

Polyvalent 23 epitope polysaccharide pneumonia vaccine induced effective protection through strain-adapted effector mechanisms as demonstrated by the different cytokine responses in mice challenged with two different strains of *Streptococcus pneumoniae*

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ABSTRACT. We used a Balb/c mouse model of pneumococcal pneumonia to investigate the protection mechanisms induced by immunization with a polyvalent 23 epitope polysaccharide pneumonia vaccine. Groups of mice were injected x 4 times s.c. within one month, with this vaccine preparation. Mice were subsequently challenged at day 45, with a lethal, intratracheal inoculum of two strains of *Streptococcus pneumoniae* – either a highly virulent and strongly immunogenic serotype 3 strain (P4241), or a less virulent and weakly immunogenic serotype 19F strain (P15986). The intratracheal *S. pneumoniae* challenge-induced lethality, antibody response, bacterial clearance, and cytokine secretions were monitored to analyze the strain-adapted effector mechanisms. Pulmonary levels of TNF α , IL-6, IL-1 β , MIP-1 α , KC, MCP-1/JE and MIP-2 cytokines were determined up to 48 hours post-infection. Survival rates were 82% and 100% among vaccinated animals challenged at day 45 with P4241, and P1598 mice respectively, and 0% in non-vaccinated mice ($p < 0.001$). Survival was associated with a rapid bacterial clearance from blood and lungs, which similar for the two strains. Immunization induced a serotype-specific antibody response. Kinetics of the cytokine profile in the lung following intratracheal inoculation with the 4241 strain was different in animals vaccinated 45 days previously, compared to naïve, control mice. Generally speaking the bacterial-induced inflammatory cytokine response induced with the 4241 strain was much weaker in vaccinated animals than in control mice. The only cytokines showing a greater increase in vaccinated mice compare to control animals were IL-1 β , KC and MCP-1. Production of TNF α and IL-6 was lower in vaccinated animals than in controls. At variance with the previous bacteria strain-induced cytokine profile, infection with the P15986 strain induced a strong inflammatory response, with a substantial increase in all the cytokine tested, which was similar in vaccinated and in naïve, control animals, except for MIP-1 α , which was the only mediator significantly more produced by vaccinated animals than by naïve, control mice following P15986 infection. The distinct cytokine profiles, which were observed in this study depending upon the two strains of *S. pneumoniae* used for challenge, demonstrated that protection against each strain was obtained through a different defence strategy.

Keywords: vaccination, inflammatory cytokine, chemokine, *Streptococcus pneumoniae*, pneumonia

Community-acquired pneumonia is the most common lethal human infection and *Streptococcus pneumoniae* remains the leading pathogen in this setting, despite the existence of effective antimicrobials [1-3]. Different *S. pneumoniae* serotypes show marked differences in pathogenicity, resistance to phagocytosis, stimulation of immune responses, and lethality rates [4-6]. Strains belonging to serotypes 1, 3 and 4 are virulent in humans [7, 8] and mice [9, 10], and are mainly associated with pneumonia and sepsis. Serotype 3 is highly resistant to phagocytosis, but is potentially immunogenic [5, 11]. In contrast, sero-

types 9, 14, 19 and 23, frequently carried in children's nasopharynx, are less pathogenic and weakly virulent in mice [6, 10]. These latter serotypes are readily phagocytosed and far less antigenic [12]. Serotype 19 can cause pneumonia in mice when given as large inoculum [10, 12]. Pneumococcal polysaccharide vaccine (Pn23) effectively protects mice against pneumococcal disease. It acts by the production of anti-capsular antibodies in a serotype manner, along with complement, leading to phagocytosis [13-17]. Immunization with bacterial polysaccharide antigens typically induces a T-cell-independent type 2 antibody

response characterized by high levels of IgM and IgG antibodies in humans and in mice [18, 19]. The impact of Pn23 vaccination on pneumococcal pneumonia has been investigated in murine pneumonia only in terms of antibody responses and mortality [20, 21]. However, its impact on the host immune response has not yet been described. Host defense against bacterial infection is characterized by the vigorous recruitment and activation of inflammatory cells, which is dependent on the coordinated expression of both pro- and anti-inflammatory cytokines. Knowledge of the expression of cytokines and chemokines, and factors involved in their action may provide information about the immune reaction responsible for elimination of bacterial infection and for immune-mediated pathology. Effective host defenses depend largely on opsonisation of the bacteria by type-specific antibodies and complement, followed by phagocytosis and killing by polynuclear leucocytes and macrophages [22, 23]. The role of chemokines on the chemotaxis of leucocytes, recruitment of polynuclear cells and monocytes inducing phagocytosis has clearly been shown [24]. Chemokines, like others cytokines, have overlapping functions and are produced by a variety of cell types. Many chemokines and their receptors play a major role in the recruitment and function of lymphocytes in lung inflammation [25, 26].

The aim of this study was to investigate the protective mechanisms induced by immunization with a polyvalent polysaccharide vaccine (Pn23) in a model of pneumococcal pneumonia induced by two different strains of *S. pneumoniae*.

METHODS

Mice

Female, 8-week-old BALB/c mice obtained from Janvier breeders (St Genest sur Isle, France) were housed in filter-topped cages with sterile litter. Food and water were given *ad libitum*. Animal studies were performed in accordance with prevailing regulations regarding the care and use of laboratory animals by the European Commission [27].

Active immunization

Groups of 10–12 mice were immunized with a 23-valent, pneumococcal polysaccharide vaccine (Pneumovax® Pasteur Vaccin, Lyon, France) containing 25 µg of each of the following serotypes: 1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20, 22F, 23F and 33F. Each injection consisted of 0.1 mL of diluted vaccine (dilution 1:10 *i.e.* 2.5 µg of each serotype) administered by the subcutaneous route (*s.c.*), on days 0, 7, 14 and 29, as previously reported [21]. Mice ($n = 10$) immunized with sterile PBS served as negative controls.

Passive immunization

Serum containing high levels of specific antibody was obtained by pooling sera from vaccinated mice. Groups of 20 mice were bled by cardiac puncture, four or five days after the last vaccine injection. Serum was collected after clotting and centrifugation. Serum was diluted 1:10 in sterile, pyrogen-free saline, and 0.3 mL of this dilution

was injected by the intraperitoneal (*i.p.*) or intravenous (*i.v.*) route one hour before bacterial challenge. Control mice received sterile saline by the *i.p.* route.

Anti-pneumococcal antibody assay

Vaccinated mice were used to determine the anti-pneumococcal antibodies in serum. Blood was collected by retro-orbital puncture and plasma was stored at -20°C until analysis. Each mouse was bled before and 6, 20 and 44 days after immunization ($n = 6$ to 10 for each point time). An enzyme-linked immunosorbent assay (ELISA) was developed to detect serotype-specific antibodies as described in the Training Manual for Enzyme-linked Immunosorbent Assay for the Quantification of *Streptococcus pneumoniae* Serotype-Specific IgG (Pn PS ELISA). In brief, microtiter plates (Immulon II, ATGC Biotechnologie, Marne la Vallée, France) were coated with purified capsular PPS 3 and PPS 19 (10 µg/mL; ATCC, Promochem, Molsheim, France) and incubated for 5 h at 37°C. Plates were first coated with methylated albumin prior to capture with PPS 3 [13–15]. All sera were preadsorbed with cell-wall polysaccharide (10 µg/mL of serum; Statens Serum Institute, Copenhagen, Denmark) at room temperature for 30 min. Absorbance was measured at 450 nm in a Titertek Multiscan Photometer (Labsystem Multiskan, Roucaire, Courtaboeuf, France). Pooled serum from 25 non-vaccinated mice served as the “negative” baseline control and a control for mouse antibodies. Results were only expressed in absorbance units when no reference serum (as 89SF for human serum) was available in mouse serum.

Bacterial strains

Two clinical isolates of *S. pneumoniae*, with different levels of virulence were used. *S. pneumoniae* P4241 (serotype 3) is an encapsulated strain. It is obtained from blood isolate and is virulent in a model of acute pneumonia (intratracheal $LD_{100} = 5 \log_{10}$ CFU/mouse), as previously reported [8, 10]. *S. pneumoniae* P15986 (serotype 19F) is a less virulent strain (intratracheal $LD_{100} = 7 \log_{10}$ CFU/mouse), originally isolated from middle-ear fluid. Both strains were provided by the French National Pneumococcal Reference Center (Dr Geslin, Creteil, France). Bacteria were grown at 37°C in brain-heart infusion broth (BHI, Biomerieux, Marcy l’Etoile, France) with 5% filtered horse serum (Sanofi Diagnostic Pasteur, Marnes la Coquette France).

Experimental pneumonia

Bacterial cultures were washed twice in 0.9% saline and resuspended in saline to the desired density, as confirmed by plating serial, 10-fold dilutions onto Columbia agar containing 5% sheep blood (Bio-Mérieux, Craponne, France) and incubation for 18 h at 37°C. We used the murine pneumococcal pneumonia model developed in our laboratory [9]. Bacterial suspension (50 µL) was instilled intratracheally into the lower respiratory tract. Fifteen animals were used per bacterial strain, and the animals were infected simultaneously in each experiment. Mice were infected with the lethal inoculum of each strain (10^5 and 10^7 CFU/mouse, for P4241 and P15986, respectively),

45 days after the first dose of vaccine. Control mice were inoculated with 50 μ L of isotonic saline. Survival rates were recorded daily for 12 days after bacterial challenge.

Bacterial clearance from blood and lungs

Groups of five animals were killed by injecting 0.2 mL of sodium pentobarbital (Dolethal, Vetoquinol Laboratories, Lure, France) 6, 24, 48 and 72 h after challenge, and were exsanguinated by cardiac puncture. The lungs were removed and homogenized in 1 mL of saline. Viable bacteria were counted in blood and lung homogenates by plating 0.1 mL of serial, 10-fold dilutions on Columbia agar containing 5% sheep blood (Bio-Mérieux, Lyon, France). Plates were incubated for 18 h at 37°C. Results were recorded as CFU per lung or per milliliter of blood. The experiment was repeated twice.

Cytokine assays

Levels of cytokines TNF α , IL-1 β and IL-6 (pro-inflammatory cytokines) and chemokines MIP-1 α , KC (murine homologue of human GRO), MCP-1 and MIP-2 (murine homologue of interleukin-8) were determined in lung homogenates of infected mice up to 48 h after challenge. Six different time-points were studied. Values for each point-time (2, 4, 6, 12, 24 and 48 hours post infection) were determined from the mean value obtained from 5 to 8 mice in one experiment, repeated with three different experiments. Whole lungs were harvested as described by Laichalk [28]. Murine ELISA kits were used to measure all cytokines, as recommended by the manufacturer (R & D Systems, Abingdon, UK). Samples were tested in triplicate. The detection limits in lung homogenates were 50, 40 and 50 pg/mL, respectively, for TNF α , IL-1 β and IL-6, and 15, 25, 30 and 15 pg/mL for MIP-2, KC, MCP-1 and MIP-1 α .

Histopathological examination

Whole lungs from four groups (n = 8 to 10 mice for each group) of immunized and non-immunized mice infected with P4241 and P15986 strains were removed 12, 24, and 48 h post-infection and fixed by immersion in 3.7% formaldehyde solution. A non-infected control group of 6-8 mice was also used. Slices were prepared from each lobe and routinely processed (dehydrated in graded ethanol, cleared in toluene, and embedded in paraffin). Sections 4 μ m thick were cut, stained with hematoxylin and eosin,

and examined with a light microscope. Infiltration by inflammatory cells (mononuclear cells, neutrophils and lymphocytes) was determined semi-quantitatively. The histological study was performed blind to the experimental group.

Statistical analysis

All data are reported as means $\pm$ standard error of the mean (SEM) were similar. All statistical analyses were performed using the Stat View 4.5 software package (Abacus Concepts, Berkeley, CA, USA). One-way or multiple analyses of variance were used to compare means within experimental groups. Survival curves were analyzed using the Kaplan-Meier method, and group-wise comparisons were made using the Mantel-Cox test. P values < 0.05 were considered to denote significant differences.

RESULTS

Determination of anti-pneumococcal antibodies

Antibody production was examined at day 44 following x 4 s.c. injections within one month, with the 23 epitope polysaccharide pneumonia vaccine started at day 0. We observed that IgM responses against serotype 3 (P4241 strain) and serotype 19F (P15986) were similar. On the same day 44, the IgG responses against serotype 3 were strong contrasting with the IgG anti-serotype 19F responses which were weak (table 1).

Survival of mice after active immunization

In contrast with the immunogenicity information deduced from the antibody response reported above, the vaccinated animals were less protected against the P4241 strain bacteria challenge serotype 3 (survival rate 82%), than against the P15986 strain bacteria challenge serotype 19F (survival rate 100%).

Analysis of the role of antibodies in protection by passive transfer in the mouse

Passive immunization (1:10 diluted serum given i.p.) protected all mice infected with strain P1598. In contrast, a serum dilution of 1:2.5 protected only 35% of P4241-infected mice when given i.p. and 58% when given i.v. Neither IgM nor IgG antibodies were sufficient to protect naive animals inoculated with the virulent serotype 3

Table 1
IgM and IgG anti-pneumococcal antibody response to serotype 3 and 19F in Balb/c mice

	Absorbance in ELISA							
	Mouse IgM antibody				Mouse IgG antibody			
	D ₀	D ₆	D ₂₀	D ₄₄	D ₀	D ₆	D ₂₀	D ₄₄
Serotype 3	0.48 $\pm$ 0.11 (n = 6)	1.68 $\pm$ 0.13 ^a (n = 7)	1.26 $\pm$ 0.19 ^a (n = 7)	1.49 $\pm$ 0.18 ^a (n = 7)	0.42 $\pm$ 0.07 (n = 6)	1.49 $\pm$ 0.13 ^{a,b} (n = 8)	1.47 $\pm$ 0.3 ^a (n = 6)	1.23 $\pm$ 0.22 ^a (n = 7)
Serotype 19F	0.41 $\pm$ 0.09 (n = 7)	0.93 $\pm$ 0.16 ^a (n = 9)	0.92 $\pm$ 0.16 ^a (n = 10)	1.28 $\pm$ 0.22 ^a (n = 9)	0.36 $\pm$ 0.04 (n = 6)	0.51 $\pm$ 0.09 (n = 7)	0.57 $\pm$ 0.09 (n = 6)	0.45 $\pm$ 0.06 (n = 8)

Responses were measured before immunization (D₀) and 6, 20 and 44 days after immunization. Serum samples were diluted 1:100 and were neutralized with PnC. Statistical comparison (Student's t test) was performed with each serotype at different time points *versus* basal level, and between each serotype at each time point.

^a p < 0.005 *versus* basal value, between S3 and S19 for IgM (D₆) and for IgG (D₂₀ and D₄₄).

^b p < 0.001 between S3 and S19 for IgG (D₆).

(P4241) strain (35% of survival rate). As mentioned above, this strain is highly resistant to phagocytosis. In contrast, IgM antibodies alone were capable of protecting all naïve animals inoculated with the serotype 19F (15986) strain (100% of survival). This strain is less virulent and can be neutralized or opsonized by IgM antibodies.

Bacterial clearance from lungs and blood

As shown in *figure 1*, vaccinated mice had significantly lower pulmonary bacterial counts than controls 48 and 72 h after challenge ($p < 0.05$). Bacteremia was detected in only one mouse (2 Log₁₀ CFU/mL, 6 hours post-infection), as compared with 100% of the control mice. In

P15986-infected mice, vaccination was associated with rapid lung clearance, without bacteremia.

Pulmonary cytokine and chemokine responses

The pulmonary kinetics of cytokines in mice infected intratracheally with strain P4241 (serotype 3) differed significantly depending on whether the animals had been vaccinated or not 45 days previously (*figure 2*). TNF α and IL-6 pro-inflammatory cytokine levels were similar in vaccinated and non-vaccinated mice during the first 12h post-infection. Their levels increased significantly in non-vaccinated mice in comparison to those found in vaccinated mice ($p < 0.05$). In contrast, IL-1 β levels were sig-

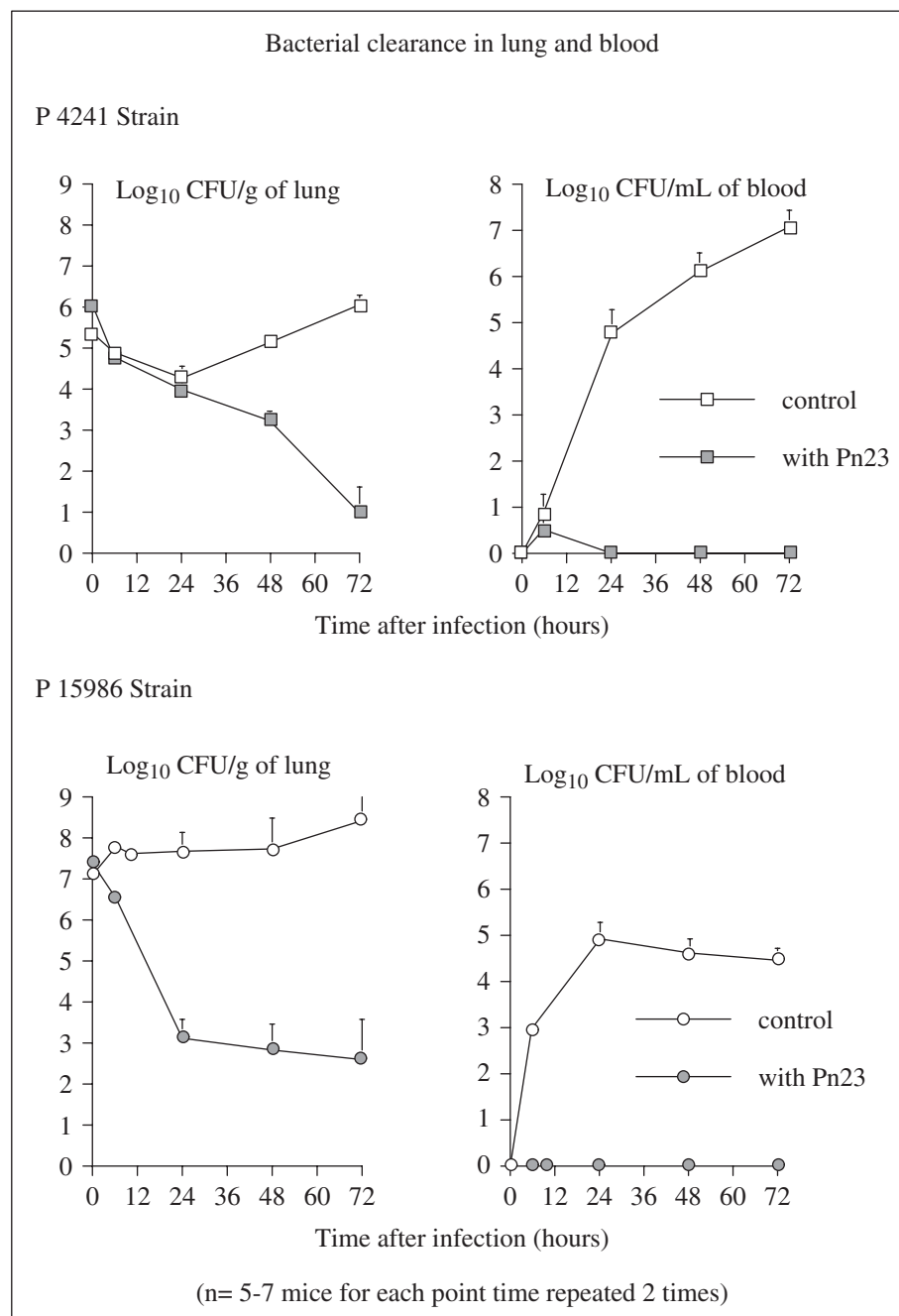
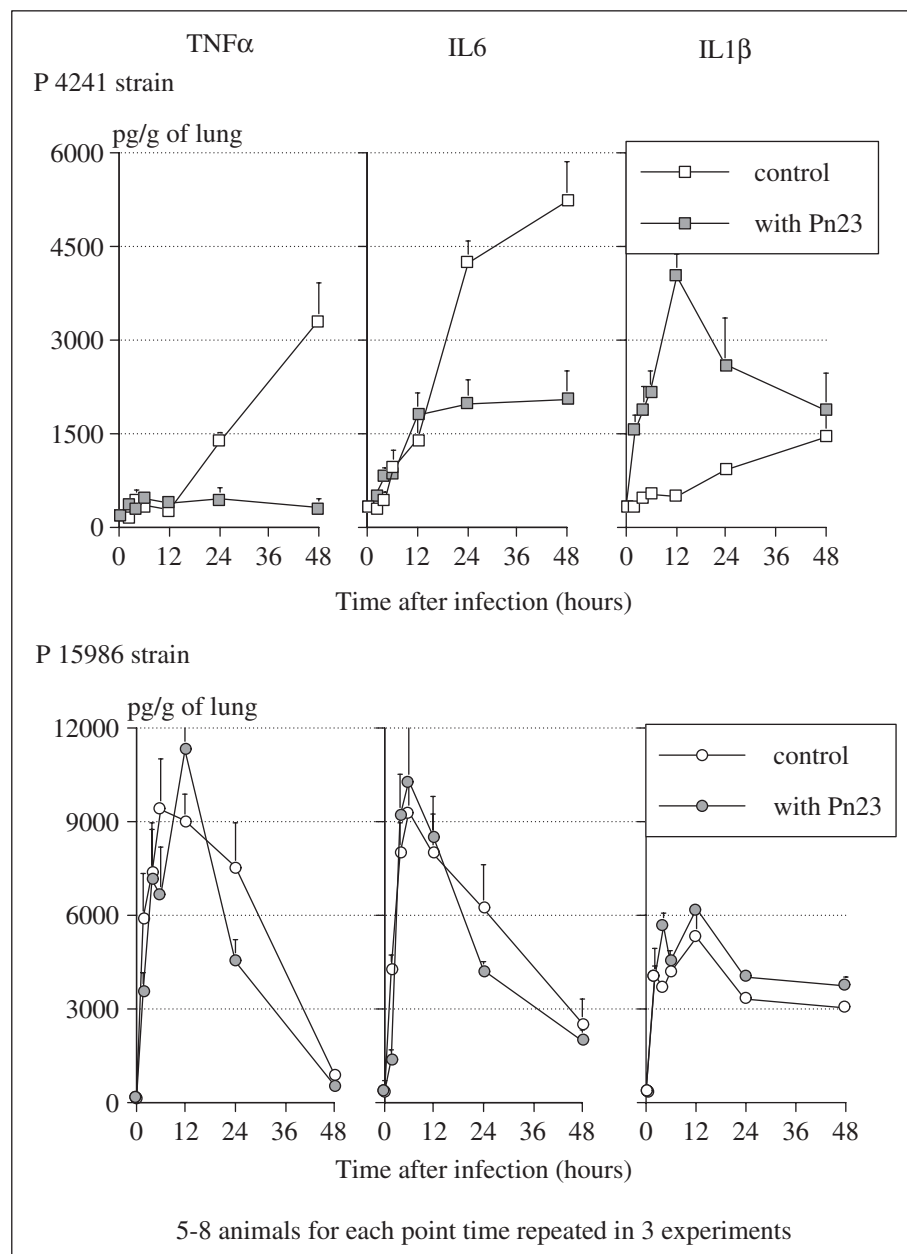


Figure 1

Bacterial clearance from lung and blood of mice challenged with a lethal inoculum of the P4241 strain (after vaccination: hatched squares; without vaccination: open squares) or with the P15986 strain (after vaccination: hatched circles; without vaccination: open circles). Each time point corresponds to the mean value from five vaccinated or non-vaccinated infected mice; this assay was repeated three times.

**Figure 2**

Pulmonary kinetics of TNF α , IL-6, and IL-1 β in mice infected with the P4241 strain (10^5 CFU/mouse) or the P15986 strain (10^7 CFU/mouse). Vaccinated mice were compared with non-vaccinated mice (controls). Each value represents the geometric mean $\pm$ SEM for five to eight mice used in each experiment, repeated three times.

nificantly higher in vaccinated mice, especially during the first 12 hours ($p < 0.0002$).

The pulmonary kinetics of cytokines TNF α and IL-6, and IL-1 β were very different in mice infected intratracheally with bacteria strain P15986 (serotype 19F) compared to the above shown results. These cytokine levels rose rapidly, peaking at 6 h for TNF α and IL-6, and at 12 h for IL-1 β and then declined. Most surprisingly, no significant difference in TNF α , IL-6 and IL-1 β levels were observed whether the animals had been vaccinated or not 45 days previously when using this P15986 bacteria strain for infection.

The pulmonary kinetics of neutrophil-derived chemokines (MIP-2 and KC) and monocyte-derived chemo-attractants (MCP-1 and MIP-1 α) are shown in figure 3. In P4241-infected mice, chemokine levels were significantly higher

in vaccinated than in non-vaccinated mice at 12 h ($p < 0.05$ for all chemokines) and at 24 h ($p < 0.05$ for MIP-2 and MIP-1 α). In P15986-infected mice, high levels of MIP-2, KC, and MCP-1 were unaffected by vaccination. Interestingly, MIP-1 α levels were significantly higher in vaccinated than in non-vaccinated mice at all time points ($p < 0.05$). Levels of pulmonary cytokines and chemokines were also determined without infection. The basal levels did not exceed the value of 300 to 400 pg/g of lung.

Histopathological examination

Histological changes in non-vaccinated, infected mice, in comparison with non-infected controls, included thickening of alveolar walls, congestive capillaries, and erythrodiapedesis. In P4241-infected mice, stronger recruitment

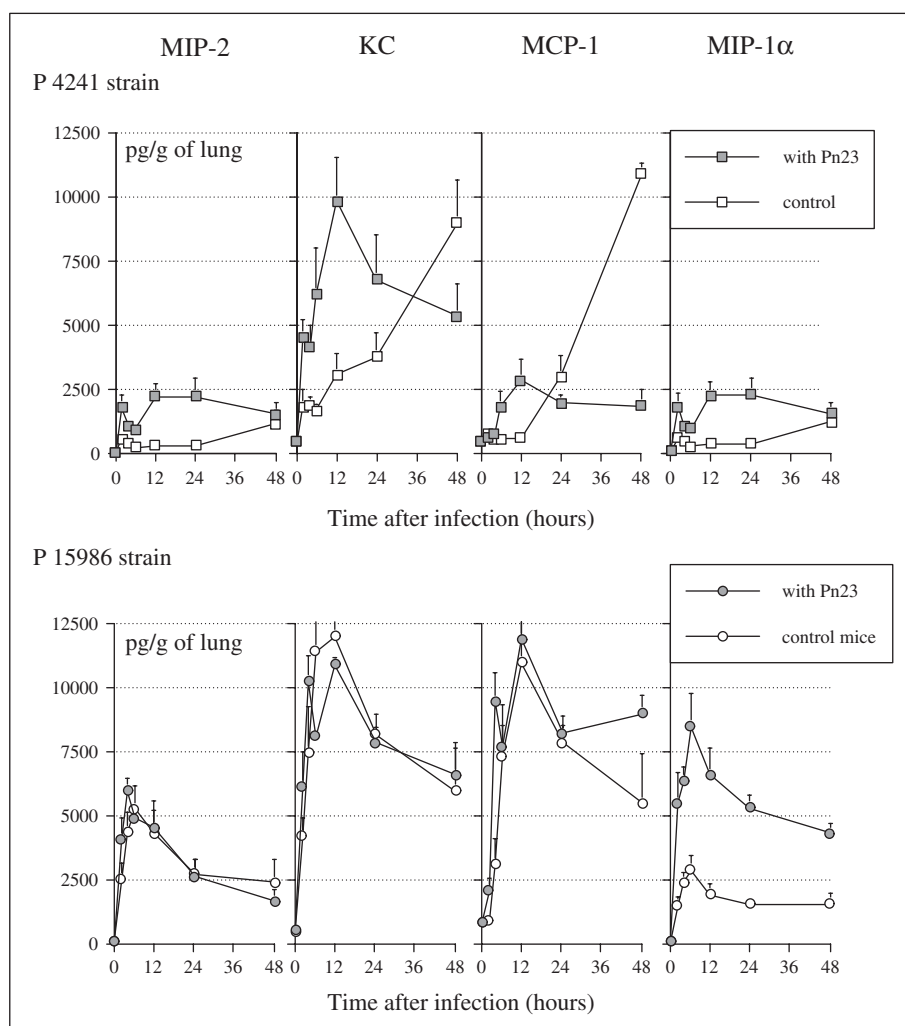


Figure 3

Chemokine profiles (MIP-2, KC, MCP-1 and MIP-1 α) in mice infected with the P4241 strain (10^5 CFU/mouse) or the P15986 strain (10^7 CFU/mouse), with or without vaccination. Vaccinated mice were compared with non-vaccinated mice (controls). Each value represents the geometric mean $\pm$ SEM for five to eight mice in each experiment, repeated three times.

of phagocytic cells was observed at 12 and 24 hours in vaccinated mice than in non-vaccinated mice (data not shown). In contrast, in mice infected by P15986, no significant differences were observed between vaccinated and non-vaccinated mice.

DISCUSSION

Immunization with the Pn23 vaccine effectively protected mice infected intracheally with two different strains of *S. pneumoniae*, with an 82-100% protection rate and rapid bacterial clearance from blood and lungs. In this study, we have shown that survival of vaccinated mice resulted from a strain-adapted effector mechanisms as demonstrated by the different cytokine profiles observed in mice challenged with two different strains of *S. pneumoniae*.

The dose of the pneumococcal vaccine used in our study, is in accordance with others reports [15, 21]. Aaberge *et al.* have shown that the range between 1.2 to 11.5 μ g of antigen induced the highest IgM anti-pneumococcal antibodies in adult Balb/c mice six days after immunization [18]. Immunization with Pn23 vaccine induced a serotype-specific antibody response for both strains. Antibodies to

serotype 19F were fully protective, confirming that humoral response appeared prominent for strain P15986 and were mainly of the IgM subtype, confirming previous studies [18]. Interestingly, full survival of serotype 3-infected mice was not obtained despite high levels of serotype 3-specific IgG and IgM antibodies found in mice immunized with the Pn23 vaccine. In a previous study, Seeland showed that protection was not obtained against serotype 3 pneumococcal strains by passive immunization despite high concentrations of serotype 3-specific IgG antibodies [12]. While serotype 3 is highly resistant to phagocytosis, we found a low protection in passively immunized mice with the P4241 strain (serotype 3). The activation of complement and phagocytosis are required for the induction of protective antibodies. Virulent pneumococcal serotypes (including serotype 1, 3, and 4) differing in their capsular polysaccharides, differ as well in the amount of covalently bound C3b deposited during opsonisation [12]. A previous study demonstrated that complement plays a central role in antibody-mediated protection against pneumococcal infection [29]. Additional studies are needed to further examine the interrelationship(s) among antibody concentration, opsonophagocytosis, antibody avidity and protection in animals.

We have previously shown in this model of pneumonia, using five different strains of *S. pneumoniae* including the P4241 and P 15986 strains, pronounced differences in the local pro- and anti-inflammatory profiles that were strain-specific. The cytokine profiles did not correlate with disease outcome. It should be noted that all animals in this study were infected with the same quantity of inoculum (10^5 CFU/mouse) [10]. In the present study, although survival rate and recovery were very similar with the two strains tested, we found profound differences in the cytokine response. For strain P4241, the profile of the cytokine response was strongly modified in vaccinated mice, especially with the early, high levels of IL-1 β . A possible role of IL-1 β in recovery and survival has been reported, promoting the infiltration of inflammatory and immunocompetent cells into the extra-vascular space, and increasing the expression of adhesion molecules [30, 31]. In addition, the early induction of chemokines KC and MCP-1 in vaccinated mice suggests a strong recruitment of neutrophil and phagocytic cells, as observed in the histological sections. In contrast, no significant difference in the levels of inflammatory mediators was noted in vaccinated and non-vaccinated mice infected with the *S. pneumoniae* strain P15986, except for MIP-1 α that was strongly expressed in vaccinated mice. The chemokine MIP-1 α has been described as being involved in the activation of the CD4 T helper cell type 2 (Th2)-dependent humoral immune response [31, 32]. This is in agreement with our results on passive immunization, indicating that humoral immunity may be considered to be an important protective pathway for P15986 strain.

Overall, our findings imply that more efficient phagocytosis of antibody-opsonized bacteria occurs in the Pn23 vaccine-immunized mice. This phagocytosis by antigen-presenting cells facilitates presentation of bacterial components, other than polysaccharides, within a short period of time. This, in turn, may lead to activation of the innate immune response-related cytokines and chemokines. This possibility is in agreement with our previous studies that demonstrated differences in cytokine expression levels, induced by different strains of bacteria, independent of capsular type [10].

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