagocytes.⁴⁹ The capacity of LAB to induce the production of cytokines is well documented,^{59,60} these mediator molecules being probably responsible for the above effect on blood and peritoneal cavity. Moreover, recent findings demonstrated in vitro that *L. lactis* NZ 9000 cells are able to upregulate the expression of MHC-II and CD86 co-stimulatory molecules in bone marrow derived dendritic cells.⁶¹ Finally, in the bone marrow cells of mice the nasal administration of *L. lactis* showed: (a) a decrease in the percentage of the post-mitotic pool (metamyelocytes, band cells and neutrophils), probably related to the release of mature neutrophils and the production of granulocytes in response to inflammatory signals;⁶² (b) an increase in bone marrow CD19⁺ cells, possibly related to the increase in serum anti-pneumococcal IgG; (c) a decrease in CD4⁺ cells, probably caused by the mobilization of cells towards the site of infection.⁴⁹ Then, preventive nasal administration of *L. lactis* at the appropriate dose was able to induce a local and systemic specific immune response.

As explained above, GEM particles have been used as a delivery system for mucosal vaccination⁶³ with intrinsic immune stimulatory properties. Investigations have shown that these particles induced the production of the proinflammatory cytokine tumor necrosis factor- α (TNF α) by macrophages as well as the maturation of dendritic cells. In addition, GEM particles were able to activate in vitro the maturation of macrophages and dendritic cells, two important cell types that act as antigen presenting cells for the initiation of the innate immune system.

As shown above, the adjuvant effect of *L. lactis* would represent an important advantage for its use as a delivery vehicle of pneumococcal proteins. Thus, nasal immunization with recombinant *L. lactis* emerges as an effective route of vaccination for both systemic and mucosal immunity against pneumococcal infection.

***Lactococcus lactis* as a nasal live vaccine.** Numerous studies have reported the successful stimulation of systemic and mucosal immunity in mice and humans with vaccines applied to mucosal surfaces.^{64,65} Gram positive food-grade bacteria such as lactococci have significant advantages over attenuated pathogens as vaccine delivery vehicles because of their inherently greater safety.

Lactococcus lactis NZ9000 is the main lactic acid bacterium employed as a vehicle of pneumococcal antigens and different pneumococcal proteins were expressed in this bacterium. Recombinant strains expressing the pneumococcal surface

protein A (PspA), the pneumococcal surface adhesin A (PsaA) and the pneumococcal protective protein A (PppA) have been obtained and their protective effects on animal models are being evaluated.

PsaA is a surface-exposed common lipoprotein detected in all known serotypes of *S. pneumoniae*. This multi-functional and immunogenic protein is a lipoprotein component of an Mn²⁺ transporter and plays a major role in both pneumococcal attachment to the host cell and virulence. In addition, PsaA induces antibodies that would be able to reduce nasopharyngeal colonization, a major prerequisite for pneumococcal pathogenesis.⁶⁶ PsaA was expressed on the surface of different species of LAB,⁶⁷ including *Lactococcus lactis*, *Lactobacillus casei*, *Lactobacillus plantarum* and *Lactobacillus helveticus* (Table 1). Nasal immunization with recombinant *L. lactis* induced very low levels of IgA (in saliva, nasal and bronchial washes) and IgG (in serum) while high levels of anti-pneumococcal antibodies were induced by the other recombinant strains. Moreover, only vaccination with recombinant lactobacilli but not with lactococci led to a decrease in *S. pneumoniae* recovery from nasal mucosa after the colonization challenge. This was probably due to the low amount of PsaA in *L. lactis* and indicates the importance of the amount of the specific antigen expressed in recombinant strains to induce an adequate humoral response in the host. The selection of the LAB to be used as vaccine vectors should be addressed considering the intrinsic properties and the appropriate dose of each strain.

Another pneumococcal protein considered as a potential candidate for vaccines is PspA. This protein plays an important role during systemic infection through the inhibition of complement deposition on the pneumococcal surface with which the pathogen evades the immune system.⁶⁸ In this sense, Hanniffy et al.⁶⁹ evaluated the protection exerted by *Lactococcus lactis* intracellularly producing PspA as a nasal mucosal vaccine in a pneumococcal infection model. The recombinant lactococcal strain was able to protect the host when sepsis and respiratory pneumococcal models were used. This finding was correlated with a shift toward a Th1-mediated immune response associated with low levels of specific antibodies. This tendency was reverted when the inactivated recombinant strain was used as a nasal vaccine and a Th2 biased response was stimulated. Humoral immunity is important in the protection against *S. pneumoniae* but recent reports have indicated that T CD4⁺ cells would also play a significant role in the host's defenses against pneumococcal infections.⁷⁰ Thus, these results represent important advances in the understanding of the immune mechanisms induced by nasally administered recombinant strains.

Adult mice models were used when the protection against *S. pneumoniae* was evaluated after immunizations with recombinant lactococcal vaccines. Considering that children are an important high risk group in pneumococcal diseases, the preventive effect of lactococcal recombinant strains in both young and adult mice was studied. Nisin-controlled gene expression (NICE) was used to develop a recombinant strain of *L. lactis* that efficiently expressed the pneumococcal protective protein A (PppA) on its surface. We assessed the effectiveness of *L. lactis*-PppA to induce local and systemic immune responses in adult (6-week

old) and young (3-week old) mice⁷¹ using an immunization protocol that included three sequential doses of *L. lactis* nasally administered. Adult and young mice induced specific anti-PppA IgM, IgA and IgG antibodies in both serum and BAL. However, the specific antibody levels were significantly higher in immunized young mice than in the adult group while no significant differences in avidity were observed. In addition, similar levels of IgG1 and IgG2a antibodies were detected in BAL and serum in the two mice groups. The two IgG subtypes would contribute to the protection against pneumococcal infection, IgG2a through optimal complement fixation⁷² and IgG1 through Fc receptor binding or by preventing pathogen attachment and mucosal colonization. Nasal immunization with recombinant *L. lactis*-PppA significantly increased the survival of young and adult mice against intraperitoneal challenge with *S. pneumoniae* compared to the controls that received PBS and non-recombinant *L. lactis*. This fact would indicate that live *L. lactis*-PppA is able to protect against systemic pneumococcal infection. The improved resistance to systemic pneumococcal challenge in passive immunization assays of naive young mice showed the protective effect of anti-PppA antibodies induced by nasal vaccination. In addition, cross-protective immunity against four pneumococcal serotypes was demonstrated. On the other hand, the host immune response was directed against the protein expressed by *L. lactis* and not against the vector, as demonstrated by the lack of antibodies against *L. lactis* in the serum or BAL of the immunized mice. An interesting contribution of this study was the comparative assessment of the humoral immune response induced when nasal recombinant immunization was started in young or adult mice. Results suggest that vaccination at an early age would be a better vaccination strategy for improved protection of the host against *S. pneumoniae* infection. However, the application of a live recombinant strain by the nasal route in humans still presents aspects that need to be resolved such as the elimination of the antibiotic resistance genes used in its selection. Induction of protective antibodies by a dead recombinant lactococcus expressing PspA was evaluated in a pneumococcal infection model.⁶⁹ Results demonstrated that a better protection was obtained with the live than with the dead recombinant bacterium. However, the immunity induced by the inactivated recombinant strains was improved by combining them with a probiotic strain. Thus, the preventive effect of the nasal administration of *L. lactis*-PppA live (LL), inactivated (D-LL) and both LL and D-LL vaccines in association with the probiotic strain (*Lactobacillus casei* CRL 431) was evaluated in young mice.⁷³ Animals that received LL and D-LL vaccines in association with Lc by the oral (O) and nasal (N) route presented the highest levels of IgA and IgG anti-PppA antibodies in bronchoalveolar lavages (BAL) and IgG in serum. However, only the groups that received LL and D-LL vaccines associated with the oral administration of the probiotic were able to prevent lung colonization by *S. pneumoniae* serotypes 3 and 14 in a respiratory infection model.⁷³ The oral administration of the probiotic strain induced an evident improvement in the host's defenses when it was associated with inactivated and live recombinant bacteria. Both the LL + Lc (O) and the D-LL + Lc (O) combinations favoured the induction not only of

protective specific antibodies but also of specific CD4⁺T cells, the full protection exerted against the pneumococcal challenge being the result of a balanced humoral and cellular immune response between the protective antibodies and the CD4⁺ Th1, Th17 and Th2 cells specific for the PppA antigen.⁷³ Although the LL + Lc (O) combination was effective in the protection against the infectious challenge, the D-LL + Lc (O) combination would be the better strategy for potential use as a nasal vaccine in humans. The results obtained show that the association of a probiotic with an inactivated vaccine would be a valuable alternative for application to human health, especially in at-risk populations. This work represents the first report of a safe and effective immunization strategy using an inactivated recombinant strain.

The potential use of GEM particles as a vaccine was also investigated with three pneumococcal antigens: the putative proteinase maturation protein A PpmA, the streptococcal lipoprotein A SlrA and the IgA1 protease IgA1p.⁷⁴ Monovalent GEM antigen vaccines (GEM particles containing PpmA, SlrA or IgA1p) elicited mucosal and systemic antibody responses in CD-1 mice intranasally immunized,⁶³ while intranasal immunization with a trivalent vaccine combination without an additional adjuvant showed significant protection against fatal pneumococcal pneumonia in mice. Immunization with a combination of PpmA-PA, SlrA-PA and IgA1p-PA (trivalent vaccine) and a combination of SlrA-PA/IgA1p-PA (divalent vaccine) were found to be much more protective against fatal challenge with *S. pneumoniae* than immunization with either of the proteins alone.⁷⁴ The GEM-based trivalent vaccine is a potential pneumococcal vaccine candidate that is expected to be safe, easy to administer and affordable to produce.

Oral Administration of *Lactococcus lactis*: Effects as Adjuvant and Carrier of Pneumococcal Antigens

Numerous studies have demonstrated that certain probiotic LAB strains can exert their beneficial effect on the host through their immunomodulatory activity.⁷⁵⁻⁷⁷ Although most research concerning probiotic-mediated enhanced immune protection is focused on gastrointestinal tract pathogens, a few recent studies have centred on whether probiotics might sufficiently stimulate the common mucosal immune system to provide protection to other mucosal sites as well.^{77,78} In this sense, our laboratory studied the potential effect of probiotics on the improvement of the immune response against respiratory pathogens using an experimental model of *S. pneumoniae* infection in adult immunocompetent mice. In our experimental model, mice were intranasally infected with *S. pneumoniae* serotype 14. After the challenge, the pathogen was detected in lung and blood samples throughout the period assayed (15 days).⁷⁹ In order to study tissue damage in lungs, biochemical and histological studies were performed in the respiratory tract of infected mice. Challenge with pneumococci significantly increased bronchoalveolar lavage (BAL) albumin concentration and lactate dehydrogenase (LDH) activity, which indicates that the infection increased the permeability of the alveolar-capillarity barrier and the cell damage in lungs.

Moreover, lung histopathological examination revealed a gradual and intense inflammatory response with progressive parenchymal involvement, including widespread cellular infiltration, passage of blood elements from capillaries to tissues, increased fibrosis in bronchial walls and vessels, hemorrhage and reduction in the alveolar airspaces.⁷⁹

Since the immunostimulating properties of LAB have been proved to be strain- and dose-dependent,⁷⁹⁻⁸³ the ability of LAB to increase resistance against *S. pneumoniae* infection was studied using several *Lactobacillus* and *Lactococcus* strains and different doses and periods of administration.^{79,84,85} Only three of the treatments assayed increased the resistance of the mice to challenge with the respiratory pathogen: administration of *Lactobacillus casei* CRL 431, a probiotic strain with widely documented immunomodulatory properties,⁷⁹⁻⁸³ of *Lactococcus lactis* NZ 9000, a strain used for the expression of heterologous proteins,⁸⁶ and of *Lactobacillus rhamnosus* CRL 1505, a new probiotic strain with optimum technological properties isolated from goat milk.⁸⁵ These studies demonstrated that these strains, administered by the oral route at the proper dose, were able to increase *S. pneumoniae* clearance rates in lung and blood, improved survival of infected mice and reduced lung injuries.

In the gut immune response induced by commensal bacteria, the antigen presentation from the luminal flora leads to the generation of large quantities of local IgA. Moreover, the increase in the number of IgA-producing cells is the most remarkable property induced by probiotic microorganisms.⁸⁷ It has been demonstrated that the IgA⁺ cells in the lamina propria of the small intestine can be increased with orally administered LAB.^{88,89} It has also been demonstrated that this effect of LAB on gut immunity can significantly increase resistance to infections due to intestinal pathogens. In summary, it is now known that certain LAB strains are capable of: (a) protecting against experimental intestinal infections in mouse; (b) enhancing the secretory IgA specific for bacterial or viral pathogens in the gut; (c) improving the efficacy of oral vaccines against intestinal infections.⁷⁶ Moreover, a common mucosal immune system exists whereby immune cells stimulated in one mucosal tissue spread and relocate to various mucosal sites. This concept implies that oral immune stimulation can induce immunity in distal intestinal mucosal sites.

When studying the capacity of *L. lactis* NZ 9000 to stimulate the IgA cycle, we found that oral treatments with this lactic acid bacterium was capable of increasing the number of IgA⁺ cells in intestine and bronchus.⁸⁴ Thus, the stimulation of the IgA cycle induced by the LAB strains could partly account for the greater resistance of the treated mice to the challenge with *S. pneumoniae*, since LAB treatment improved the production of anti-pneumococcal IgA in the airways.

Our experiments demonstrated that *L. lactis* NZ9000 treatment also improved the production of anti-pneumococcal IgG in BAL. This fact could be related to the stimulation of APCs cells in the lung that induces T-cell activation and B cell clonal expansion and differentiation into IgG⁺ antibody-secreting plasma cells. Bearing the above in mind, we studied the activation of the recruited macrophages and the levels of TNF α in BAL after the

challenge with the pathogen. Mice treated orally with *L. lactis* NZ9000 showed higher values of BAL TNF α and percentages of NBT⁺ cells than untreated mice, which would indicate that these treatments would be capable of improving macrophage-mediated antigen presentation.⁸⁴ Preventive treatments with LAB were able to increase the levels of IL-6 and TNF α in the respiratory tract after challenge with *S. pneumoniae*.^{79,84} These results would indicate that oral treatment with LAB would be capable of improving antigen presentation mediated by pulmonary dendritic cells. At present, our laboratory is working to demonstrate this effect.

Recombinant *Lactococcus lactis* PppA elicited specific anti-PppA mucosal and systemic antibodies after oral immunization. Adult immunocompetent mice orally immunized with 10⁸ recombinant *L. lactis* PppA cells/mouse/day for 5 consecutive days (the optimal cell dose with adjuvant properties⁸⁴) induced the production of specific anti-PppA IgM, IgG and IgA in BAL and serum.⁹⁰ The levels, avidity and opsonophagocytic activity of respiratory and systemic specific IgA and IgG anti-PppA antibodies, but not those of specific IgM, were significantly improved with boosting.⁹¹ The efficacy of the boosting immunization scheme to afford protection against *S. pneumoniae* infection was evaluated in adult mice challenged with pneumococcus of serotypes 3, 6B, 9, 14, 18, 19 and 23F.⁹¹ These serotypes were selected taking into account that in our country they are the ones most often associated with invasive disease, greatest prevalence being shown by serotype 14.⁹² The four serotypes studied were capable of infecting adult mice. *S. pneumoniae* serotype 3 was the most virulent, followed by serotypes 14 and 6B, while serotype 23F was less virulent.⁹¹ In these studies, adult mice immunized twice with *L. lactis* PppA showed significantly lower lung bacterial cell counts than their respective control groups. Moreover, vaccination with *L. lactis* PppA was able to prevent blood dissemination of serotypes 6B, 14 and 23F and enabled the elimination of serotype 3 from blood on day 5 post-infection.⁹¹

Most experimental vaccines designed to prevent pneumococcal infections have been studied in infection models with adult immunocompetent mice with a view to their future application to high risk populations (children, the elderly and immunocompromised individuals). However, pneumococcal infectious disease is a major cause of human infant mortality and, therefore, one of the major challenges in vaccinology is the development of products that are able to induce protective immunity during early life. In this sense, it was found that oral immunization protocols with *L. lactis* PppA that are effective in inducing protective immunity in adult mice are able to protect young mice against pneumococcal respiratory infection.⁹¹ Oral administration of recombinant lactic acid bacteria *L. lactis* PppA induced the production of specific antibodies both in the intestinal tract and at the systemic level⁹¹ of young mice. The efficient stimulation of the gut mucosal immune system by *L. lactis* PppA was evidenced by the increase in the number of IgA⁺ cells in the intestine and by the production of specific anti-PppA IgA antibodies in the intestinal fluid, while the efficient stimulation of the systemic immune response after vaccination with *L. lactis* PppA was evidenced by the detection of specific anti-PppA IgG antibodies in

the serum. Recent in vitro studies showed that *L. lactis* NZ9000 has intrinsic adjuvant properties since it is able to stimulate the maturation of dendritic cells derived from bone marrow and to induce the production of cytokines with adjuvant properties such as IL-1 β .⁹³ These properties and the adjuvant activities of *L. lactis* NZ9000 demonstrated in vivo in our laboratory would explain the efficacy of the oral immunization with *L. lactis* PppA to stimulate the specific immune response against the *S. pneumoniae* antigen in young mice. The analysis of IgG subtypes showed that *L. lactis* PppA immunization stimulated a mixture of the Th1 and Th2 response, which agrees with our previous studies in adult mice where oral immunization with *L. lactis* PppA was able to induce the production of IL-4 and INF- γ producing spleen cells.⁹¹ Recent in vitro studies also showed that *L. lactis* NZ9000 is able to stimulate the production of both IL-12 and IL-10 by bone marrow derived dendritic cells.⁹³

Oral immunization of young mice with *L. lactis* PppA was also able to induce the production of specific IgA- and IgG-type antibodies in the respiratory tract. The mucosal and systemic antibodies induced by the oral immunization with *L. lactis* PppA afforded protection to young mice against an *S. pneumoniae* respiratory infection, although their bacterial counts in lung and hemocultures after challenge with different serotypes of the pathogen were significantly higher than the ones previously found in adult mice.⁹¹ Thus, oral immunization of the young mice with *L. lactis* PppA was able to increase resistance to infection with the four serotypes of *S. pneumoniae*, although the protective capacity of the experimental vaccine was different for each serotype. Immunization decreased colonization in lung, prevented bacteremia of serotypes 6B, 14 and 23F and decreased serotype 3 counts.⁹¹

The protective effect in distant mucosal sites mediated by oral vaccines could be ascribed to the existence of a common mucosal immune system. When the mucosal immune response is induced, primed T and B cells migrate through the lymphatic system and enter the peripheral blood circulation via the thoracic duct. Extravasation of the immune cells occurs not only in the gut lamina propria but also in other mucosal sites such as the respiratory tract.⁹⁴ Thus, this homing pathway of primed lymphoid cells from the inductive sites in Peyer's patches to distant mucosal sites after antigen stimulation could be exploited

to design recombinant *L. lactis*-derived oral vaccines that could afford protection against respiratory pathogens.⁷⁷

Conclusions

Search for new vaccination strategies that are inexpensive and can afford protection against different serotypes of *S. pneumoniae* is a worldwide priority. The safe status and the intrinsic adjuvant properties of some LAB represent an interesting alternative in the development of mucosal vaccines to fight this important pathogen. Two pneumococcal capsular polysaccharides and different pneumococcal proteins have so far been expressed in *L. lactis* and the effectiveness as antigen vehicles of some of these recombinant strains has been evaluated using animal models. However, comparison of the different vaccine delivery and adjuvant systems is difficult for several reasons that include antigen nature, dose and cellular location of the recombinant (cytoplasmic versus bound to the cell membrane versus bound to the cell wall), intrinsic antigenicity and immunomodulatory properties of the bacterial carrier, mouse strain and immunization protocol. Nasal immunization of young and adult mice with recombinant LAB is able to induce an effective specific immune response at both airways and systemic sites, which suggests that the nasal route is the best alternative for protection against a pneumococcal infection using *L. lactis* as an adjuvant and antigen delivery vehicle. Before the potential transfer of live vaccine to human health, one important aspect that needs to be resolved with live recombinant strains is the elimination of the antibiotic resistance genes used in its selection.

Inactivated vaccines have emerged as an excellent short-term alternative for clinical use. Although the immunogenicity of inactivated vaccines is reduced compared to the immunogenicity elicited by live vaccines, their administration associated with a probiotic strain has been proposed as an alternative strategy to successfully overcome this disadvantage, resulting in an even better proposal than the live vaccine by itself.

The variety of antigens evaluated, the progress made so far in the immune parameters involved in the protective effect of recombinant vaccines and the safety considerations of their application in humans suggest that the use of recombinant LAB as an antigen vehicle represents a tangible short-term option to control pneumococcal infections.

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