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REVIEW

Serotype-independent pneumococcal vaccines

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Abstract *Streptococcus pneumoniae* remains an important cause of disease with high mortality and morbidity, especially in children and in the elderly. The widespread use of the polysaccharide conjugate vaccines in some countries has led to a significant decrease in invasive disease caused by vaccine serotypes, but an increase in disease caused by non-vaccine serotypes has impacted on the overall efficacy of these vaccines on pneumococcal disease. The obvious solution to overcome such shortcomings would be the development of new formulations that provide serotype-independent immunity. This review focuses on the most promising approaches, including protein antigens, whole cell pneumococcal vaccines, and recombinant bacteria expressing pneumococcal antigens. The protective capacity of these vaccine candidates against the different stages of pneumococcal infection, including colonization, mucosal disease, and invasive disease in animal models is reviewed. Some of the human trials that have already been performed or that are currently ongoing are presented. Finally, the feasibility and the possible shortcomings of these candidates in relation to an ideal vaccine against pneumococcal infections are discussed.

Keywords *Streptococcus pneumoniae* · Vaccine

Abbreviations

AOM	Acute otitis media
BAL	Broncholaveolar lavage
CBP	Choline-binding protein
CFA	Complete Freund's adjuvant
CTB	Cholera toxin B subunit
CWPS	Cell wall polysaccharide
DTP_w	Diphtheria, tetanus and whole cell pertussis vaccine
LAB	Lactic acid bacteria
LT	*Escherichia coli* heat labile toxin
OPA	Opsonophagocytic killing assay
PC	Phosphorylcholine
PCV	Pneumococcal conjugate vaccine
PS	Capsular polysaccharide
sIgA	Secretory IgA
WCV	Whole cell pneumococcal vaccine
wP	Whole cell pertussis vaccine

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Introduction

Streptococcus pneumoniae, or pneumococcus, is an important human pathogen, causing diseases such as sinusitis, otitis media, pneumonia, meningitis, and bacteremia. It has been estimated that 14.5 million episodes of severe pneumococcal disease occur each year worldwide, yielding 826,000 deaths of children aged 1–59 months [1]. The highest incidence is found in developing countries, especially in Africa and Southeast Asia. Furthermore, otitis media is the most common cause of visits to pediatricians in developed countries.

The polysaccharide capsule is the most important virulence factor of *S. pneumoniae*, and differences in the composition of the capsule are the basis for the classification of pneumococci into more than 90 serotypes [2–5]. There are two types of licensed vaccines against invasive pneumococcal disease, both based on the generation of antibodies against capsular polysaccharides (PS). Anti-PS antibodies

can opsonize the bacteria in a serotype-specific manner, leading to complement-dependent phagocytosis. The first generation vaccine is composed of PS from the 23 most prevalent serotypes in the United States and in Europe (Pneumovax® 23; Merck) and is indicated for the elderly. Since the response to the PS is T-independent, this vaccine does not induce memory. Its use in children is not indicated due to low immunogenicity. Conflicting data on the efficacy of this vaccine have been reported and, in fact, the use of the 23-valent PS vaccine even for the elderly has been questioned. In the United Kingdom, for instance, the use of the 23-valent PS vaccine is no longer recommended by the Joint Committee on Vaccination and Immunisation (JCVI) [6], due to the lack of evidence of a decrease in invasive disease despite widespread use since 2003 [7].

The second generation vaccines are indicated for use in children and are composed of PS conjugated to carrier proteins, which results in a T-dependent antibody response to PS. The 7-valent vaccine (Prevnar®; Wyeth/Pfizer) (PCV7) was licensed in 2000 and contains PS from serotypes 4,6B, 9V, 14, 18C, 19F, and 23F conjugated to the non-toxic derivative of diphtheria toxin CRM_{197}. In the United States, PCV7 has led to a decrease in pneumococcal invasive disease [8], and a decline in colonization by pneumococci has also been reported [9, 10]. Reduction in colonization has led to diminished circulation of the bacteria in the community, reducing disease in non-vaccinated individuals by herd immunity. In fact, the Centers for Disease Control and Prevention (CDC) has estimated that 2/3 of the impact of this conjugate vaccine in the United States can be attributed to herd immunity [11]. However, the widespread use of PCV7 has led to an increase in disease caused by non-vaccine serotypes through a phenomenon known as serotype replacement [12–15]. Serotype replacement in colonization has also been described [16, 17]. In the United States, the non-PCV7 serotypes 3, 7F, and 19A are now the major causes of invasive pneumococcal disease in children under 5 years of age [13, 18]. While PCV7 has led to a sustained decrease in pneumococcal invasive disease in all age groups in the United States [19], the impact of replacement disease in other countries seems to be higher [20]. PCV7 was introduced in the childhood immunization program in the UK in 2006, and the total cases of invasive pneumococcal disease in those less than 5 years of age have dropped from approximately 550 cases in 2006 to 400 cases in 2010, while the total number of invasive pneumococcal disease cases in those over 5 years of age did not change significantly between 2005–2006 (5,514 cases) and 2007–2008 (5,496 cases) [21]. Moreover, the overall meningitis incidence in children in France did not change despite high rate of PCV7 coverage, with 264 cases in 2001–2002 and 244 cases in 2007–2008 [22]. In order to circumvent the problem of serotype replacement, two new conjugate vaccines were licensed in 2009–2010. Prevnar 13® (Pfizer) (PCV13), first licensed in the United States, includes six additional serotypes (1, 3, 5, 6A, 7F, and 19A) conjugated to CRM_{197}. Synflorix™ (GSK) (PCV10), first licensed in Europe, is composed of PS from serotypes 1, 4, 5, 6B, 7F, 9V, 14, and 23F conjugated to protein D from non-typable *Haemophilus influenzae*, PS from serotypes 18C conjugated to tetanus toxoid, and 19F conjugated to diphtheria toxoid.

Though these new licensed vaccines are expected to increase coverage of strains that are currently more prevalent, new serotypes can emerge as important disease causes in the long run. Furthermore, vaccines composed of several different PS-conjugates are very expensive to produce and many countries will not be able to afford them. The development of alternative vaccines is thus still a priority. The different stages of pneumococcal infection that can be targeted by these new vaccines are discussed in this review. Furthermore, the possibility of using protein vaccines, whole cell pneumococcal vaccines, and recombinant bacteria expressing pneumococcal antigens for the induction of serotype-independent protection against pneumococcal infection is also addressed.

Colonization, mucosal disease, and invasive disease

Streptococcus pneumoniae is part of the commensal microbiota of the nasopharynx. The percentage of children colonized with pneumococci varies in different regions of the world [23], with the highest rates found in developing countries with reports of 80–90 % of children colonized in the first year of life in some countries in Africa [24]. The duration of carriage declines with age and young adults show carriage rates that average 20–30 % in the United States [25]. Pneumococcal colonization proceeds to disease only in particular situations, such as a respiratory viral infection.

Streptococcus pneumoniae causes mucosal disease, namely sinusitis, otitis media, and pneumonia, and invasive disease, such as meningitis and bacteremia (Fig. 1). Although pneumonia is not considered an invasive disease by itself, 40–50 % of cases were reported to have associated bacteremia [26, 27]. Since pneumococcal infection can occur in several different niches of the human host, one critical question in the development of new vaccines is which of these niches should be targeted.

Pneumococci were shown to display phase variation in colony morphology between opaque and transparent phenotypes [28]. Opaque strains have thicker capsules and have been shown to have enhanced virulence in systemic infection of mice [29], while transparent strains have thinner capsules and are related to more efficient colonization [28]. Whereas the thicker capsule of opaque variants provides resistance to complement deposition and phagocytosis during systemic infection, the exposure of adherence

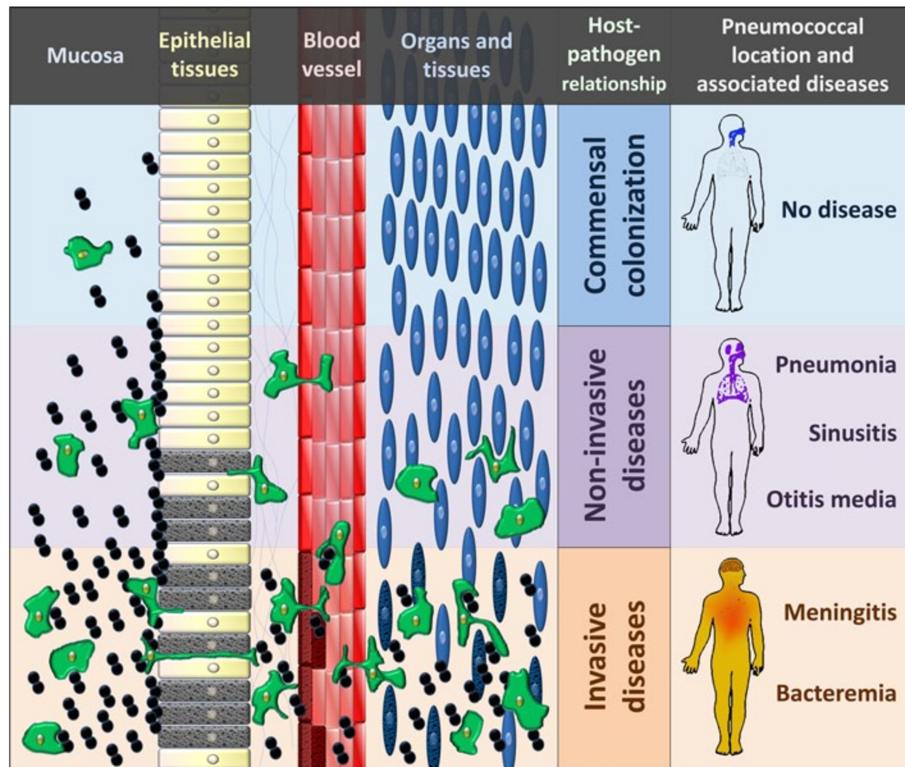

Fig. 1 Commensal and pathogenic interactions of *Streptococcus pneumoniae* with human hosts. Commensal colonization of the upper respiratory tract occurs several times in life and is normally asymptomatic. The process triggers an inflammatory local response that is acute and controls the bacterial density and clearance. During the carrier state of the individual, transmission from hosts to hosts occurs (*upper panel*). Occasionally, the density of colonization may increase and pneumococci may invade mucosal sterile niches, such as the lungs and the middle ear, resulting in robust inflammatory responses at these locations. The development of pneumonia, sinusitis or otitis media are observed in these conditions (*middle panel*). The expression of virulence factors contribute for the evasion from the immune system. If the infection is not controlled, it may progress with the invasion of the bloodstream and other tissues by the bacteria, leading to more severe conditions such as bacteremia and meningitis (*lower panel*)

factors on the surface of the transparent variants facilitates colonization of the nasopharynx. More recently, it has been proposed that chain size may also give selective advantage during either systemic infection or colonization. The growth of pneumococci as diplococci has been proposed to be advantageous during invasive disease due to reduced surface for complement deposition [30], whereas longer chains have been proposed to provide selective advantage for adherence to epithelial surfaces during colonization [31]. No genetic mechanism has been discovered for instances of phase variation in *S. pneumoniae*. Since different virulence factors seem to be important during colonization and invasive disease, it is important to keep in mind that vaccines against each of these stages of bacterial infection may have to target different antigens.

Conjugate vaccines induce serotype-specific protection against colonization and invasive disease, but lower protection against otitis media and pneumonia has been reported. The Northern California Kaiser Permanente trial has shown 97 % efficacy for PCV7-serotypes against invasive disease, but only 7 % for all episodes of acute otitis media (AOM) [32]. A trial in Finland further showed 6 % efficacy for all episodes of AOM, 34 % for all pneumococcal AOM, and 57 % for AOM caused by PCV7-serotypes [33]. As for pneumonia, a review of clinical trials has discussed the difficulties in the determination of vaccine efficacy and showed that conjugate vaccine efficacy against radiographycally confirmed pneumonia or primary endpoint pneumonia ranged from 17 to 37 % [34]. The reduced efficacy against otitis media and pneumonia may indicate that protection against mucosal disease involves mechanisms other than solely anti-PS antibodies.

Conjugate vaccines induce anti-PS antibodies that are specific for each serotype (with little cross-reactivity) and that opsonize the bacteria, leading to phagocytosis. In fact, the in vitro opsonophagocytic killing assay (OPA) using differentiated HL-60 cells is the standard test for the analysis of functional antibody activity induced by conjugate vaccines [35]. While the induction of anti-PS antibodies in vaccinated individuals has been shown to correlate with the

protection against invasive disease, the naturally acquired immunity seems to involve other mechanisms. The peak of incidence of pneumococcal disease is around the first year of life and declines thereafter for all serotypes. If protection were correlated with serotype-specific anti-PS antibodies, one would expect this decline to vary between serotypes. Furthermore, this reduction in susceptibility to invasive disease appears to precede the natural development of anti-PS antibodies in non-immunized children [36].

The induction of serum antibodies against pneumococcal proteins found in all serotypes could be the mechanism by which children become resistant to infection. Data from Finland [37] and Kenya [38] showed that there is an increase in antibody concentrations against some pneumococcal proteins by the beginning of the second year of life, which are induced by carriage of pneumococci. In an experimental human challenge model with the intranasal inoculation of *S. pneumoniae* [39], protection against carriage was correlated with pre-existing serum antibodies against pneumococcal surface protein A (PspA), but not to PS. Though antibodies against each individual protein may be less efficient in opsonization than anti-PS antibodies, a response directed against several protein antigens may have a compensatory effect, affording broad protection in a serotype-independent manner.

A role for antibodies against pneumococcal teichoic acid, the cell wall polysaccharide (CWPS), in protection against invasive disease has also been proposed. Phosphorylcholine (PC) is a component of teichoic acid and is responsible for much of the inflammation caused by pneumococcal infection, since it is the ligand for C-reactive protein. A mouse monoclonal antibody to PC has been shown to protect mice from intravenous and intraperitoneal challenges with pneumococci from different serotypes [40]. Moreover, passive transfer of human IgG against PC has been shown to protect mice from invasive pneumococcal infection [41].

Natural exposure to pneumococci has been shown to induce salivary IgA antibodies against pneumococcal proteins [42] and PS [43] in children. However, the protective role of these antibodies in mucosal tissues remains unclear. Data obtained in immunized mice support an important role for secretory IgA (sIgA) in the nasopharynx for the protection against pneumococcal colonization [44, 45]. Furthermore, subcutaneous immunization of mice has been shown to be protective against colonization when the more efficient complement-fixing isotype IgG2a was induced [46]. In humans, antibody isotypes IgG1, IgG2, and IgG3 activate complement efficiently and only IgG4 does not. So the induction of complement-fixing antibodies through intramuscular immunization with conjugate vaccines could be the mechanism by which conjugate vaccines protect children against carriage [45].

Data obtained in mice have shown that antibodies play a limited role in the clearance of primary pneumococcal colonization [47]. Subsequently, it was shown that antibody-deficient mice exposed nasally to live pneumococci [48, 49] or to a heat-killed non-encapsulated pneumococcal strain [49] were protected against colonization re-challenge. On the other hand, mice deficient in $CD4^+$T cells as well as mice depleted of $CD4^+$ T cells through antibody treatment at the time of the intranasal challenge were not protected against colonization [49]. These results indicate an effector role for $CD4^+$ T cells in the protection against colonization acquired through previous exposure to pneumococci. It was later determined that intranasal immunization with pneumococci in fact accelerates clearance, with infiltration of neutrophils in the nasopharyngeal mucosa. $CD4^+$ T cells secreting IL-17A (T_H17 cells) have been shown to be critical for protection in this mouse model, and it was proposed that this cytokine would act by enhancing phagocytic activity of neutrophils [50]. IL-17-A has been shown to act both in the clearance of a primary infection through recruitment of monocyte/macrophages and in the clearance of previously colonized animals through activation of neutrophil influx [51].

Clearance of bacterial infections in the lungs is highly regulated in order to control the growth of the pathogen without excessive tissue injury [52]. Primary host defense involves phagocytosis through alveolar macrophages with a low inflammatory response. When higher bacterial loads are reached, there is an increase in secretion of pro-inflammatory cytokines, such as TNF-α and IL-8, resulting in the recruitment of neutrophils. T cells are also recruited into the lungs and are believed to promote alveolar macrophage activation through secretion of IFN-γ.

Thus, antibodies against PS have been shown to be the effectors of protection of conjugate vaccines against invasive disease, but the natural protection against pneumococcal infections probably involves additional mechanisms. A role for antibodies against proteins and CWPS has been suggested. $CD4^+$ T cells secreting IL-17A and IFN-γ are probably also involved in the protection against colonization and pneumonia, respectively. Given that morphological variation in invasive and colonizing pneumococcal strains has also been described, vaccines designed for protection against invasive and mucosal disease have to take into account differences both in the virulence factors expressed by the pathogen and in the effector host immune response in each of these niches.

As already mentioned, the greatest proportion of the protection elicited by conjugate vaccines is believed to be due to herd immunity through reduced colonization of children. Still, this protection against colonization brings the downside of opening the niche for colonization not only by other pneumococcal serotypes but also by other species that are

potentially pathogenic. Colonization of the nasophanryx by *Staphylococcus aureus*, *Haemophilus influenzae*, *Moraxella catarrhalis*, and *Neisseria meningitis* can be altered by elimination of pneumococci. In fact, an increase in the isolation of *S. aureus* has been reported in children with recurrent AOM immunized with PCV7 [53]. Furthermore, persistent higher rates of nasopharyngeal carriage of *S. aureus* and *H. influenzae* were observed in young children and their parents after PCV7 implementation in the Netherlands. Carriage rates of *M. catarrhalis* remained unaltered [54]. Vaccines targeting nasopharyngeal colonization may thus have a relevant impact on disease caused by other pathogens and continuous surveillance is necessary.

Animal models of colonization, mucosal disease and invasive disease

Since the pneumococcus is considered to be primarily a human pathogen, all the different animal models of pneumococcal infection have some limitations. One important shortcoming of the use of these animal models relies on some specific interactions between pneumococcal virulence factors and human proteins, such as pneumococcal IgA protease, which is highly specific and cleaves only human IgA1 [55]. Here, we will discuss some of the mouse models most commonly used to evaluate alternative pneumococcal vaccines against invasive disease, colonization, and non-bacteremic pneumonia. Mouse models of meningitis and otitis media and a non-human primate model of pneumonia will be shortly addressed. Extensive reviews on animal models of pneumococcal infection in different species, including rats, rabbits, and chinchillas have been published [56–58].

Results from the several studies are sometimes difficult to compare, since the research groups usually use different pneumococcal strains as well as different mouse lineages. Commonly used mouse strains include the inbred BALB/c, C57BL/6, and C3H lineages and the outbred MF1 and CD-1 lineages. CBA/CAHN-XID ("CBA/N") mice are also used as a model for pneumococcal infection. These mice do not make natural antibodies to polysaccharides, including teichoic acid [59], and are thus more susceptible to pneumococcal infection [60].

Mouse models of bacteremic fatal disease use the intravenous, intraperitoneal, and intranasal routes for inoculation of the bacteria [57]. Vaccine efficacy in these models can be assessed through recovery of bacteria from blood samples or from the lungs. Survival can be scored when the animals reach the moribund state and have to be euthanized. Intravenous and intraperitoneal challenges are performed through the injection of bacteria in the tail vein and in the peritoneal cavity of mice, respectively. Though the intraperitoneal challenge model uses an inoculation route that does not mimic the natural course of infection or disease, it has long been used to assess the efficacy of polysaccharide vaccines. The intranasal lethal challenge is performed through the inoculation of large volumes (40–50 μL) of the bacterial inoculum into anesthetized animals, which ensures the aspiration of the pneumococci into the lungs. From there, the bacteria invade the bloodstream, leading to sepsis and death of mice. Since neonates and infants are at increased risk of pneumococcal disease, early-life mouse intranasal models of lung aspiration have also been described to evaluate vaccine efficacy [61, 62]. There is a strong association between capsular serotype and virulence in mice. Types 3 and 4 and group 6 pneumococci have been shown to cause fatal infection of mice when inoculated intravenously or intraperitoneally, while type 1 strains have been shown to be marginally virulent. Type 14 and group 19 and 23 strains have been shown to be avirulent [63]. Since there are differences in the susceptibility in each mouse lineage, 300 CFU and 10^6 CFU of the pneumococcal strains were inoculated in CBA/N and BALB/c mice in this study, respectively.

In contrast to invasive disease, most pneumococcal strains are able to colonize the nasopharynx of mice. The technique most commonly used is to develop colonization of the nasopharynx with minimal aspiration into the lungs by dropping a small volume of the bacterial inoculum (10 μL) with a micropipette into unanesthetized animals [58]. The bacteria can then be recovered through the retrograde tracheal wash collected from the nares or from the homogenized nasal tissue. It was shown that nasal colonization of mice involves two populations of pneumococci: a transparent-phase population loosely associated with the nasal surface, which is released through nasal wash, and an invading opaque population more intimately associated with or within the nasal mucosa and submucosa, which is recovered from the nasal tissue [64].

Mouse models of pneumonia have been described using intratracheal, intranasal, and aerosol inoculation. Most of them cause pneumonia followed by sepsis and are therefore not suitable models for non-bacteremic pneumonia. An interesting mouse model of a self-resolving pneumonia without bacteremia using serogroup 19 strains has been described [65, 66]. In this model, anesthetized CBA/N mice were inoculated with a large volume (40 μL) of the bacterial inoculum. Five days after the challenge, bacteria could be recovered from nasal washes and lungs, but not from blood. Colonization of the nasopharynx and infection of the lungs was observed till day 35 post-challenge. Interestingly, though the inoculum contained roughly 50 % of transparent and opaque strains, the bacteria recovered from the nasal washes were virtually all transparent, whereas the majority of pneumococci from the lungs were opaque. Moreover, histological examination of the lungs removed at 6–8 days after challenge showed signs of acute pneumonia.

Alveoli contained neutrophils, monocytes, and necrotic debris. At later time points, lungs showed signs of healing [65]. Capsular serotype 14 and serogroup 23 strains can also be used in this model [57]. A non-human primate study with a rhesus macaque model for pneumococcal pneumonia has also been described and proposed to be used in pre-clinical trials [67]. In this model, the inoculum challenge of a serotype 19F strain was delivered to the lower respiratory tract via bronchoscopy of anesthetized animals. The clinical course of disease mimicked many aspects of pneumococcal infection of the lower respiratory tract in humans, with elevated levels of neutrophils and pro-inflammatory cytokines in the bronchoalveolar lavage (BAL) fluid. Bacteria were recovered from BAL, but not from blood samples.

There are two types of meningitis models in mice: one involves the direct infection by the intracerebral or intracisternal routes and the other is induced by intraperitoneal or intranasal infection [56]. Meningitis models are generally used to study the disease itself or virulence factors of the bacteria. Since meningitis is preceded by colonization of the nasopharynx, by invasion of the bloodstream, or by sinuses/middle ear infection, protection against meningitis is not commonly analyzed as a vaccine efficacy endpoint in animal models.

Chinchillas have been historically used as a model for otitis media and have several advantages, such as the inoculation of a small bacterial inoculum into the middle ear or to the nasopharynx and the development of local disease without disseminated infection. An extensive review on the chinchilla otitis media model has been published [68]. A review on mouse models for the evaluation of mucosal vaccines against otitis media has also been published [69], and it emphasized the role of sIgA antibodies in the protection against otitis media.

Antigens tested in animal models

Several antigens have been tested in animal models as candidates for a pneumococcal vaccine that elicits serotype-independent protection and we will describe the results of some of the most promising candidates here (Table 1; Fig. 2). Due to space limitations, not all antigens found in the literature will be described.

Vaccine candidates identified by conventional methodologies

Several proteins have been proposed to be used as vaccine against pneumococcal infections. The first antigens were identified through classical methodologies, such as the screening of monoclonal antibodies, and include pneumolysin (Ply), pneumococcal surface protein A (PspA), pneumococcal surface protein C (PspC), and pneumococcal surface antigen A (PsaA). Antibody responses to these four protein antigens have been described in children and were related to pneumococcal exposure [37, 42, 70]. Recently, new antigens have been identified through high-throughput screenings. The described antigens include virulence factors and adhesins that target the different stages of bacterial infection and that are mostly surface-exposed. Virulence factors can act by subverting the host response, leading to immune evasion, while adhesins are important for the attachment of the pathogen to host tissues. Pneumococci display three major groups of surface proteins: lipoproteins, LPXTG consensus sequence-carrying proteins that are covalently linked to the cell wall peptidoglycan via sortases, and choline-binding proteins (CBPs) that are non-covalently linked to the choline-residues of the CWPS [71, 72]. Recently, it was shown that large extracellular domains of integral membrane proteins can be exposed at the surface of pneumococci and that proteins can also be bound to the surface of the bacteria through interaction with these exposed extracellular domains [73]. Ply shows hemolytic activity and was one of the first virulence factors identified in *S. pneumoniae*. It is a cholesterol-binding protein that forms pores in eukaryotic membranes [74]. Ply was recently shown to activate the innate immune system through interaction with TLR4 [75] and activation of the NLRP3 inflammasome [76, 77]. It was first described that the intraperitoneal immunization with a partially inactivated Ply adjuvanted with Complete Freund's Adjuvant (CFA) increased survival time of outbred mice after an intranasal lethal challenge with a serotype 2 strain [78]. Toxoids with reduced hemolytic activity, such as PdA and PdB, were later generated by point mutation of Ply and shown to protect mice immunized with proteins adjuvanted with CFA or alum from lethal challenges with pneumococcal strains from different serotypes [79, 80].

PspA was identified through the screening of monoclonals generated by the immunization of CBA/N mice with a heat-killed non-encapsulated strain (R36A) [81, 82]. The analysis of the clones that were not reactive with PC revealed that two of the monoclonals reacted with the surface protein PspA, and provided protection against the intravenous challenge with serotype 2 (D39) and 3 (WU2, A66) strains. PspA is a CBP with a variable N-terminal region that is exposed on the surface of the bacteria [83]. PspA interferes with the host immune response during invasive disease by avoiding the activation and deposition of complement on the bacterial surface [84, 85]. During mucosal infection, PspA protects the bacteria from killing by apolactoferrin [86]. Sequence analysis of PspA was used to classify the different variants in 3 families [87]. Since the majority of the strains expresses family 1 or family 2 PspAs, it was proposed that a vaccine composed of one molecule of each of these families would be able to afford broad coverage [88, 89], though

Table 1 Serotype-independent pneumococcal vaccines tested in animal models

Antigen	Adjuvant	Animal model	Challenge strain	Challenge model	Outcome	Reference
Protein antigens						
Native PLY (i.p.)	CFA/IFA	Prince Henry mice	NCTC7466 (St 2)	i.n. lethal	↑anti-Ply abs; ↑ survival time	[78]
PdA (Ply cys_{428} ▸ gly) (i.p.)	CFA/IFA	BALB/c	D39 (St2)	i.p.	↑anti-Ply abs; ↑survival	[79]
PdB (Ply trp_{433} ▸ phe) (i.p.)	CFA and $AlPO_4$	Quaquenbush (Q/S) and MF1	Various (Sts 1, 3, 4, 5, 6, 7F, 8, 18C)	i.p.; i.n. lethal	↑anti-Ply abs; ↑survival time; ↑survival	[80]
rPspA fragments (i.p.)*	CFA	CBA/N	WU2 (st 3)	i.v.	↑anti-PspA abs; ↑survival	[93]
rPspA fragments (strains BG9739, L81905 or DBL5) (i.p.)	CFA/IFA	CBA/N	A66.3 (st 3); WU2 (st 3); DBL6A (st6A); BG7322 (st 6B); DBL5 (st 5); BG9739 (st 4); L81905 (st 4)	i.v.	↑anti-PspA abs, ↑survival time; ↑survival	[90]
Native PspA/rPspA fragments (i.p./s.c.)	None/CFA	CBA/N; BALB/c	WU2 (st 3); A66.1 (st 3)	i.v. or i.p.	↑anti-PspA abs; ↑survival	[94]
Native PspA (i.n.)	CTB	CBA/N; BALB/c	A66.1 (st 3); L82016 (st 6B); BG7322 (st 6B); BG8826 (st 23F)	i.v. or i.p. or i.t. or i.n.	↑anti-PspA IgA and IgG; ↑survival; ↓carriage	[95]
rPspA (i.n.)	IL-12	BALB/c	TJ0983 (st 14); A66.1 (st 3)	i.n. colonization; i.p.	↑anti-PspA IgG1; IgG2a and IgA; ↓colonization; ↑survival	[162]
Family 1 or 2 rPspAs (i.p)	Aluminum hydroxide	BALB/c	A66.1 (st3, fam1 PspA), ATCC6303 (st3, fam2 PspA)	i.n. lethal	↑anti-PspA abs; ↑C3 deposition; ↑survival	[91]
DNA vaccine expressing PspA (i.m.)	none	C57Bl/6	0603 (st 6B)	i.n. colonization	↑anti-PspA IgG1 and IgG2a; ↑IFN-γ; ↑complement deposition; ↓colonization	[46]
rPspA	Whole cell pertussis or DTPw	BALB/c	0603 (st 6B); ATCC6303 (st 3); A66.1 (st 3)	i.n. colonization and i.n. lethal	↑anti-PspA IgG1; IgG2a and IgA; ↓colonization; ↑survival	[163]
rPspA (i.n.)	DNA expressing Flt3 ligand	C57Bl/6; CBA/N	EF3030 (st 19); WU2 (st 3)	i.n. colonization or i.v.	↑anti-PspA IgA and IgG; ↓carriage; ↑survival	[44]
rPspC (i.p.)	$AlPO_4$ or $AlPO_4$ + MPLA	BALB/c	D39 (st 2)	i.p.	↑anti-PspC abs; ↑survival	[110]
rPspC (i.n.)	CTB	CBA/N	PLN-A ($D39^{Ply-}$, st 2)	i.n. colonization	↑anti-PspC IgG; ↓colonization	[109]
Sp36, Sp46, Sp91, Sp101, Sp128, Sp131 (N4 strain, st 4) (s.c.)	CFA/IFA	C3H/HeJ	SJ2 (st 6B)	i.p.	↑abs against the antigens, ↑survival	[133]
rPcpA (s.c.)	Aluminum hydroxide	CBA/N	TIGR4 (st 4); TJ0893 (st 14); L82016X (st 6B); EF9303 (st 23F)	i.v. or i.n.	↓lung infection; ↑survival time	[127]

Table 1 continued

Antigen	Adjuvant	Animal model	Challenge strain	Challenge model	Outcome	Reference
rStkP, rPcsB (s.c.)**	Alum	C3H/HeNHsd	PJ1259 (6B); EF3030 (st 19)	i.p.; i.n.	↑anti-StkP; -PcsB IgG, ↑survival, ↓lung colonization	[134]
rPhtA, rPhtB, rPhtD[a] (s.c.)	CFA/IFA	BALB/c; C3H/HeJ	SJ2 (st 6B); EF6796 (st 6A); EF5668 (st 4); WU2 (st 3); N4 (st 4)	i.p.	↑abs; ↑binding to pneumococcal surfaces; ↑survival	[113]
rPhtD (i.m. or i.n.)**	AS02 (i.m.) or LT(i.n.)	OF1; BALB/c; CBA/J	43 (st 3); D39 (st2); CDC (st 4); CDC (st 6B); 2737 (19F)	i.n. lethal; i.n. colonization lung colonization	↑anti-PhtD IgG, ↑survival, ↓nasal and lung colonization	[116]
RrgB321 fusion protein (i.p.)	Aluminum hydroxide	BALB/c; CD1	TIGR4 (st 4); Finland 12 (st 6B); SME15 (st 35)	i.p. or i.v.	↑anti-RGB abs; ↑OPA; ↓bacteremia, ↑survival	[121]
rGlpO (i.p.)	Imject Alum[e]	CD1	WCH43 (st 4)	i.p.	↑anti-GlpO abs, ↑survival time	[137]
Combinations of antigens						
rPsaA or rPsaA + rPspA (i.n.)	CTB	mice	E134 (st 23) L82016 (st 6B)	i.n. colonization	↑anti-PsaA and -PspA IgG and IgA; ↓carriage	[100]
rPsaA + rPspA + PdB (Ply $trp_{433} \blacktriangleright$ phe) (i.p.)	Imject Alum[e]	BALB/c	D39 (st 2); WCH43 (st 4)	i.p.	↑anti-PsaA, -PdB, –PspA abs, ↑survival time	[128]
rPiuA, rPiaA, rPiuA + PiaA[b] (i.p.)	Imject Alum[e]	BALB/c	D39 (st 2)	i.p.	↑anti-PiuA and anti-PiaA IgG, ↑survival	[119]
rPsaA + rPspA + PdB (Ply $trp_{433} \blacktriangleright$ phe) (s.c.)[c]	Imject Alum[e]	CBA/N	L82013 (st 19); EF3030 (st 19)	i.n.	↑anti-PsaA, -PdB; –PspA IgG, ↓lung infection	[65]
rPiuA + rPiaA (i.n./i.p.)	CT (i.n.)/ Imject Alum[e] (i.p.)	CBA/CA	JSB1 (st1)	i.n. lethal	↑anti-PiuA, -PiaA IgG and IgA, ↑survival	[118]
rPspA, rPspC, rPdB, rPhtE, rPhtB or combinations (i.p.)[d]	Imject Alum[e]	BALB/c; CD1	D39 (st 2); WCH16 (st 6A)	i.p.	↑IgG against the antigens, ↑survival time	[129]
rPsaA + rPspC + Pdt (i.n.)	CT	C57Bl/6	0603 (st 6B)	i.n. colonization	↑IL-17; ↓colonization	[141]
rPhtD + dPly (i.m.)	AS02	rhesus macaque	ATCC6319 (st 19)	i.b. lethal	↑abs; ↑survival; ↓pneumonia	[117]
Cellular vaccines; other components and live vectors						
WCV (i.n, s.c., s.l. or oral)**	CT, CTB, LT or mLT (i.n.) Aluminum hydroxide (s.c)	C57Bl/6 Sprague–Dawley rats	0603 (st 6B); CT882328 (st 14); TN82328 (st 23F); WU2 (st 3); DBL5 (st 5)	i.n. colonization, aspiration-sepsis (mice), i.th. (rats)	↑ IL-17 ↑abs; ↓colonization; ↓sepsis	[138, 139, 143, 144]
CWPS (i.n.)	CT or CTB	C57Bl/6	0603 (st 6B); WU2 (st 3)	i.n. colonization, aspiration-sepsis	↑IL-17; ↓colonization; ↓sepsis	[140]
PsaA-Pdt fusion protein + CWPS (i.n. or s.c.)	CT/Aluminum hydroxide	C57Bl/6	0603(st 6B);TIGR4:19F (st 19F); WU2 (st 3)	i.n. colonization, aspiration-sepsis	↑ IL-17; ↑IgG against the antigens; ↓colonization ↓sepsis	[142]

Table 1 continued

Antigen	Adjuvant	Animal model	Challenge strain	Challenge model	Outcome	Reference
Salmonella expressing PspA, PspA fusion proteins, PspC, PsaA or PspA + PspC (oral or i.n.)**	None	BALB/c; C57Bl/6	WU2 (st 3); 3JYP2670 (st 3); A66.2 (st 3) D39 (st 2); L81905 (st 4)	i.p.; i.v.; i.n. colonization; i.n. lethal	↑anti-PspA IgG and IgA, ↑IL-4 and IFN-γ; ↑survival	[147–149, 152, 153]
LAB expressing PsaA (i.n.)	None	C57Bl/6	0603 (st 6B)	i.n. colonization	↑anti-PsaA IgG and IgA ↓colonization	[155]
L. lactis or *L. casei* expressing PspA (i.n.)	None	CBA/ca; BALB/c	TIGR4 (st 4); A66.1 (st 3) ATCC6303 (st 3)	i.p.; i.n. lethal	↑anti-PspA IgA and IgG, ↑complement deposition; ↑survival time	[157–159]
L. lactis expressing PppA (i.n.)	None	Swiss	T14 (st 14); AV3 (st 3) AV6 (st 6B); AV14 (st 14); AV23 (st 23F)	i.p.; i.n.	↑anti-PppA IgG and IgA, ↑survival; ↓bacteria in lungs	[160]

i.b. intrabroncheal, *i.n.* intranasal, *i.p.* intraperitoneal, *i.t.* intratracheal, *i.th.* intrathoracic, *i.v.* intravenous, *s.c.* subcutaneous, *s.l.* sublingual, *abs* antibodies, *OPA* opsonophagocytosis

[a] Best results were observed with rPhtD

[b] Best results were observed with the combination of PiuA and PiaA

[c] Best results were observed with the combination of PspA and PdB

[d] Best results observed with the combinations of PdB and PspA, PdB and PspC or PspA and PspC

[e] Aluminum hydroxide + magnesium hydroxide

*The PspA antigen has already been tested in humans [165, 166]

**Formulations based on these vaccines were recently tested or are currently being investigated in clinical trials

broad protection with a single PspA molecule has also been described [90, 91]. Full-length native PspA and a recombinant N-terminal PspA fragment have been shown to protect immunized mice from the intravenous challenge with different pneumococcal strains [92–94]. Immunization with native PspA afforded protection of CBA/N mice against the intravenous challenge with a serotype 3 strain (WU2) even without the use of any adjuvant [94]. The intranasal immunization of mice using cholera toxin B subunit (CTB) as adjuvant protected BALB/c mice against systemic infection following intravenous, intratracheal, and intraperitoneal lethal challenges with a serotype 3 strain (A66). CBA/N mice were also protected against carriage with serotype 6B and 23F strains [95]. It has been proposed that sIgA plays a necessary role in the protection against colonization in mice immunized intranasally with PspA [44]. PspA has a conserved proline-rich region (PRR) located at the C-terminal of the variable region, and PRR was proposed to be used as an alternative antigen with potentially broader cross-reactivity than the variable N-terminal fragments of PspA usually tested [96, 97].

PsaA was also identified through the recognition by a monoclonal antibody produced through the immunization of mice with R36A [98]. PsaA is a conserved lipoprotein that belongs to the ABC-type protein complex that transports Mn^{+2} and was shown to play a role in pneumococcal adhesion through an indirect effect [99]. The intranasal immunization with lipidated PsaA adjuvanted with CTB was shown to protect mice against carriage with serotype 6B and serogroup 23 strains [100].

PspC was identified based on the homology with PspA [101]. It was described independently by several research groups, receiving different names: choline-binding protein A (CbpA) [102], *S. pneumoniae* secretory IgA binding protein (SpsA) [103], factor H-binding inhibitor of complement (Hic) [104], and C3-binding protein [105]. It is a multifunctional protein, capable of interacting with complement through binding to C3 [105] and to Factor H [104, 106, 107], and also acting as an adhesion molecule through interaction with sIgA [103] and to the laminin receptor [108]. The nasal immunization of CBA/N mice with PspC adjuvanted with CTB was shown to protect mice against an intranasal challenge with a serotype 2 strain (Ply mutant of D39) [109], while the intraperitoneal immunization of BALB/c mice with PspC using different adjuvants (alum with or without monophosphoryl lipid A) afforded protection against

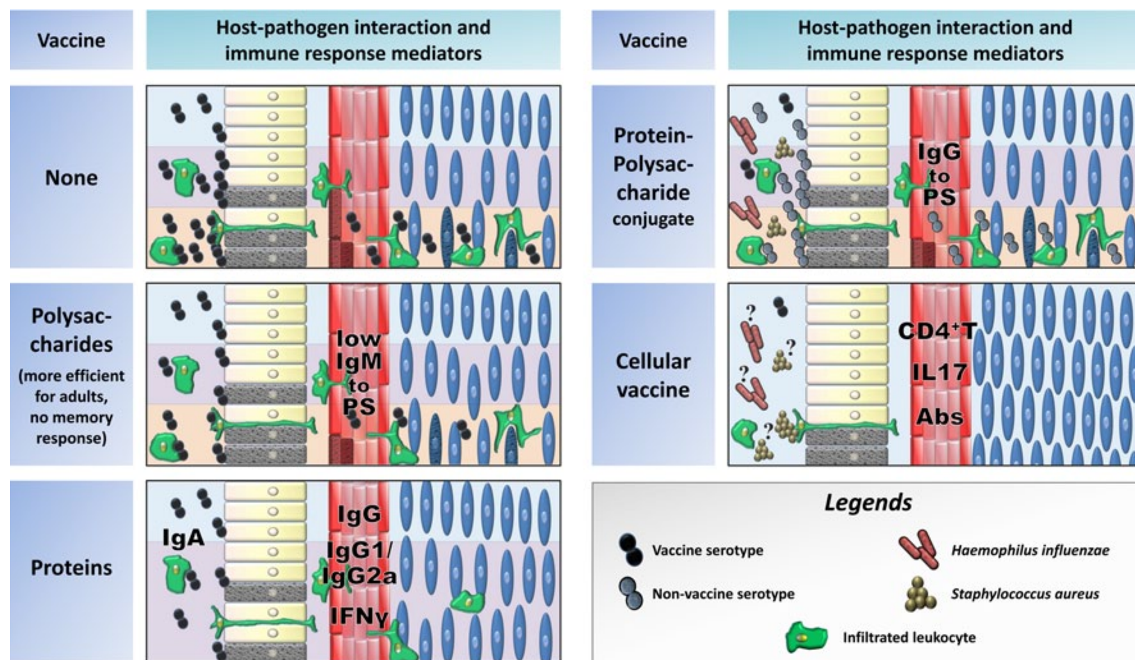

Fig. 2 Host–pathogen interactions during vaccine interventions. In non-vaccinated individuals, the three types of interaction may eventually occur (colonization, non-invasive disease, and invasive disease) (*upper left panel*). Polysaccharide (*PS*) vaccines induce low levels of systemic IgM against the antigens. These responses are more effective in adults but there is no memory response, leading to a temporary effect of the vaccine (*middle left panel*). Most of the protein vaccine candidates were shown to be immunogenic, inducing mucosal and systemic antibodies, as well as cellular immune responses, depending on the antigen, route of inoculation, and adjuvant used. The effectiveness of these vaccines against colonization and invasive diseases varies according to the antigen tested, and, usually, combinations of antigens tend to be more effective (*lower left panel*). PS-protein conjugate vaccines induce IgG against PS and are very effective against colonization and invasive diseases caused by pneumococcal vaccine-serotypes. The reduction in colonization opens a niche in the mucosa for other bacteria. As a result, an increase in diseases caused by pneumococcal non-vaccine serotypes and other respiratory pathogens is observed (*upper right panel*). The pneumococcal cellular vaccine (WCV) is a promising strategy that has been shown to induce T_H17 responses that are very effective against pneumococcal colonization. In addition, the induction of antibodies by this vaccine affords protection against invasive diseases. WCV was shown to induce protection against several pneumococcal serotypes in animal models. The elimination of pneumococcal colonization has to be carefully evaluated since colonization by other respiratory pathogens may emerge (*middle right panel*)

an intraperitoneal challenge with D39 [110]. Similarly to PspA, PspC shows variability between strains [101, 111], but broad cross-reactivity of antibodies induced against a single PspC variant has been described [112].

Different groups have tested proteins containing the conserved histidine triad motif (HXXHXH) as vaccine antigens. The proteins received different names, including pneumococcal histidine triad (PhtA, PhtB, PhtD, and PhtE) [113], pneumococcal histidine protein (PhpA, PhpB, and PhpC) [114] and BVH-3 and BVH-11 (BVH-11, BVH-11-2, and BVH-11-3) [115]. Subcutaneous immunization of C3H and BALB/c mice with PhtD (BVH-11-2) adjuvanted with CFA/IFA was shown to be protective against an intraperitoneal challenge with serotypes 3 (WU2), 4, 6A, and 6B strains [113]. Intramuscular immunization with PhtD formulated with the adjuvant system AS02 (oil-in-water emulsion containing monophosphoryl lipid A and QuilA) was later shown to protect OF1 mice from intranasal lethal challenges with serotype 2 (D39), 3, and 4 strains and to reduce lung colonization with a serotype 19F strain in CBA/J mice. Intranasal immunization of BALB/c mice with PhtD using *Escherichia coli* heat-labile toxin (LT) as adjuvant also provided protection against colonization with serotypes 2 (D39), 4, and 6B strains [116]. The rhesus macaque model was further used to evaluate a vaccine formulation containing a formol-detoxified pneumolysin (dPly) and pneumococcal histidine triad protein (PhtD) using the AS02 adjuvant system [117]. Animals were challenged with a 19F strain, and the immunized group showed higher survival than the control group, with lower bacterial loads in BAL samples. Protection was partly mediated by anti-dPly and anti-PhtD antibodies.

PiuA and PiaA are lipoprotein components of *S. pneumoniae* iron uptake ABC transporters that have been shown to be surface-exposed and conserved among different pneumococcal isolates [118]. Intraperitoneal immunization of

BALB/c mice with PiuA and PiaA using alum as adjuvant was shown to be protective against an intraperitoneal challenge with a serotype 2 strain (D39), with the highest survival rates found for animals injected with both antigens [119]. CBA/CA mice primed through the intranasal immunization with PiuA and PiaA using CT as adjuvant, followed by an intraperitoneal booster with alum, were further shown to be protected against an intranasal lethal challenge with a serotype 1 strain [118]. Passive protection with antibodies from immunized animals was also shown [118–120] and anti-PiuA and anti-PiaA antibodies were shown to act by increasing opsonophagocytosis rather than inhibiting iron transport [120].

The intraperitoneal immunization of BALB/c mice with a fusion protein containing the three variants of RrgB, the major backbone component of *S. pneumoniae* pilus 1, adjuvanted with alum has been shown to induce protection of BALB/c mice against an intraperitoneal challenge with a serotype 4 strain (TIGR4). Immunized CD1 mice were also protected against the intravenous challenge with TIGR4 and serotypes 6B and 35B strains. Antibodies to this fusion protein have been shown to enhance complement-dependent opsonophagocytosis of the bacteria and to afford passive protection against an intraperitoneal challenge with TIGR4 [121]. Importantly, this was the first report of pneumococcal protein antigens that were able to induce antibodies with opsonophagocytic killing comparable to conjugated polysaccharides. This result could be due to the greater exposure of the pilus beyond the capsule when compared to other protein antigens. Moreover, the presence of several RrgB subunits covalently linked forming the pilus structure might enhance binding of antibodies and opsonophagocytic killing. Pilus islet 1 (PI-1) is present in 30 % of strains and 50 % of the antibiotic-resistant strains [122, 123], indicating that a vaccine using RrgB might be important for the control of antibiotic-resistant strains. A second pilus islet (PI-2) has been described and is found in 16 % of clinical isolates [124]. Pilus 1 expression was recently shown to be biphasic, with distinct populations of cells with either undetectable or prominent pilus expression [125, 126].

A proposal to avoid problems of replacement is to induce protection against pneumococcal disease without altering colonization. This can be achieved by the use of vaccine antigens that are only expressed during the disease phases of infection. Expression of pneumococcal choline binding protein A (PcpA) is induced only at low manganese concentrations, such as those found in the blood and lungs. Immunization with PcpA using alum as adjuvant was shown to provide protection of CBA/N mice against pneumonia caused by serotype 6B, 14, 19F, and 23F strains and also against an intravenous challenge with a serotype 4 strain (TIGR4). Significant protection against colonization with a 19F strain was not observed [127]. Although it seems reasonable that an antigen that does not alter colonization will avoid replacement disease and will not lead to alterations in the nasopharyngeal microbiota, the fact that the majority of the reduction in invasive disease elicited by the conjugate vaccines is achieved through herd immunity brings doubt to the potential efficacy of this approach.

Formulations containing multiple antigens

An efficient vaccine based on protein antigens will probably have to be composed by a mixture of proteins and many groups have thus tested different formulations in mouse models. The use of a combination of antigens rather than one single antigen would be important to overcome problems such as antigen variability, differences in expression among strains, and also during different stages of infection, and the possibility of immune evasion.

The intranasal immunization with recombinant lipidated PsaA, PspA, and PdB using CTB as adjuvant was tested for protection against colonization with 6B and 23 strains. PsaA was shown to elicit better protection than PspA against carriage, whereas PdB did not alter colonization. Immunization with a mixture of PsaA and PspA elicited the best protection [100]. Protection against intraperitoneal challenge with serotype 2 (D39) and serotype 4 strains was also analyzed in BALB/c mice immunized intraperitoneally with PsaA, PspA, and PdB using alum as adjuvant. The median survival times were significantly longer for animals immunized with the combination of PdB and PspA than with any of the antigens alone [128]. The protection in the model of focal pneumonia with a serogroup 19 strain in CBA/N mice was also tested in animals immunized subcutaneously with PsaA, PspA, and PdB adjuvanted with alum. PspA and PdB inoculated individually led to a reduction of bacteria recovered from the lungs, but the most dramatic effect was observed for the immunization with a combination with PspA and PdB [65]. The intraperitoneal immunization of BALB/c and CD1 mice with PdB, PspA, PspC, PhtB, and PhtE using alum as adjuvant was also tested against the intraperitoneal challenge with serotypes 2 (D39) and 6A strains. It is interesting to note that the use of two different mouse lineages and two pneumococcal strains for the challenge gave sometimes conflicting results. Still, in many cases, immunization with multiple proteins afforded the best protection. The median survival times for mice immunized with combinations of PdB and PspA, PdB and PspC, or PspA and PspC were higher than for animals immunized with single antigens [129].

Another interesting approach based on a mixture of proteins as a vaccine is the use of CBPs. CBPs have a

biologically active module and a choline-binding module that anchors these proteins non-covalently to the choline residues of teichoic acid. The number of CBPs varies in different strains (approximately 15) and some of them are highly variable [71, 130]. Choline is essential for pneumococcal growth, but can be substituted by different aminoalcohols, such as ethanolamine. Pneumococci grown in chemically defined medium with ethanolamine and without choline grow in long chains and do not undergo autolysis. Furthermore, CBPs are released by the bacteria and can be recovered from the culture supernatant [94, 131]. CBA/N and BALB/c mice immunized intraperitoneally with CBPs without adjuvant were shown to be protected against an intravenous challenge with a serotype 3 strain (WU2). PspA was shown to be the most important antigen, but other CBPs also contributed to protection [94]. Besides the advantage of being a mixture of different protection-eliciting proteins, a formulation based on CBPs would be similar to other licensed vaccines composed of antigens purified from culture supernatants.

High-throughput screenings of protein antigens

Different approaches have been used to screen pneumococcal proteins for their potential as vaccine candidates. The analysis of the whole genome sequence of a serotype 4 strain identified 130 open reading frames encoding proteins with secretion motifs or similarity with predicted virulence factors. The subcutaneous immunization of C3H mice with 108 of these proteins using CFA as adjuvant identified 6 antigens that protected animals against the intraperitoneal challenge with a serotype 6B strain. Two proteins contained a signal peptidase motif, two were cell wall anchored serine proteinases and the last two were the choline-binding proteins LytB and LytC. LytB and LytC are important cell wall hydrolases [132]. All the 6 proteins were shown be present in several strains and to be immunogenic during human infection [133].

The screening of *E. coli* display libraries with sera from exposed individuals or convalescent patients led to the identification of 140 pneumococcal antigens. Based on several in vitro assays, 18 novel candidates were used to immunize C3H mice using CFA as adjuvant. Four proteins were able to provide protection against an intravenous challenge with a serotype 6B strain. Two of these antigens (PcsB—protein required for cell wall separation of group B streptococcus, and StkP—serine/threonine protein kinase) were further used for the immunization of mice (using alum as adjuvant) and were shown to be protective in models of sepsis (intraperitoneal challenge with a serotype 6B strain and intranasal challenge with a serotype 1 strain) and pneumonia (intranasal challenges with serotype 3 and serogroup 19 strains). PcsB and StkP are highly conserved among clinical isolates [134]. PcsB is a putative peptidoglycan hydrolase [73, 135], while StkP plays an important role in regulating cell wall synthesis and controls correct septum progression and closure [135, 136]. PcsB was recently shown to be attached to the surface of pneumococci through interaction with the large extracellular domain of the FtsXSpn integral membrane protein [73]. Since protection against colonization was shown to involve a T_H17 response, an interesting strategy would be the use of antigens that elicit secretion of IL-17A. An expression library was thus screened for the induction of secretion of IL-17A by $CD4^+$T cells isolated from mice immune to pneumococcal colonization. From the several selected clones, three proteins (SP2108, SP0148, and SP0882) were shown to provide protection against colonization and may be used in a vaccine formulation [11]. Interestingly, two of the selected antigens have not been previously described as antibody targets, showing that this approach can lead to the discovery of totally new vaccine antigens.

Recently, the genome-wide in vivo transcriptomic analysis of bacterial genes upregulated in the brain of intranasally infected mice led to the identification of a previously uncharacterized protein, ∝-glycerophosphate oxidase (GlpO) [137]. The immunization of outbred CD1 mice with GlpO adjuvanted with alum was shown to protect mice against an intraperitoneal challenge with a serotype 4 strain. Furthermore, mice passively immunized with anti-GlpO antibodies showed a lower ratio of bacterial numbers in the brain versus blood than control mice after challenge, indicating that these antibodies could be blocking progression of pneumococci from the blood to the brain. GlpO plays a role in pneumococcal meningitis, probably through the cytotoxic effects of the generation of H_2O_2 from glycerol and also through the promotion of adherence to the cerebrovascular endothelium. Antibodies generated against GlpO can act both through the neutralization of its activity as well as through the promotion of opsonophagocytic killing of the bacteria.

Whole cell and live recombinant vaccines

A whole cell pneumococcal vaccine (WCV) was proposed by Malley and collaborators some years ago [138]. This is a killed unencapsulated bacterium that was first tested as a nasal vaccine in mice. The absence of capsule allows the exposure of protein antigens on the pneumococcal surface to the immune system and, thus, protection conferred by this vaccine can be serotype-independent. In fact, nasal immunization with WCV, using CT or CTB as adjuvant, was shown to confer protection against nasal colonization with a variety of pneumococcal serotypes in mice [139], as well as against a lethal challenge with a serotype 3 strain in rats [138]. Further studies on the mechanisms of protection

against nasal colonization of mice elicited by WCV have described the role of T_H17 cells. Protection was observed in μMT−/− mice, which are deficient for the production of antibodies. Depletion of $CD8^+$ T lymphocytes in vaccinated mice showed no impact on protection whereas depletion of $CD4^+$T lymphocytes abolished the effect of WCV on pneumococcal colonization [49]. A strong negative correlation between the density of pneumococcal colonization in the nasopharynx of mice vaccinated with WCV and the levels of IL-17A in the blood was shown; with non-detectable numbers of pneumococci in animals with high levels of this cytokine. In addition, protection was no longer observed in mice lacking the receptor for IL-17A [50]. In an in vitro assay, IL-17A was shown to enhance killing of pneumococci by neutrophils even in the absence of antibodies, which could be a mechanism for the protection conferred by WCV in μMT−/− mice. T_H17 cells were also shown to be the effectors in the protection against pneumococcal nasal colonization in mice immunized with the purified CWPS, using CT as adjuvant. The zwitterionic charged motif of the pneumococcal CWPS provides a character to the polysaccharide that enables its presentation by B cells, via MHC class II, to T cells, resulting in the induction of IL-17A [140]. Nasal immunization of mice with CWPS also resulted in protection against a lethal challenge with a serotype 3 pneumococcal strain, although the mechanism here seems to be related to the induction of systemic and mucosal antibodies against CWPS [140].

The involvement T_H17 cells in protection against pneumococcal carriage stimulated the search for new vaccine formulations and antigens that could induce such response. A screening of antigens that could stimulate T_H17 cells from mice immunized with WCV identified two new pneumococcal proteins (SP2108 and SP0148) that can induce protection in the colonization model, as mentioned before [11]. Among the commonly studied pneumococcal antigens, a nasal formulation containing PsaA, PspC, and PdT in the presence of CT as adjuvant elicited the secretion of IL-17 by $CD4^+$T cells in mice and protected against nasal colonization [141]. Despite the induction of antibodies against the three proteins, a significant decrease in colonization was also observed in μMT−/− mice, showing that protection was also independent of antibodies. In an approach to enhance immunity, Lu and collaborators have tested a conjugate vaccine composed of a PsaA-PdT fusion protein and the CWPS, using CT as adjuvant, by the nasal route. This vaccine was shown to induce high levels of antibodies and IL17-A responses against the three antigens (PsaA, PdT, and CWPS) and protection was shown in both models of nasal colonization and fatal aspiration pneumonia in mice [142]. Interestingly, the authors also tested a protocol for subcutaneous immunization with the conjugate, using alum as adjuvant, as the use of CT or derivatives may present a risk in humans. Protection against the nasal colonization and the fatal aspiration pneumonia challenges was also observed in this case [142].

More recently, the strategies pursued by Malley and collaborators to adequate WCV for human studies were reported. These include the use of an unencapsulated strain which expresses a non-hemolytic derivative of pneumolysin and is autolysin-negative (RM200 strain) [143]. Different methods for killing the bacteria were tested, with beta-propiolactone being the chosen agent [143, 144]. Besides the previous use of beta-propiolactone for the production of human vaccines, the method allowed the retention of antigens in the killed bacteria, increasing the immunogenic potential of the preparations. Following a recommendation of the World Health Organization [145], the vaccine was also tested through parenteral routes of immunization, using aluminum hydroxide as adjuvant. Among the important considerations that led to a move from nasal to parenteral immunization were the lack of safe adjuvants for nasal immunization and the homogeneity of the doses in infants eventually presenting copious nasal mucus [144]. In addition, other mucosal routes such as the oral or the sublingual would require considerable higher amount of antigen [143]. The final protocol was shown to confer protection against nasal colonization and lethal respiratory challenges in mice. The antibodies induced by the subcutaneous immunization of mice with WCV adsorbed to aluminum hydroxide showed cross-reactivity with different pneumococcal serotypes. The vaccine also induced T_H17 responses against different pneumococcal isolates [146]. All these results supported the approval of a phase 1 clinical trial for WCV that is currently ongoing.

Live recombinant vectors can be useful alternatives for the induction of complex immune responses against heterologous antigens. In addition, they are usually administered through mucosal routes, providing immunity at these sites. In the case of pneumococcal vaccines, the use of *Salmonella*-derived vectors was extensively studied for the presentation of protein antigens such as PspA, PspC, or PsaA [147–149]. The main challenge in this approach is to guarantee the attenuation of the live vector while maintaining the immunogenicity against the target antigen. With this in mind, Curtiss and collaborators have engineered numerous *Salmonella* strains for the development of safe and effective mucosal pneumococcal vaccines. Attenuated pathogens frequently present lower ability to infect and colonize the hosts, when compared to their wild-type counterparts, impairing the immune responses against the target antigen. In order to circumvent this obstacle, they have constructed *Salmonella typhimurium* strains with regulated delayed attenuations. In such recombinant strains, the attenuated phenotype is dependent on the absence of mannose or arabinose in the environment. As a result, when

the vaccine strains are grown in the presence of these components, their invasive and colonizing abilities, by the time of immunization, are similar to the observed for a wild-type strain. However, after a few cell divisions in the host, the attenuated phenotype is acquired [147]. Oral immunization of mice with these *Salmonella* strains expressing PspA elicited strong immune responses against the antigen that were characterized by the induction of mucosal and systemic anti-PspA antibodies as well as PspA-specific secretion of IL-4 and IFN-γ by splenocytes. Protection against an intraperitoneal challenge with the WU2 pneumococcal strain (serotype 3) was increased in mice immunized with the *S. typhimurium* strains bearing the regulated delayed attenuations and PspA, when compared to mice immunized with commonly attenuated *S. typhimurium* strains expressing PspA. Passive transfer experiments showed that immunity was conferred by sera or spleen cells from immunized mice, confirming the induction of both humoral and cellular responses [147]. In addition, to overcome eventual problems of toxicity due to constitutive expression of heterologous antigens, the researchers also developed a system for regulated delayed antigen synthesis, in which the expression of the protein of interest (PspA was also tested in these strains) is repressed in the presence of arabinose. Therefore, once the vaccine strain encounters the host environment, PspA expression is released, stimulating the immune system [150]. Improvements of the immune responses were also achieved by the use of different signal sequences, to address the antigens to the periplasm or the extracellular compartments [149]. To address the problem of vaccine coverage against strains expressing different PspAs, fusion proteins composed of N-terminal fragments of PspAs from family 1 and 2 have been expressed. The resulting vaccine strains were tested through the oral route in mice and induced balanced IgG1:IgG2a responses. The anti- PspA antibodies reacted with different pneumococcal strains expressing heterologous PspAs, and protection was observed in mice challenged by the intraperitoneal, intravenous or intranasal routes with three different pneumococcal strains [151]. Besides PspA, the *Salmonella* system was also tested for the presentation of PspC and PsaA. Immune responses against both PspC and PspA, as well as protection against mice models of lethal infection and nasal colonization, respectively, were observed [148, 149]. More recently, studies described the construction of new *S. typhimurium* strains able to deliver multiple antigens. This system was tested for the expression of PspA and PspC and immunization of mice with the recombinant strain resulted in improved protection against different pneumococcal challenge models [152]. The data obtained with the *S. typhimurium* strains have driven the construction of recombinant *Salmonella typhi* strains carrying similar characteristics for attenuation and antigen expression.

In addition to the modifications described here, mutations in two major *S. typhi* antigens, the O-antigen and the Vi capsule, were also performed, intending to reduce host immune responses against *Salmonella*. These strains were tested for attenuation by the evaluation of survival in the presence of human blood and peripheral mononuclear cells as well as in the environment. The immune responses were tested in mice immunized with the *S. typhi* strains expressing PspA [153]. The results supported a Phase 1 clinical trial with three different *S. typhi* strains expressing PspA.

Another interesting strategy for the production of live recombinant vaccines against *S. pneumoniae* is the use of lactic acid bacteria (LAB)-based vectors. The main advantages of this approach rely on the safety status of these bacteria and the administration of the vaccine at mucosal sites. In recent decades, increasing knowledge on the genetic engineering of LAB has provided tools for the development of diverse expression systems, although the advances cannot be compared with the knowledge on *Salmonella*-based systems. LAB is a group of very diverse bacteria that encompasses strains that do not colonize human or animal mucosal tissues and strains that may remain for days before being eliminated from mucosal sites. Because of this, and the diversity of the surface molecules, differential modulation of the immune system can be observed. The first report on the expression of pneumococcal antigens in lactic acid bacteria was with an inducible expression system based on the lac operon from *Lactobacillus casei* [154]. Both PsaA and PspA antigens were successfully expressed in this system, but immunization of mice with the recombinant *L. casei* strains did not produce significant results. A constitutive expression system that could be used in different LAB strains was used to express the PsaA antigen in *Lactococcus lactis* and in three different lactobacillus strains, *L. casei*, *L. plantarum*, and *L. helveticus*, which were then used for nasal immunization of mice. The levels of mucosal and systemic anti-PsaA antibodies induced by vaccination varied depending on the bacterial vector, but all lactobacilli-based vaccines successfully reduced pneumococcal nasal colonization. No correlation between the levels of antibodies and the reduction in pneumococcal numbers in nasal mucosa was observed, suggesting that cellular immune responses could be participating in protection [155].

In an attempt to test other vaccine formulations against pneumococcal colonization, the same expression system was used for the expression of PspC in *L. casei*. Although nasal immunization of mice did not produce detectable levels of mucosal and systemic antibodies, a significant reduction in pneumococcal nasal colonization was observed. Further evaluation showed the induction of anti-PspC antibodies in the respiratory mucosa of mice immunized with

L. casei-PspC, after the pneumococcal challenge, indicating that the vaccine primed the immune system [156].

The PspA antigen was also expressed in *L. lactis* using a nisin-inducible expression system. Nasal immunization of mice with this strain induced a balanced IgG1:IgG2a response against PspA and resulted in increased mean survival time after an intraperitoneal challenge and increased overall survival after a lethal respiratory challenge with a serotype 4 pneumococcal strain [157]. Similar results were obtained with the nasal immunization of mice with a *L. casei* strain expressing PspA constitutively. The vaccine was able to induce systemic antibodies against PspA that were able to bind and to induce the deposition of complement on the surface of different pneumococcal strains in vitro. Partial protection was observed after an intraperitoneal challenge with a serotype 3 strain [158]. Further characterization of the protective immune responses elicited by *L. casei* expressing PspA indicated that the presentation of the antigen by the bacteria during immunization induced antibodies with good ability to bind to the pneumococcal surface. Despite lower antibody levels induced by *L. casei*-PspA, when compared with the levels induced by the recombinant protein, flow cytometry analysis showed a similar capacity of the sera to bind to the pneumococcal surface and to induce complement deposition. It was hypothesized that the antibodies induced by the different vaccines (*L. casei* or recombinant protein) may recognize different epitopes [159]. Consistent with the induction of T_H1 responses, mice vaccinated with *L. casei*-PspA displayed significantly higher levels of antigen-specific IFN-γ secretion in the lungs, 13 h after a pneumococcal respiratory challenge. The protective potential was tested through a lethal respiratory challenge with a serotype 3 strain and, once again, resulted in significant but partial protection (around 40 % survival, the same percentage observed in the previous work with *L. lactis* or *L. casei*). Thus, although the LAB systems seem to be an attractive alternative for the development of pneumococcal vaccines, improvements are still necessary. The most successful report to date was observed for a *L. lactis* vaccine expressing the pneumococcal protective protein A (PppA), an antigen that has been implicated in pneumococcal colonization and systemic disease. Mice immunized with *L. lactis*-PppA were protected against an intraperitoneal challenge with a serotype 14 strain (60–70 % survival) and against an intranasal lethal challenge with the same strain (around 60 % survival). Passive immunization experiments confirmed the role of antibodies against the lethal pneumococcal challenges. Since PppA is an antigenically conserved protein, the authors also analyzed the protective potential of *L. lactis*-PppA against different pneumococcal isolates. The results showed reduction of bacterial burden in the lungs and in the blood of mice challenged with serotypes 2, 6B, 14 and 23F [160].

Adjuvants

The PS conjugate vaccines currently in use have aluminum salts in their composition, which provide the enhancement of antibody responses against pneumococcal polysaccharides. Aluminum salts were also the choice of adjuvants for parenteral administration of pneumococcal proteins in animals [128, 134]. However, a variety of other adjuvants were already combined to pneumococcal antigens. Mucosal formulations, including protein vaccines and the WCV, were often tested with derivatives of the *Vibrio cholera* and *E. coli* toxins [100, 140]. Still, concerns about the safety of these molecules for human use cannot be neglected.

Several data in the literature support the rational proposal of adjuvants for new pneumococcal vaccines. It is now well established that T_H17 responses are effectors in the protection against pneumococcal colonization both in acquired and innate immunity, resulting in the recruitment of PMN cells to the site of infection [49–51]. On the other hand, antibodies have been shown to be key mediators of protection against invasive disease in animal models, elicited by different vaccines [110, 146]. However, some data show that the mechanisms may not be exclusive. For instance, as already mentioned, sIgA was shown to be the effector in the protection against nasal colonization induced by a PspA mucosal vaccine [44]. In addition, preferential induction of complement fixing IgG2a by a DNA vaccine expressing PspA correlated with protection against nasal colonization [46]. Thus, besides T_H17 responses, antibodies against major surface antigens, such as PspA, can induce protection in the colonization model.

In a similar way to the results observed against nasal colonization, modulation towards a T_H1 character, with low IgG1:IgG2a ratios and antigen-specific secretion of IFN-γ was shown to optimize the immune responses against invasive pneumococcal challenge models. This was clearly observed by the use of DNA vaccines expressing PspA [161] and by a PspA nasal vaccine adjuvanted with interleukin-12 (IL-12) [162]. Live vaccine vectors such as *Salmonella* and LAB, discussed above, also have the potential to induce T_H1 responses [151, 159].

In a recent study, the potential use of the whole cell pertussis vaccine (wP) as adjuvant to PspA was described. The formulation was shown to induce very high levels of anti-PspA antibodies, with balanced IgG1:IgG2a ratios, when administered through the nasal route in mice. The PspA-wP vaccine conferred protection against pneumococcal nasal colonization with a serotype 6B strain and against a respiratory lethal challenge with a serotype 3 strain. Although the nasal PspA-wP vaccine also induced a rapid influx of immune cells to the airways of mice after the respiratory lethal challenge, antibodies seem to be the key mediators of protection in this model. Passive immunization experiments

with sera from immunized mice conferred survival to 75 % of naïve mice after the challenge [163]. In addition, depletion of $CD4^{+}T$, $CD8^{+}T$, or B lymphocytes in immunized mice, during the challenge, did not impair the protection conferred by the vaccine, indicating that circulating antibodies would be sufficient for protection [164]. It was also very interesting to note that the combination of PspA with DTP_w (diphteria, tetanus and whole cell pertussis vaccine) through the subcutaneous route was very effective in protecting mice against the respiratory lethal challenge with two serotype 3 strains [163]. These results open the possibility to combine DTP_w with pneumococcal protein antigens, mostly for application in developing countries where wP is administered to children.

Antigens tested in human trials

Some human clinical trials have been performed to evaluate serotype-independent pneumococcal vaccines. PspA was the first protein antigen tested in humans [165, 166]. Intramuscular immunization of human volunteers with PspA adjuvanted with alum led to the induction of serum anti-PspA IgG antibodies that were reactive with heterologous recombinant and native PspA molecules [166]. Furthermore, post-immune sera were able to passively protect CBA/N mice against intravenous challenge with pneumococci from different serotypes expressing heterologous PspAs [165].

Interest in testing new vaccines in humans has faded after this first clinical trial, possibly due to the licensure of PCV7 in 2000 and its dramatic success in reducing vaccine-type invasive disease. More recently, interest in testing alternative vaccines has risen again due to the emergence of disease caused by non-vaccine serotypes, and new clinical trials have been performed or are ongoing. Most of the results of the trials themselves are not yet available in the literature and only data on the pre-clinical animal models have been published in some cases.

Two conserved pneumococcal antigens, PcsB and StkP, which were selected by the screening of display libraries expressing fragments of the pneumococci proteome with sera from individuals exposed to pneumococci or convalescent from pneumococcal diseases, comprised a formulation that was tested in a phase 1 clinical trial in 2010. These antigens were shown to confer protection in animal models of lethal sepsis and pneumonia [134]. The final formulation tested in the clinical trial also contained PsaA and alum as adjuvant (IC47; Intercell, Vienna, Austria). The study was designed to assess safety, immunogenicity, and dose responses of IC47, given through the intramuscular route in healthy subjects. In addition to the protective efficacy shown in animal models, blood samples collected from healthy individuals from different ages displayed antibodies that reacted with the three antigens, PcsB, StkP, and PsaA. PBMCs from these individuals were also stimulated to secrete mostly T_H17 and T_H1 cytokines upon contact with each of the three antigens [167].

A phase 1 clinical trial for the evaluation of three *S. typhi* vectors expressing PspA (developed by the group of Dr. Roy Curtiss) was performed in 2011 (ClinicalTrials.gov Identifier: NCT01033409). A single oral dose of the *S. typhi*-vectored pneumococcal vaccines in different concentrations was tested in healthy adults. The main objective of this study was to compare the vectors with respect of their abilities to induce mucosal and systemic antibody responses against PspA and to assess safety and tolerability. The study was sponsored by the Arizona State University, USA and was completed in 2011.

The WCV produced by the group of Dr. Richard Malley (Children's Hospital, Harvard Medical School, USA) is currently being evaluated in a phase 1 clinical trial (Clinical Trials.gov Identifier: NCT01537185). In this study, healthy adults are receiving three injections of the vaccine with alum as adjuvant. The groups were composed of three different doses of 100, 300, and 600 μg of the vaccine to assess safety and tolerability. In addition, humoral immune responses will be determined by ELISA. The estimated date for the completion of this study sponsored by PATH was August 2012.

Formulations containing PhtD with or without adjuvant, given in two intramuscular injections, have been tested in phase 2 clinical trials to study safety, reactogenicity, and immunogenicity (Clinical Trials.gov Identifier: NCT00307528). Combinations of PhtD and dPly with or without PCV10 using $AlPO_4$ as adjuvant have also been tested in phase 2 clinical trials (EudraCT number: 2009-012701-19), and the evaluation of the co-administration of PhtD, dPly, and PCV10 with the DTPa-HBV-IPV/Hib vaccine in a phase 2 clinical trial is currently ongoing (EudraCT number: 2010-019730-27).

Finally, results from three phase I clinical trials were recently published by a same group (Sanofi Pasteur and Covance Clinical Research Unit). Studies were performed to assess the safety and immunogenicity in healthy adults of the following vaccines adjuvanted with aluminum hydroxide: PhtD (6, 25, or 100 μg) [168], the genetically mutated pneumolysin protein PlyD1 (10, 25, or 50 μg) [169], and monovalent PcpA (25 μg) and bivalent PcpA-PhtD (10, 25, or 50 μg of each antigen) vaccines [170]. All formulations were shown to be safe and immunogenic, and repeated vaccination significantly increased antibody levels.

An experimental human carriage model was described some years ago, with the intranasal inoculation of strains from serotypes 6B and 23F [39]. More recently, this model was used again now including the study of responses in

saliva, nasal washes, and BAL samples collected through bronchoscopy [171]. Volunteers challenged but not colonized were shown to have increased levels of antibodies against pneumococcal proteins in nasal washes (IgG and IgA) and BAL (IgG) samples, but not in the serum. Moreover, increase in anti-PS antibodies was not detected [172]. Baseline levels of IgG to protein or PS were not associated with protection against carriage. Carriage induced increased mucosal and serum IgG levels to several proteins and PS, resulting in increased opsonophagocytic activity. It was thus proposed that carriage in healthy adults is a mechanism that sustains effective immunity against pneumococcal disease (Ferreira D.M. and Gordon S.B., personal communication). The inclusion of the analysis of the mucosal response can bring important information for the understanding of the mechanisms of protection against carriage and acquired immune responses after colonization. The use of this model has also been proposed to evaluate new vaccine candidates. Furthermore, interactions with other potentially pathogenic species like *S. aureus*, *H.influenzae*, *M. catarrhalis*, and *N. meningitidis* can be assessed [173]. Though human studies are very complicated in terms of logistics and costs, these data are essential for the study of pneumococcal disease and should be further explored.

Final remarks

Diseases caused by *S. pneumoniae* represent a major public health problem, associated with high morbidity and mortality among children and the elderly, especially in developing countries. Several efforts are being made to develop a vaccine that could overcome the shortcomings of the PS-conjugate vaccines, and it is important to define what would be desired for an ideal vaccine against this microorganism. Such a vaccine has to be affordable, effective against all the diseases, and, ideally, should not allow the colonization of the nasopharyngeal microbiota by new opportunistic pathogens.

The number of serotypes that can be included in a PS-conjugate pneumococcal vaccine has probably reached a limit of cost-effectiveness, with the possible licensure of a new vaccine covering 15 serotypes. Currently, most of the developing countries cannot afford this kind of vaccine. In this regard, the alternative vaccine candidates based on protein antigens, whole cell pneumococci, and recombinant bacteria expressing pneumococcal antigens presented in this review are expected to have much lower production costs.

Streptococcus pneumoniae is able to colonize the nasopharynx and, depending on the host conditions, it can induce several diseases. Without any doubt, protection against invasive disease has to be prioritized, followed by pneumonia. Protection against otitis media would also be desirable. Since there is a direct link between pneumococcal disease and colonization, it is expected that a vaccine should also target the colonization process. Several of the vaccine candidates described here meet the requirements of eliciting protection against invasive disease, pneumonia, and colonization in animal models. As discussed, vaccine formulations have been tested by several groups using different challenge models, which makes it very difficult to compare results and define which would be the best candidate. Data on clinical trials are now necessary for further evaluation of these vaccines. Testing new formulations will have an additional complication where the PS-conjugate vaccines are already in use and very large numbers of individuals will have to be immunized in order to evaluate efficacy in clinical trials. Alternatively, in these locations, pneumococcal protein antigens could be used as carriers for conjugate vaccines or could simply be added to conjugate vaccine formulations to increase strain coverage. Such formulations would still have the problem of excessive cost for developing countries.

One of the complications in the development of new vaccine formulations is the decision to target the colonization of the nasopharynx by pneumococci through immunization, which can be a double-edged sword. The widespread use of the PS-conjugate vaccine has led to a rapid decrease in vaccine-type disease, which is believed to be mostly due to herd immunity. The diminished colonization of vaccinated children has thus led to a decline in the circulation of vaccine-type pneumococci in the population, which in turn caused a reduction in disease. The downside of reduced colonization was the increase in colonization by non-vaccine serotypes and by other species, such as *S. aureus* and *H. influenzae*. It thus seems that a vaccine against pneumococcal diseases has to target colonization to be effective at the population level, but there will always be the possibility of increase in colonization by other potentially pathogenic microorganisms in the non-sterile niche of the nasopharynx. Commensal colonization of human upper respiratory airways by pneumococci would ultimately be the ideal scenario for a peaceful co-existence. However, during co-evolution, most of the advantages acquired by the bacteria for a successful colonization and spread to new hosts (such as the expression of virulence factors and the induction of local inflammatory responses) are also responsible for the eventual disease state [174]. The major challenge for the new proposed vaccines is to prevent disease without causing any significant misbalance in the commensal state of this or other respiratory pathogens. For all the strategies described here, this remains to be demonstrated.

Streptococcus pneumoniae is a naturally transformable organism, and a final complicating factor for the development of new vaccines is the plasticity of the pneumococcal genome, with high rates of recombination. The analysis of the complete genomes of isolates from a multidrug resistant

lineage has recently shown that this plasticity permits the adaptation to clinical interventions over short time scales. Moreover, some loci were shown to display higher levels of recombination, possibly due to the diversifying selection by the immune system. The pneumococcal population could thus respond very rapidly to the introduction of some of the new pneumococcal vaccines currently under development [175], and the emergence of escape strains could turn the development of vaccines against pneumococcal diseases into an endless story. Broad protection could be achieved through the use of conserved antigens as well as through the inclusion of different variants of important virulence factors that are under the diversifying pressure of the immune system, either in multicomponent vaccines or more complex formulations such as whole bacteria. Furthermore, both antibody and cellular immune responses will have to be induced in order to obtain protection against the different stages of pneumococcal infection, which could be achieved through the use of adequate adjuvants.

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